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JOURNAL ARTICLES

[1] Glenn, G.M., Younce, F.L. and Pitts, M.J.1991. Fundamental physical properties characterizing the hardness of wheat endosperm. Journal of Cereal Science 13: 179-194.

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Scientific Research

Phenotypic and molecular identification of the non-pathogenic lactic acid bacteria isolated from the local sheep yogurt

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ABSTRACT

With the growing tendency of people to consume organic dairy products, there is always a risk of pathogenic microbial strains in unhygienic locally prepared products. Local sheep yogurt as an organic product is one of the most valuable dairy products in Iran. The aims of this study were molecular and phenotypic identification of lactic acid bacteria (LAB) isolated from sheep yogurt from Hamadan and investigation of their pathogenic characteristics. The LAB isolated from yogurt samples were identified phenotypically and molecularly by PCR amplification of the 16S rDNA region and subsequent sequencing. The pathogenic and safety characteristics of the isolates including antibiotic resistance, blood hemolysis, amino acid decarboxylase, DNase, and gelatinase activities were then investigated. Forty-seven bacteria were isolated from yogurt samples, and only 22 gram-positive isolates reacted negatively to catalase. Based on the results, the isolates were molecularly characterized as 4 *Lactobacillus helveticus*, 5 *Enterococcus mori*, 2 *Streptococcus salivarius subsp. thermophilus*, 5 *Lactobacillus kalixensis*, and 6 *Lactobacillus delbrueckii subsp. bulgaricus*. Phenotypic identification also confirmed the isolates assigned to *S. thermophilus* and *L. bulgaricus* by molecular identification. The evaluation of the pathogenic characteristics of the species associated with yogurt production, *S. thermophilus* and *L. bulgaricus*, confirmed that two isolates (HL1, HL2) of *L. bulgaricus* are safe as starter culture for yogurt production.

1- Introduction

Due to the different climatic conditions in Iran, the breeding of domestic animals, including sheep, is widespread and the dairy products obtained from them find many customers in the country. Sheep yogurt is more nutritious than cow yogurt as it has a higher content of fats, calcium and proteins. The unique taste and flavor of sheep yogurt is influenced by various factors, including the type of raw materials, the inherent microbial lactic acid flora, the production methods, and various additives that vary from region to region. Especially for the production of sheep's yogurt, which is mostly done locally and traditionally, the undefined microbial flora of sheep's yogurt is used as native starter culture, whereas in industrial yogurt production from cow's milk, defined safe starter cultures are often used. These starter cultures were previously tested for their non-pathogenic. Therefore, there are always concerns regarding the consumption of local sheep yogurt, as it contains an unknown microbial flora that has not been tested for non-pathogenic and may carry pathogenic genes. Pathogenicity parameters of bacteria include antibiotic resistance, hemolysin, amino acid decarboxylase, DNase and gelatinase activities (1). DNase activity is an indicator of bacterial pathogenicity as it hydrolyzes cellular DNA molecules, and bacterial strains used in fermented products should be free of it (2). Biogenic amines are produced during the decarboxylation of amino acids by microbial activity and can cause health problems such as diarrhea, vomiting, palpitations and headaches in consumers. One of the producers of these biogenic amines in fermented products are lactic acid bacteria (LAB) (3). The enzyme gelatinase is able to hydrolyze gelatin (found in connective tissues) through two reactions, first into polypeptide compounds and then into amino acids. Various bacterial species such *as Staphylococcus aureus*, some Vibrio spp., *Pseudomonas aeruginosa*, and *Bacillus subtilis* are gelatinase-positive. Gelatinase positivity is one of the indicators of bacterial pathogenicity and the microorganisms used in fermentation should be gelatinase-negative (4). The absence of antibiotic-resistant genes in starter cultures of fermented products such as yogurt is also important. This is because the consumption of these products introduces antibiotic-resistant gene-carrying microbial flora into the consumer's body (5). Another important parameter in the use of starter cultures is the absence of hemolytic activity, i.e. the lack of ability to hydrolyze blood cells (6). The isolation and identification of indigenous LAB species from traditional dairy products is necessary to exploit the unique sensory properties of these indigenous strains. In addition to evaluating the technological potential of the strains, their non-pathogenic should also be confirmed. The aim of this study is to isolate and identify the safe LAB found in traditional sheep yogurt in Hamedan.

2. Material and methods

1.1. Sampling of yogurt and Isolation of LAB

Three samples of sheep yogurt were collected in rural areas of Hamadan city in Iran under sterile conditions and brought to the laboratory at 4°C (7). The samples were serially diluted to 10^7 in Ringer's solution and surface cultures were established on M17 agar (Merck, Germany) and MRS agar (Merck, Germany). Subsequently, these culture media were incubated anaerobically by anaerocult type A (Merck, Germany) for 48 hours at 39 °C for M17 agar and 45 °C for MRS agar. In the next step, various colonies were purified by streaking on agar plates. The purified isolates were examined by microscopic observation, Gram staining, and catalase test (8-10).

1.2. Measurement of lactose of milk samples and pH of yogurt samples

The lactose in the milk samples and the pH value in the yogurt samples were measured using a milk analyzer and a digital pH meter respectively.

1.3. Phenotypic identification of isolates at the genus level

The Gram-positive and catalase-negative isolates were screened at the genus level for their growth potential under various conditions, including sodium chloride concentrations of 6.5% and 18%, temperatures of 10°C, 15°C, 30°C, 45°C, pH= 4.4 and pH=9.6, and the ability to produce carbon dioxide gas from glucose. Homofermentative cocci strains that can grow at 30°C and not at 10°C, pH= 9.6, and 6.5% sodium chloride were confirmed as Streptococcus (11). Gram-positive Bacillus strains with negative catalase activity that can grow at 45°C, pH= 4.4 and 6.5% sodium chloride and cannot grow at pH= 9.6, 18% sodium chloride and 15°C and do not produce carbon dioxide gas from glucose were confirmed as homofermentative Lactobacillus (12).

1.4. Phenotypic identification of isolates at the species level

Streptococcus and Lactobacillus spp. confirmed to genus level in the previous phase were further identified to species level using biochemical tests according to the guidelines of the manual (13).

1.5. Molecular identification of isolates at the species level

The DNA of cocci and Bacillus strains was extracted according to Ruiz-Barba, Maldonado-Barragán and Jiménez Díaz (14). In this method, a portion of the colonies of the isolates was dissolved in 100 µL of sterile deionized water and 100 µL of isoamyl alcohol and chloroform (Merck, Germany) solution was added at a ratio of 1:24. The mixture was shaken for 5 seconds and centrifuged at 16000 g for 5 minutes. The upper phase was used for the DNA template. For amplification of the 16S rRNA gene by PCR, 1 µL of each primer (B27F and U1492R) (Bioneer, Korea) at a concentration of 10 picomoles/µl, 12.5 µL of master mix (SinaClon, Iran), 2.5 µL of DNA and 8 µL of sterile deionized water (SinaClon, Iran) were used. After activation at 94°C for 5 min, the PCR reaction consisted of the following temperature-time program for 40 cycles: Denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min. Then a final extension step was performed at 72°C for 10 min and a final cooling step at 4°C for 5 min (15). The PCR products were sent for sequencing (Macrogen, Korea), and the sequences obtained were analyzed using the BLAST program on the NCBI website to identify the isolates.

1.6. Assessment of non-pathogenic properties of LAB isolates

In order to evaluate the safety properties of LAB, only bacilli and cocci isolates identified as *L. bulgaricus* and *S. thermophilus* were investigated.

1.6.1. Antibiotic resistance assessment of isolates

Cocci bacteria on M17 broth and Bacillus on MRS broth were activated. After preparing bacterial suspensions with a turbidity of 0.5 McFarland, the cocci isolate on M17 agar and the Bacillus isolates on MRS agar were surface cultured with a sterile swab. Antibiotic disks containing tetracycline, amoxicillin, penicillin, chloramphenicol, erythromycin, vancomycin, and ampicillin were then placed on the agar surface to determine the antibiotic resistance of the isolates. The plates were then incubated at 37°C for 48 hours, and the absence or presence of bacterial growth around the disks indicated

the sensitivity or resistance of the bacteria to the respective antibiotics (16, 17).

1.6.2. Assessment of hemolytic activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was streaked onto blood agar medium containing 5% sheep blood (Darvash Co, Iran) using a sterile loop. The plates were then incubated at 37°C for 48 hours. After incubation, hemolysis around the colonies was examined to determine hemolytic activity.

1.6.3. Assessment of decarboxylase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was inoculated onto lysine decarboxylase and ornithine decarboxylase media (Darvash Co, Iran) using a sterile loop and incubated at 37°C for 24 hours. The formation of a purple color indicated positive decarboxylase activity, while a yellow color indicated negative results.

1.6.4. Assessment of DNase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was streaked linearly onto DNase agar medium (Darvash Co, Iran) using a sterile loop and incubated at 37°C for 24 hours. After incubation, 3% hydrochloric acid solution was poured over the agar surface. The formation of a halo around the colonies indicated positive DNase activity.

1.6.5. Assessment of gelatinase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was inoculated to a depth of about one centimeter on gelatin agar medium (Darvash Co, Iran) and incubated at 37°C for 24 hours. After incubation, the test tubes were brought to 4°C. The presence of gelatin as a liquid in the tube after 2 to 3 hours indicated positive gelatinase activity.

1.7. Statistical analysis

The lactose of milk samples and the pH value of the yogurt samples and the pathogenic tests including antibiotic resistance, DNase, decarboxylase, gelatinase and hemolytic activities were performed in 3 replicates. The mean diameter of the growth inhibition zone, which was determined from the antibiotic sensitivity of the bacteria, and the standard deviation were calculated.

3. Results and Discussion

1.8. Measurement of lactose and pH

According to the results, the pH of Hamadan sheep yogurt was 4.1±0.13, and the lactose content of raw milk was 4.4 ± 0.2.

1.9. Phenotypic identification of isolates

A total of 47 bacteria were isolated from 3 yogurt samples, 22 of which were gram-positive and catalase-negative. Only the bacteria identified at genus level as Lactobacillus (HL1, HL2, HL3, HL7, HL9, HL10) and Streptococcus (HS1, HS2) were phenotypically identified to species level. The results of phenotypic identification of LAB isolates in yogurt at species level (Tables 1 and 2) showed that *Lactobacillus delbrueckii* subsp. *bulgaricus* is the predominant lactic acid flora in Hamadan sheep yogurt and not Streptococcus. This finding is consistent with the results of Davati and Hesami (18). Several factors may contribute to the reduction of *Streptococcus salivarius* subsp. *thermophilus* in Hamadan yogurt. One of these factors is the presence of autolyzing

strains of this species in the yogurts of the western regions. Sandholm and Sarimo (19) and Thomas and Crow (20) reported that in yogurts with lower lactose content and lower pH, the rate of autolysis of strains of *S. thermophilus* is higher. There is also the possibility of autolysis of some Streptococcus strains in Hamadan yogurt, which may lead to the loss of these strains and consequently to their non-cultivation and recovery. This is because, according to the results of chemical tests, the lactose content and pH value in Hamadan sheep's yogurt are lower than usual. According to Davati and Hesami (21), the microbial flora of a local doogh (ayran) produced in the western regions of Iran was identified as the following species using 16s metagenomics analysis: *Lactobacillus equicursoris* 24.8%, *Lactobacillus delbrueckii* 51.81%, *Lactobacillus apis* 3.61%, *Lactobacillus ultunensis* 2.79%, *Lactobacillus taiwanensis* 0.86%, *Lactobacillus gigeriorum* 0.85%, *Pediococcus argentinicus* 0.42%, and 13.64% unclassified bacteria. Since doogh itself is a type of product containing salt and yogurt, the absence of Streptococcus species was probably due to their high autolysis rate in the yogurts of the western regions.

Table 1. Phenotypic Diagnosis of *Lactobacillus delbrueckii* subsp. *bulgaricus*

Identified isolate	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Isolate code	HL1, HL2, HL3, HL7, HL9, HL10
Growth at	
15 °C	–
45 °C	+
Acid production from	
Galactose	–
Lactose	+
Maltose	–
Mannitol	–
Mannose	–
Melibiose	–
D-raffinose	–
Sucrose	–
$C0_2$ production from glucose	–

+: 90% or more strains are positive, **-:** 90% or more strains are negative

Table 2. Phenotypic Diagnosis of *Streptococcus salivarius* subsp. *thermophilus*

Identified isolate	*Streptococcus salivarius* subsp. *thermophilus*
Isolate code	HS1, HS2
Growth at	
2% NaCl	–
15 °C	–
30 °C	+
45 °C	–
Acetoin production	+

Diacetyl production	+
Citrate	–
Acid production from	
Arabinose	–
Galactose	d
Glucose	+
Fructose	+
Mannose	+
Maltose	+
Lactose	+
Sucrose	+
Melibiose	-
Raffinose	d
Rhamnose	–
production from glucose $C0_2$	–

+: 90% or more strains are positive, **-:** 90% or more strains are negative, **d:** 11-89% are positive.

1.10. Molecular identification of isolates

The data from 16S rDNA gene sequencing for the LAB isolates of Hamadan yogurt are shown in Table 3 (Figure 1). The length of the sequenced gene was 1500 bp. Based on the results, the isolates were molecularly characterized as 4 *Lactobacillus helveticus*, 5 *Enterococcus mori*, 2 *Streptococcus salivarius subsp. thermophilus*, 5 *Lactobacillus kalixensis*, and 6 *Lactobacillus delbrueckii subsp. bulgaricus*. The diversity of lactic flora in local yogurts from the western regions of Iran has been studied previously. According to Davati (22), the presence of *Pediococcus acidilactici, Enterococcus faecium, Lactobacillus paraplantarum, Enterococcus durans, Lactobacillus delbrueckii, Lactobacillus fermentum*, and *Lactobacillus johnsonii* in yogurt produced by Alvand nomads was confirmed. The diverse lactic flora in Hamadan sheep yogurt is influenced by secondary contamination of the product, different microbial flora and the breed of sheep supplying the milk. Sheep milk can be affected by secondary contamination from sewage-contaminated pastures and animal manure (23).

Table 3. Molecular identification of isolates

Identified species	%Identity in NCBI	Isolate code
Lactobacillus delbrueckii subsp. bulgaricus	100	HL1, HL2, HL3, HL7, HL9, HL10
Streptococcus salivarius subsp. *thermophilus*	99	HS1, HS2
Lactobacillus helveticus	98	HL4, HL5, HL6, HL8
Lactobacillus kalixensis	98	HL11, HL12, HL14, HL16, HL19
Enterobacter mori	99	HE 13, HE 15, HE 17, HE18, HE 20

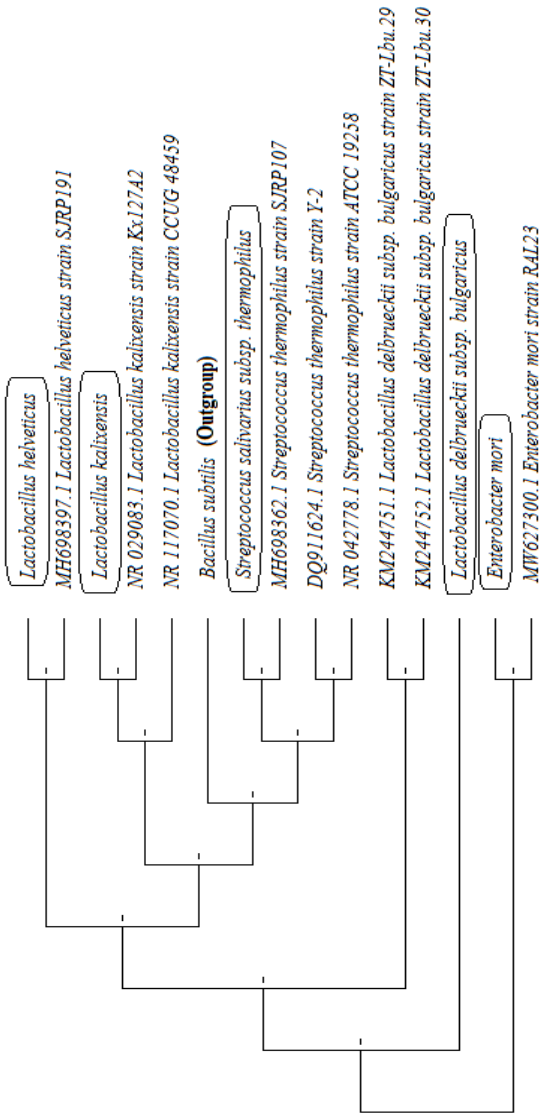

Figure 1. Phylogenetic tree of LAB isolated from sheep's yogurt and comparison with some sequences of same species (Registered in NCBI) and Outgroup

Based on studies conducted worldwide, the genus Lactobacillus has shown the highest prevalence (86.96%) among LAB in dairy products (24). In line with these findings, Bhardwaj, Puniya, Sangu, Kumar and Dhewa (25) reported that *Lactobacillus casei* (24.35%) and *Lactobacillus acidophilus* (17.37%) were the dominant microbial flora in Dahi, a traditional dairy product in India. Similarly, in our study, the predominant lactic flora in Hamadan yogurt belonged to the genus Lactobacillus. However, it should be noted that the lactic flora of local dairy products varies depending on the type of product, production method, additives, and climatic conditions. Several studies have been conducted on the microbial flora of local Iranian yogurts. In this context, Bonyadi, Mojarrad Khangah, Qanbarov, Gojezadeh and Dalili Oskuee (26) reported *Lactobacillus delbrueckii* and *Lactobacillus plantarum* as the predominant flora in traditional yogurts from rural areas in East Azerbaijan province, Iran. Jafari, Shariatifar, Khaniki and Abdollahi (27) reported *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactococcus*

lactis subsp. *lactis*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus casei* in traditional yogurt in Fars province, Iran. Hajimohammadi Farimani, Habibi Najafi, Fazly Bazzaz, Edalatian, Bahrami, Flórez, et al. (28) isolated *Streptococcus thermophilus* (34) and *Lactobacillus delbrueckii* (the subsp. *bulgaricus* and *lactis*) from Iranian traditional yogurt from different areas of Khorasan-e-Razavi region, Iran.

1.11. Non-pathogenic properties of LAB isolates

One of the most important criteria in the selection of starter cultures for industrial fermentation is high technological potential and safety (lack of pathogenicity). Factors indicating bacterial pathogenicity include antibiotic resistance, DNase, decarboxylase, gelatinase and hemolytic activities. The enzyme DNase hydrolyzes the DNA molecules of organisms (29). In addition, LAB can produce biogenic amines in fermented foods with decarboxylation activity. Consumption of biogenic amines can cause headaches, toxic effects on humans, diarrhea, palpitations, and vomiting (3). Gelatin, one of the most important components of vertebrate connective tissue, is degraded by gelatinases. Gelatinases are extracellular metalloendopeptidases that break down gelatin into polypeptide compounds and then into amino acids (mainly alpha types). In addition to gelatine, gelatinases are also able to hydrolyze fibrinogen, casein, and collagen. Microorganisms used in the fermentation of food should be free of gelatinases (4). Table 4 shows the results of the investigation of the pathogenic and safety properties of strains, including antibiotic resistance, hemolytic, DNase, gelatinase, ornithine decarboxylase, and lysine decarboxylase activities.

Table 4. Pathogenic properties of LAB isolates

Species	Ornithine decarboxylase	Lysine decarboxylase	DNase activity	Antibiotic resistance (diameter (mm) of inhibition zone of microbial growth)	Hemolytic activity	Gelatinase activity
***L. bulgaricus* (HL1)**	Negative	Negative	Negative	Negative 5±0.4	Negative	Negative
***L. bulgaricus* (HL2)**	Negative	Negative	Negative	Negative 4±0.1	Negative	Negative
***L. bulgaricus* (HL3)**	Negative	Negative	Negative	Ampicillin	Negative	Negative
***L. bulgaricus* (HL7)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***L. bulgaricus* (HL9)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***L. bulgaricus* (HL10)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***S. thermophilus* (HS1)**	Positive	Positive	Negative	Negative 7±0.1	Negative	Negative
***S. thermophilus* (HS2)**	Positive	Positive	Negative	Negative 8±0.2	Negative	Negative

According to our results, all strains were free of hemolytic, DNase and gelatinase activities. However, regarding ornithine decarboxylase and lysine decarboxylase activities, only Streptococcus spp. was positive. In addition, *L. bulgaricus* (HL7),

L. bulgaricus (HL9) and *L. bulgaricus* (HL10) were resistant to vancomycin, while *L. bulgaricus* (HL3) was resistant to ampicillin. Several studies have reported the resistance of Lactobacillus to antibiotics, especially vancomycin, and the decarboxylase activity of LAB. Bernardeau, Vernoux, Henri-Dubernet and Guéguen (30) reported Lactobacillus species with decarboxylase activity and resistance to antibiotics, especially vancomycin. Perin, Miranda, Todorov, de Melo Franco and Nero (31) reported positive antibiotic resistance, DNase, decarboxylase, gelatinase, and hemolytic activities in many LAB strains in goat milk. Domingos-Lopes, Stanton, Ross, Dapkevicius and Silva (32) showed that most LAB strains of artisanal Pico cheese reacted positively to resistance to aminoglycoside antibiotics and nalidixic acid, while they reacted negatively to histamine and DNase production. They also reported strains with gelatinase and alpha-hemolytic activities. Omafuvbe and Enyioha (33) reported Lactobacillus species including *L. acidophilus*, *L. fermentum*, and *L. casei* with amino acid decarboxylase activity that can produce ornithine, lysine, and tyrosine amines. Therefore, in the present study, most LAB strains isolated from Hamadan yogurt were safe compared to other studies that have investigated the presence of unsafe LAB strains in various dairy products around the world.

6. Conclusion

The presence of unsafe indigenous LAB strains in local dairy products is due to the diversity of the genome of the microbial flora in these products. Local dairy products from different regions have inherent bacterial strains that are specific to the geographical area and may differ from other similar species in terms of pathogenic parameters. Based on our study results and similar research, the probable presence of autolytic Streptococcus spp. in local yogurt from western Iran is suggested. The results of phenotypic identification were approximately consistent with molecular identification. However, there may be unrecovered LAB spp. in Hamadan yogurt that were damaged due to environmental stressors and cannot be cultured. When investigating the non-pathogenic of LAB isolated from Hamadan yogurt, *L. bulgaricus* (HL1, HL2) are recommended as safe starter cultures for industrial yogurt production.

Acknowledgments: We gratefully appreciate the cooperation of all the current study participants.

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مقاله علمی_پژوهشی

شناسایی فنوتیپی و مولکولی باکتری‌های اسید لاکتیک غیربیماری‌زای جدا شده از ماست گوسفندی محلی

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چکیده

با افزایش تمایل مردم به مصرف محصولات لبنی ارگانیک، همواره خطر سویه‌های میکروبی بیماری‌زا در محصولات محلی وجود دارد. ماست گوسفندی محلی به عنوان یک محصول ارگانیک از باارزش‌ترین محصولات لبنی در ایران است. هدف از این مطالعه تشخیص فنوتیپی و مولکولی باکتری‌های اسید لاکتیک جدا شده از ماست گوسفندی همدان و بررسی خواص بیماری‌زای آن‌ها است. جدایه‌های لاکتیکی از نمونه‌های ماست از نظر فنوتیپی و مولکولی با تکثیر ناحیه 16S rDNA توسط واکنش PCR و به دنبال آن توالی‌یابی شناسایی شدند. خصوصیات بیماریزایی و ایمنی جدایه‌ها شامل مقاومت به آنتی‌بیوتیک، همولیز سلول‌های خونی، فعالیت‌های آمینواسید دکربوکسیلازی، DNase، و ژلاتینازی بررسی شدند. ۴۷ باکتری از نمونه‌های ماست جدا شدند و تنها ۲۲ جدایه گرم مثبت و کاتالاز منفی بودند. براساس نتایج، جدایه‌ها از نظر مولکولی به عنوان ۴ *لاکتوباسیلوس هلوتیکوس*، ۵ *انتروکوکوس موری*، ۲ *استرپتوکوکوس سالیواریوس* زیرگونه *ترموفیلوس*، ۵ *لاکتوباسیلوس هلوتیکوس* و ۶ *لاکتوباسیلوس دلبروکی* زیرگونه *بولگاریکوس* تشخیص داده شدند. شناسایی فنوتیپی همچنین حضور جدایه‌هایی که متعلق به تولید ماست بودند را تایید کرد. ارزیابی خواص بیماری‌زایی گونه‌های مرتبط با تولید ماست، *استرپتوکوکوس سالیواریوس زیرگونه ترموفیلوس* و *لاکتوباسیلوس دلبروکی زیرگونه بولگاریکوس*، تایید کرد که تنها دو جدایه از *لاکتوباسیلوس دلبروکی زیرگونه بولگاریکوس* (HL1, HL2) به عنوان کشت آغازگر برای تولید ماست ایمن هستند.

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Scientific Research

Antioxidant activity study and GC-MS Profiling of Bamboo Seed Variety

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ABSTRACT

Extensive research has been conducted on the antioxidant properties of bamboo seed extract, particularly from the Bamboo (*subfamily Bambusoideae*) Linn plant. Due to their high antioxidant content, bamboo seeds have garnered interest for their potential health benefits in combating age-related chronic illnesses such as diabetes, cancer, Alzheimer's, Parkinson's, and cardiovascular disease. The consumption of bamboo-derived products on a daily basis may play a role in reducing the risk of developing these conditions. Antioxidants play a crucial role in the food and pharmaceutical industries, as they help counteract free radicals that can degrade products during processing and storage. The chemical components of the ethanol extract of Bamboo seed variety were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS), identifying twenty-four compounds. These included compounds such as 9,12-Octadecadienoic acid (Z,Z)-, Hexadecanoic acid ethyl ester, and Tetradecanoic acid -12-methyl- methyl ester, which were found in both *Bambusa arundinacea* and *Dentrocalamus strictus*. Furthermore, Linoleic acid ethyl ester and Octadecanoic acid were predominant in *Bambusa arundinacea*, while (E)-9-Octadecenoic acid ethyl ester, n-Hexadecanoicacid, Oleic Acid, and 12-Methyl-E,E-2,13-octadecadien-1-ol were found in *Dentrocalamus strictus* only. Other phytonutrients were also present in bamboo seed varieties, indicating their potential use in the treatment of various diseases as antioxidants and as a viable rice substitute.

1- Introduction

Grass plants classified as bamboos belong to the Bambusoideae subfamily and Poaceae family. They are predominantly found in tropical and subtropical regions worldwide and are known for their high sustainability [1,2]. Globally, there are around 121 genera and approximately 1662 species of bamboo [1], with about 31.5 million hectares of bamboo plantations [3]. The highest diversity of bamboo species is concentrated in Asia, closely followed by South America and Africa [1,4]. Brazil boasts a vast natural bamboo collection, with over 200 species spread across 180,000 km^2 [4,5].

Due to its versatility across various fields, bamboo cultivation has gained international acclaim [6]. For instance, bamboo can be utilized in furniture production by flattening culms into boards [7] and in the construction sector, where leaves and ashes can be used as a supplement to cement [5]. Moreover, bamboo finds applications in the pharmaceutical industry, food sector [8], handicrafts [9], cycling frames, and medicines.

Notably, bamboo fibers are marketed as Jelucel® BF, Nutriloid® Bamboo Fiber, and CreaFibe in various countries [4]. Additionally, the Ministry of Health in the People's Republic of China highlights the use of bamboo as an additive in food and pharmaceutical products [10].

Bamboo leaves are rich in protein, calcium, iron, and magnesium, while being low in thein and caffeine. They have been employed in fortifying different food items biologically to enhance their nutritional content, reduce harmful acrylamide formed during processing, or extend shelf-life and enhance flavor [6,11–13]. These leaves can serve as food additives, flavorings, and preservatives in various food categories.

The potential of bamboo has attracted interest from the food, nutraceutical, cosmeceutical, and pharmaceutical industries. This paper aims to explore the antioxidant properties of bamboo and conduct a Fatty acid analysis of a bamboo seed variety isolated from the ethanolic extract using Gas Chromatography (GC) with Mass Spectrometry (MS).

2.Materials and Methods

1.1.Plant specimen and collection

Bamboo seed is an underutilized species in India. The bamboo seed varieties of (*Bambusa arundinacea* and *Dentrocalamus strictus*) grown in Kerala were obtained from the Department of Forest Service in Salem, ground into flour, and used for analysis. The analytical grade chemicals and solvents used in this study were sourced from Sigma Aldrich and E. Merck in Germany.

1.2.Preparation of plant materials

The seeds were carefully dried in a controlled environment to prevent direct sunlight exposure, cleaned, and manually winnowed to eliminate dust and other foreign particles before being used for additional analysis. The selected varieties of bamboo paddy were authenticated by Prof. P. Jayaraman, Director of the Institute of Herbal Botany, Plant Anatomy Research Center, in Chennai, Tamil Nadu.

1.3.Antioxidant Activity evalution

1.3.1. 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity (DPPH)

The antioxidant activity of the bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-K)

was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of [14]. The sample extracts at various concentrations (20 - 100 µg) was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as:

% DPPH radical scavenging activity = (Control OD-Sample OD / Control OD) × 100

1.3.2. Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was used to estimate the reducing capacity of the sample [15]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ [2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine] solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3$. $6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37 °C. 900 µl FRAP reagent was mixed with 90 µl water and 10 µl of the extract of bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-K). The reaction mixture was incubated at 37 °C for 30 minutes and the absorbance was measured at 593 nm.

1.3.3. Metal Chelating Activity

The chelating of ferrous ions by the extract of bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-K) was estimated [16]. Briefly, 50 µl of 2 mM $FeCl_2$ was added to sample extracts. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The results were expressed as EDTA equivalent.

1.3.4. Hydroxyl Radical Scavenging Activity

The scavenging activity of the extract of bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-K) on hydroxyl radical was measured according to the method of Klein [17]. Different concentrations of the extract (20 - 100 µg) were added with 1ml of iron-EDTA solution (0.13 % ferrous ammonium sulfate and 0.26 % EDTA), 0.5 ml of EDTA solution (0.018 %), and 1ml of Dimethyl sulfoxide (DMSO) (0.85 % v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22 %) and incubated at 80-90 °C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1ml of ice-cold TCA (17.5 % w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The hydroxyl radical scavenging activity (%) (HRSA) was calculated as

%HRSA (%) = (Control OD-Sample OD / Control OD) × 100

1.3.5. Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging activity of the extract of bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-k) was measured according to the method of Sreejayan and Rao [18]. 3 ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered

saline (pH 7.4) was mixed with different concentrations (20-100 µg) of solvent extracts and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent (1 % sulfanilamide, 0.1 % naphthylethylene diamine dihydrochloride in 2 % H_3PO_4) was added. The absorbance of the chromophore formed was read at 546 nm. Percentage of nitric oxide radical scavenging activity of the sample was calculated as

Nitric oxide radical scavenging activity (%) = (Control OD-Sample OD / Control OD) x 100

1.3.6. Superoxide Radical Scavenging Activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich [19]. The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (20 – 100 µg) of bamboo kernel flour (Ba-T, Ba-K, Ds-T and Ds-K) extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as

Inhibition (%) = (Control OD-Sample OD / Control OD) x 100

1.4.Gas chromatography-Mass spectroscopy condition

The total of bamboo seed varieties of *Bambusa arundinacea* and *Dentrocalamus strictus* grown in Tamil Nadu and kerala was analyzed by GC/MS. Preparation of extract: 2 µl of the ethanolic extract of bamboo seed was employed for GC/MS analysis. Instruments and chromatographic conditions: GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-5MS (5% Diphenyl /95% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 2µl was employed, injector temperature 250°.

The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C/min, then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The name, molecular weight and structure of the components of the test materials were ascertained [20].

3.RESULTS AND DISCUSSION

1.5.Gas chromatography-Mass spectroscopy

The present investigation was carried out to determine the components present in ethanolic extracts of bamboo seed varieties by GC-MS. The analysis gave the GC-MS Chromatogram which indicated a mixture of compounds with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the ethanol

extract of bamboo seed variety are presented in Fig. 1.

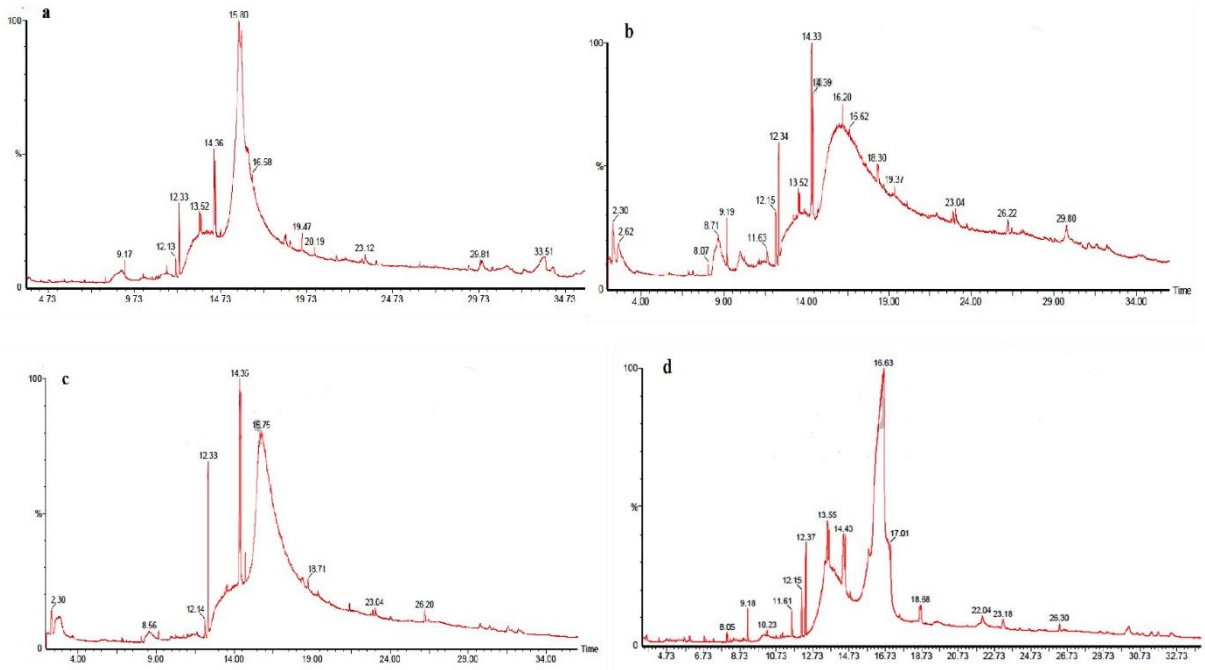

Fig. 1. (a-d): GC-MS chromatogram of Ba-T, Ba-K, Ds-T and Ds-K kernel flour

Table1. Activity of the compound

Name of the compound	Activity
Lactose	Preservative
à-D-Glucopyranoside,O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	Preservative
Tetradecanoic acid, 12-methyl-, methyl ester	Antioxident, cancer preventive, cosmentic, hypocholesterolemic, lubricant and nematicide.
Hexadecanoic acid, ethyl ester	Antioxident, hypocholesterolemic, nematicide, pesticide, anti androgenicflavor, hemolytic and 5-alpha reductase inhibitor.
Linoleic acid ethyl ester	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepato protective, nematicide, insectifuge, antihistaminic, antieczemic antiacne, 5-alpha reductase inhibitor, antiandrogenic,antiarthritic,anticoronary and insectifuge.
Octadecanoic acid	Antibacterial
9,12-Octadecadienoic acid (Z,Z)-	Antiinflammatory, hypocholesterolemic, cancer preventive, nematicide, insectifuge, antihistaminic, antieczemic, antiacne.
(E)-9-Octadecenoic acid ethyl ester	Raises VLDL and Lowers HDL Cholesterol

n-Hexadecanoic acid	Antioxidant, Hypochloesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Haemolytic, 5-Alpha reductase inhibitor
12-Methyl-E,E-2,13-octadecadien-1-ol	Antibacterial
Oleic Acid	Cancer preventive, Anemiagenic, Insectifuge, Antiandrogenic, Dermatitigenic.
[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	Hemolytic, pesticide, Skin irritant, hypocholesterolemic
7-Methyl-Z-tetradecen-1-ol acetate	Anti-cancer, antiinflammatory, hepatoprotective
Z-10-Tetradecen-1-ol acetate	Unsaturated alcoholic compound
Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	Antibacterial
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	No activity
Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy	No activity
Dodecane, 2,6,10-trimethyl-	Antioxidant, Antibacterial, COX-1
Hexadecane	No activity
Heptadecane	No activity
Squalene	Antioxidant, Antitumor
2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-	Antimicrobial
Cholestan-3-ol, 2-methylene-, (3á,5à)-	Antimicrobial Antiinflammatory Anticancer ,Diuretic Antiasthma Antiarthritic
á-Sitosterol	Antimicrobial, Anticancer, Antiarthritic, Antiasthma Diuretic, Anti-inflammatory

The peak of the chromatogram (75.82, 77.58, 86.56 and 71.86%), the major component was identified as 9,12-Octadecanoic acid (Z,Z) in *Bambusa arundinacea* grown in Tamil Nadu and kerala, *Dentrocalamus strictus* grown in Tamil Nadu and Kerala with molecular 280. Similar results were observed in leaves of *Cleome chelidonii (L.) Linn var. (CC)* Synonyms / other Latin name are Polanisia chelidonii DC, [family: Capparaceae] most places throughout the India and Tropical and warm temperate regions. It is grown as perennials throughout dry seasons and it contain the Compounds like 9,12,15-Octadecadienoic acid (Z,Z,Z)-, methyl ester (20.61%), 9,12-Octadecenoic acid (Z,Z)-, methyl ester (3.10%), n – decanoic acid (0.69%), Hexadecanoic acid and Squalene (0.55%). More than 35 compounds have been identified (Parimalakrishnan *et al*., 2015). Followed by Octadecanoic acid, n-

Hexadecanoic acid, Linoleic acid ethyl ester,(E)-9-Octadecenoicacid ethyl ester, Hexadecanoic acid ethyl ester, 12-Methyl-E,E-2,13-octadecadien-1-ol and Tetradecanoic acid, 12-methyl-,ester and Oleic acid were present in bamboo seed varieties with different peak levels and molecular weight and these fatty acids are play the varies beneficial roles to human beings and it was explained in Table 1. The similar results were also observed in cereals of wheat, rye and barley also contain Linoleic acid, oleic acid and palmitic acid studied by Zhou *et al*.,(1996) [21] reported that the major fatty acids (FA) in spring and winter wheat cultivars shows the presence of linoleic, palmitic and oleic acids, whereas α-linolenic and stearic acids are minor components and According to Kanimozhi and Ratha Bai (2012) reported that this analysis revealed that ethanolic extract of *Coriandrum sativum* contains 9-Octadecenoic Acid (Z)- ethyl ester(56.68%), Linoleic Acid ethyl ester(13.64%), Ethyl Hexadecanoate(7.69%), Alpha.-Monoolein(6.66%) in a high percentage [22]. The most prevailing compound 9-Octadecenoic Acid (Z)- ethyl ester (ethyl oleate) used as a solvent for pharmaceutical drug reparation, it acts as a drug for intramuscular drug delivery, in some cases to prepare the daily doses of progesterone in support of pregnancy and Cristina Botinești*ean et al*., (2012) observed that the tomato seed oil by Gas Chromatography combined with Mass Spectrometry [23]. Tomato seed oil that was used for analysis has been obtained by cold pressed extraction method. It is known that individual fatty acids can be identified by GC because of their different retention times, the samples of tomato seeds oil were testifier to bring them into a vaporous phase, transforming the fatty acid from tomato seed oil into fatty acids methyl esters. The results showed that the major component of tomato seed oil was linoleic acid (48,2%), followed by palmitic acid (17.18%) and oleic acid (9.2%), all the fatty acids were expressed in methyl esters. It can be concluded that tomato seed oil is an excellent source of essential fatty acids omega-6 (linoleic acid) and omega-9 (oleic acid). The other component like sugars, Esters, Acetate, Alkanes and Aldehyde are also identified with different peaks levels in bamboo seed varieties and it was act as Antibacterial, Preservative and hypocholesterolemia effect. Lin *et al.*, (2010) stated that the most abundant aldehyde in indica and japonica rice varieties was hexanal and nonanal; ketone compound was 6-10,14 trimethyl 2 penta decanone [24]. Among identified phytochemicals, Dodecane, 2,6,10-trimethyl- and Squalene were possessing antioxidant Activity. Recently it has been found that Squalene possesses chemo-preventive activity against the colon carcinogenesis [25]. Cholestan-3-ol, 2-methylene-, (3á,5à)- and á-Sitosterol are antiarthritic and anticancer agents. The geographical location and agronomical characters are the major reason for present or absent of above-mentioned components within the variety. Bajwa et al.,(2021) studied major phenolic compounds identified in shoots are ferulic acid, p-coumaric acid, caffeic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, syringic acid, and chlorogenic acid while major sterols are β-sitosterol, campesterol, stigmasterol, cholesterol, ergosterol, and stigmastanol [26]. According to Upadhyay *et al*., (2010) studied the phytochemical analysis of *lawsonia inermis*. L leaf and

revealed that color, number and leaf size showed morphological variation in the *Lawsonia inermis* populations; these are strongly influenced by environmental factors [27]. Morphological variation is apparently the result of an adaptive response to the environment; for example, variation in growth traits and phonological traits is associated with a latitudinal and altitudinal range or by contrasting climatic conditions. The observed trend of morphological variation made mention of adaptation to the contrasting micro-edaphic conditions prevailing for these groups and this was supported by the significant correlation with soil physicochemical characteristics. The greater discrimination power of adaptation micro edaphic conditions compared to the geographical regions of origin of accession in this study clearly indicated the greater importance of environmental factors (soil texture, soil chemical characteristics, and annual rainfall) than geographical location, in discriminating populations.

1.6.Antioxidant Profile

Antioxidants are the substances that inhibit oxidation. Cells in the human body may function poorly or die if oxidation occurs. To prevent free radical damage, body has a defense system of antioxidants. Antioxidants are the molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged [28]. Antioxidants can react with free radicals during the oxidation process by acting as a reactive species, scavenger and liberating catalysts, so antioxidants can be used to reduce the oxidative process but they are not 100 % effective [29]. Bamboo has been used over centuries by the humans both in daily life and for medicinal purpose in China and other Asian countries. The earliest scientific evidence for use of bamboo in traditional medicine dates back to 1963 [30]. This marked the beginning of the use of bamboo as medicine which was followed by series of research carried out by different workers [31–37]. *B. arundinacea* possess several characteristic identifying features in their pharmacognostical as well as physicochemical profiles. Phytochemical analysis exhibited vital information regarding the bio-constitutents present in the seeds which implies their possible therapeutic potential [38]. The bioactivities of some constituents present in the seeds can ensure a likely utilization of the seeds in the manufacturing of medicines in future. There was no significant investigation on antioxidant activity of bamboo paddy and its byproducts. Hence the antioxidant activity profile of dehusked bamboo kernel flour of two bamboo varieties was studied through DPPH radical scavenging activity, FT-IR assay, hydroxyl radical scavenging activity, nitric oxide radical scavenging activity, superoxide radical scavenging activity and metal chelating ability.

1.6.1. DPPH Radical Scavenging Activity

The DPPH free radical method has been used extensively to evaluate reducing substances, based on the reduction of ethanolic DPPH solution in the presence of a proton donating substance, resulting in the formation of diamagnetic molecules [39]. DPPH is commercially available nitrogen centered stable free radical which is destroyed by a free radical scavenger. The method is based on the measurement of the loss of deep purple colour of DPPH after reaction with the test compound functioning as a proton radical scavenger or hydrogen donor [40]. The DPPH radical

scavenging activity of Ba-T, Ba-K, Ds-T and Ds-K kernel flour is depicted in Table 2 and Fig. 2 .

TABLE 2: ANTIOXIDANT AND RADICAL SCAVENGING ACTIVITY (IC_{50} VALUE) OF BAMBOO KERNEL FLOUR

Antioxidant Assay	Standards	Ba-T	Ba-K	Ds-T	Ds-K
DPPH IC_{50} (µg/ml) Std: Ascorbic acid	27.23±0.82*	46.04±0.06^{ax}*	79.62±1.83^{bx}*	55.45±2.39^{ay}*	52.57±0.04^{by}*
FRAP IC_{50} (µg/ml) Std: Ascorbic acid	39.11±0.18*	85.99±2.25^{ax}*	80.85±2.56^{ax}*	59.14±3.32^{ay}*	52.12±9.32^{ay}*
Hydroxyl radical scavenging IC_{50} (µg/ml) Std: Ascorbic acid	44.24±1.42*	56.16±0.00^{ax}*	52.02±0.04^{bx}*	64.08±0.00^{ay}*	59.72±0.61^{by}*
Nitric oxide radical scavenging activity(µg/ml) Std: Ascorbic acid	25.23±1.10*	25.15±4.60^{ax}	26.17±0.04^{ax}	35.48±0.00^{ay}*	31.77±0.00^{ay}*
Superoxide radical scavenging activity(µg/ml) Std: Ascorbic acid	34.16±0.09*	36.28±0.00^{ax}*	35.71±0.00^{bx}*	44.47±0.03^{ay}*	40.15±0.04^{by}*
Metal chelating ability (mg EDTA/g) Std: Ascorbic acid	45.23±0.01*	50.47±0.01^{ax}*	47.87±0.01^{bx}*	57.39±0.00^{ay}*	53.48±0.00^{by*}

Values are the average of three determinants. Different alphabets in superscript (a, b) indicates significant geographical difference between means at $p<0.05$ using LSD test; (x, y) indicates the significant varietal difference between the means at $p<0.05$ using LSD test; * indicates significant difference between the sample means and standard mean at $p<0.05$ using LSD test.

TABLE 3: DPPH RADICAL SCAVENGING ACTIVITY OF BAMBOO KERNEL FLOUR AT VARIOUS CONCENTRATIONS

Concentration(µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K
20	22.52±0.04	23.09± 1.78	16.09±0.00	20.08±0.04

40	42.45±1.41	42.45±1.41	29.11±0.08	34.15±0.04
60	72.21±7.05	72.19±7.03	55.18±18.50	70.24±0.04
80	78.78±0.70	78.78±0.70	70.35±0.04	73.36±0.04
100	89.80±5.88	89.80±5.88	79.50±0.04	82.52±0.00

Value indicates the percentage of inhibition of DPPH radical

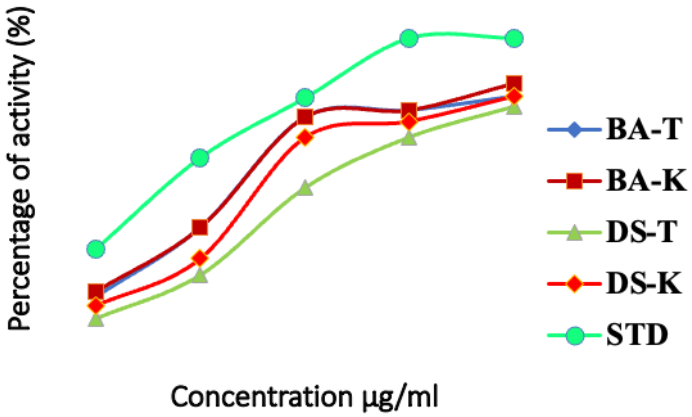

Fig. 2: DPPH activity in aqueous extract of Ba-T, Ba-K, Ds-T and Ds-K

DPPH radical scavenging activity was studied at 20-100 µg/ml concentration (Table 3 and Fig. 2) and the different concentration of aqueous extract of Ba-T (Y=0.814×+12.54), (Y=0.814×+12.49) and (Y=0.813×+12.50); Ba-K (Y=0.798×+13.84), (Y=0.798×+13.72) and (Y=0.799×+13.76); Ds-T (Y=0.840×+2.291), (Y=0.839×+2.261), and (Y=0.841×+5.673); Ds-K (Y=0.820 x+6.909), (Y=0.820 x+6.841) and (Y=0.821 x+6.769) (Fig. 2) was linearly correlated with superoxide radical scavenging activity. According to IC_{50} value of DPPH activity (Table 3), the significantly highest activity was ($p<0.05$) found in Ba-T and Ba-K when compared to Ds-T and Ds-K. Moko *et al* (2014) stated that the lower IC_{50} value indicated the strong capability of samples to catch free radical of DPPH [41]. The scavenging activity of bamboo kernel flour was significantly low when compared to standard ascorbic acid. Biswas *et al* (2011) observed the IC_{50} value in *Jaladhi 1* rice variety was at the range of 23.23 µg/ml that exhibit higher DPPH radical scavenging activity which was comparable to the present study of bamboo seed varieties [42]. The variation in the DPPH radical scavenging activity among bamboo seed varieties could be attributed to the variation in the composition and secondary metabolites of crops based on the genetic diversity and variations [43,44]. Different extracts of bamboo leaves proved to possess radical scavenging, metal chelating, ferric reducing and nitric oxide scavenging capability. The present study has verified the usefulness of bamboo leaves as effective antioxidant and a rich source of phenol and flavonoids [45].

Pujiarti *et al*., (2020) studied that higher antioxidant activity was shown by the 70% ethanol-soluble extract of *G. verticillata*, followed by D. asper, and the G. verticillata leaf extract exhibited effective DPPH radical-scavenging activity [46]. It contained high amounts of TPC and TFC.

1.6.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric iron ($Fe^{3+)}$ in the presence of antioxidants, which are reductants with half-reaction reduction potentials above Fe^{3+}/Fe^{2+} [47]. The FRAP activity of bamboo kernel flour was increased with concentration (Table 4 and Fig 3). The different concentration of aqueous extract of Ba-T (Y=0.521×+4.668), (Y=0.510×+5.064) and (Y=0.556×+3.916); Ba-K (Y=0.538×+4.866), (Y=0.63×+1.128) and (Y=0.589×+2.215); Ds-T (Y=0.743×+3.714), (Y=0.725×+6.061) and (Y=0.712×+11.16); Ds-K (Y=0.729x+6.239), (Y=0.643x+15.22) and (Y=0.554x+26.54) (Fig 3). The FRAP of Ba-T and Ba-K was significantly ($p<0.05$) low when compared to Ds-T and Ds-K kernel flour. The difference between varieties may be the difference in phenolic contents (polyphenolics and anthocyanin extracts) and/or electron-donating activity [48]. As per the IC_{50} value, the Ba-T and Ba-K showed high reducing capacity (50 %) of ferric ion at lowest concentration and it was significant at $p<0.05$. Table 4 explained that highest level of phenolics was observed in *Bambusa arundanacea* variety than *Dentrocalamus strictus* variety. The FRAP of bamboo kernel flour was significantly lower than the content ascorbic acid. Tundis *et al*., (2023) studied that bamboo leaves of phyllostacys edulis J.Houz has anti-inflammatory and antioxidant properties of BL and BS, corroborating their different potential applications in the nutraceutical, cosmetic and pharmaceutical industries [49].

TABLE 4 : FERRIC REDUCING ANTIOXIDANT POWER OF BAMBOO KERNEL FLOUR AT VARIOUS CONCENTRATION

Concentration (µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K
20	13.16±1.44	14.48±0.45	18.21±0.01	25.96±7.06
40	23.11±2.24	27.25±4.22	35.15±6.22	39.16±5.74
60	26.12±0.03	41.80±4.71	58.52±4.06	63.21±3.50
80	43.58±1.94	60.49±4.59	65.23±0.16	69.15±1.37
100	57.9±4.03	72.17±2.91	75.86±1.78	75.19±2.85

Value indicate the percentage of ferric reducing power

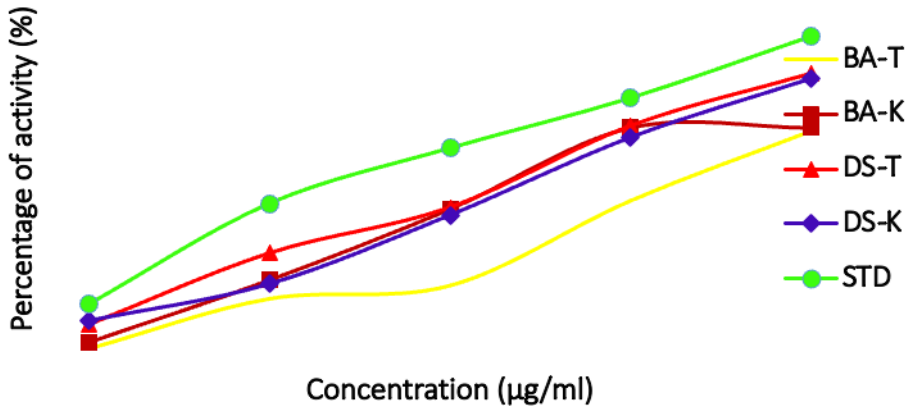

Fig. 3: FRAP activity in aqueous extract of Ba-T, Ba-K, Ds-T and Ds-K

1.6.3. Hydroxyl Radical Scavenging Activity

Hydroxyl radical is the most reactive free radical in the biological system and it has been regarded as the highly damaging to almost every molecule found in the biological system. It can conjugate with nucleotides in DNA and cause strand breakage which leads to ultimately mutagenesis, carcinogenesis and cytotoxicity [50]. The hydroxyl radical scavenging activity of bamboo kernel flour was gradually increased with increased concentration and the studied varieties of bamboo kernel flour were significantly ($p<0.05$) less effective than ascorbic acid in destroying the hydroxyl radicals (Table 5) and (Fig 4). The different concentration of aqueous extract of Ba-T (Y=0.599×+16.36), (Y=0.599×+16.36) and (Y=0.599×+16.36); Ba-K (Y=0.604×+18.56), (Y=0.604×+18.56) and (Y=0.605×+18.55); Ds-T (Y=0.610×+10.91), (Y=0.610×+10.91) and (Y=0.610×+10.910); Ds-K (Y=0.610x+13.78), (Y=0.610x+13.78) and (Y=0.610x+13.13) (Fig. 4). According to the IC_{50} value, the Ba-K showed highest inhibition activity at lowest concentration followed by Ba-T, Ds-K and Ds-T (Table 4.15). The IC_{50} value of non-germinated rice variety of superjami (48.57±0.71) reported by Im Chung (2016) was comparable to IC_{50} value of Ba-K and Ba-T. Colombo *et al.*, (2024) studied that rice (*Oryza sativa* L.) production and consumption is increasing worldwide and many efforts to decrease the substantial impact of its byproducts are needed. In fact, rice byproducts are rich in secondary metabolites (phenolic compounds, flavonoids, and tocopherols) with different types of bioactivity, mainly antioxidant, antimicrobial, antidiabetic, and anti-inflammatory, which make them useful as functional ingredients [51].

TABLE 5: HYDROXYL RADICAL SCAVENGING ACTIVITY OF BAMBOO KERNEL FLOUR AT VARIOUS CONCENTRATION

Concentration (µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K

20	31.16±0.00	33.25±0.00	26.11±0.00	29.18±0.00
40	39.27±0.00	42.36±0.00	34.22±0.00	37.26±0.04
60	50.34±0.00	52.45±0.00	45.32±0.00	47.40±0.00
80	60.44±0.00	62.57±0.00	55.43±0.00	58.51±0.00
100	80.55±0.00	83.64±0.00	76.54±0.00	79.60±0.00

Value indicates the percentage of hydroxyl radical scavenging ability

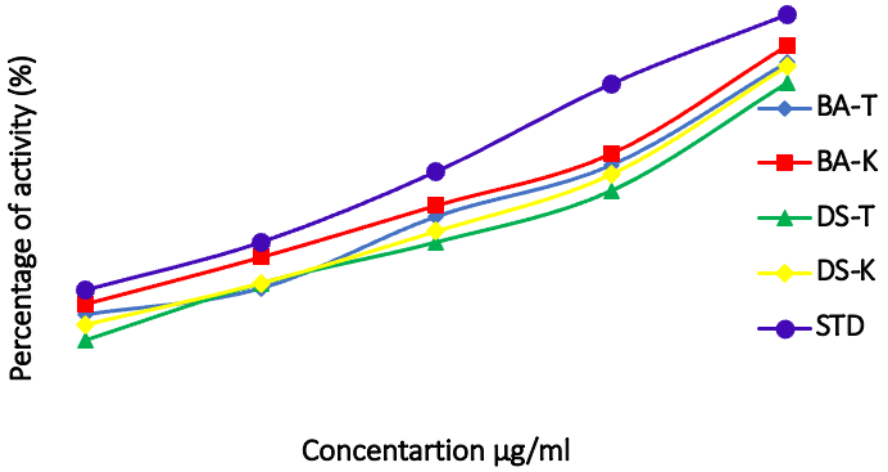

Fig.4: Hydroxyl radical scavenging activity in aqueous extract of Ba-T, Ba-K, Ds-T and Ds-K

1.6.4. Nitric Oxide Radical Scavenging Activity

Nitric oxide has also been involved in a variety of biological functions including neurotransmission, vascular homeostatic, antimicrobial and antitumor activities [18,52–57].

The nitric oxide radical scavenging activity was performed by comparing with ascorbic acid standard. The nitric acid radical scavenging activity was high in Ba-K (94.76±0.04) at 100 µl concentration followed by Ba-T, Ds-K and Ds-T (Table 6 and Fig. 5). The different concentration of aqueous extract of Ba-T (Y=0.714×+29.16), (Y=0.714×+29.15) and (Y=0.720×+31.17); Ba-K (Y=0.720×+31.17), (Y=0.720×+31.17) and (Y=0.720×+31.17); Ds-T (Y=0.720×+24.45), (Y=0.720×+24.45) and (Y=0.719×+24.48); Ds-K (Y=0.700X+27.76), (Y=0.700X+27.76) and (Y=0.7X+27.75) (Fig. 4). The IC_{50} value of bamboo kernel flour represented in Table 6 and reveals that *Bambusa arundanacea* variety exhibited strongest nitric oxide radical scavenging activity and equal to ascorbic acid standard, while *Dentrocalamus strictus* variety exhibited significantly low inhibition activity than Ba-K, Ba-T.

TABLE 6: NITRIC OXIDE RADICAL SCAVENGING ACTIVITY OF BAMBOO KERNEL FLOUR AT VARIOUS CONCENTRATION

Concentration (µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K

20	32.25±0.00	34.37±0.00	27.06±0.00	30.16±0.00
40	64.37±0.00	67.44±0.06	61.16±0.00	63.26±0.04
60	83.44±0.00	84.56±0.04	78.27±0.00	80.35±0.00
80	88.54±0.00	90.70±0.00	84.38±0.00	86.45±0.00
100	91.65±0.00	94.76±0.04	87.46±0.04	88.56±0.00

Value indicates the percentage of nitric oxide radical scavenging ability

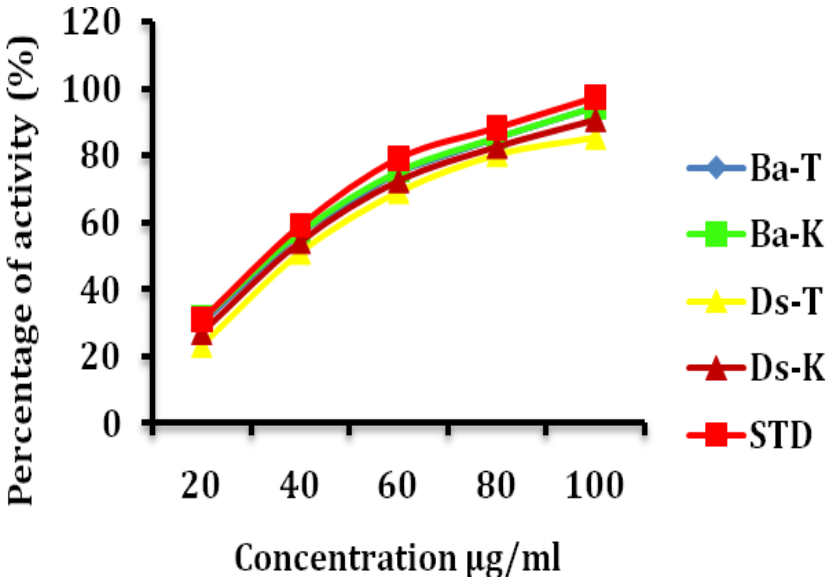

Fig. 5: Nitric oxide radical scavenging activity in aqueous extract of Ba-T, Ba-K, Ds-T and Ds-K

1.6.5. Superoxide Radical Scavenging Activity

The SOD-like activity measures the ability of the extract to catalyze the conversion of superoxide radicals into hydrogen peroxides, providing a defence mechanism against oxidative damage [58]. The superoxide radical scavenging activity of was increased significantly ($p<0.05$) (Table 7 and Fig. 6) as concentration of the extract increased.

TABLE 7: SUPEROXIDE RADICAL SCAVENGINGACTIVITY OF BAMBOO KERNEL FLOUR AT VARIOUS CONCENTRATION

Concentration (µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K
20	30.23±0.00	31.23±0.00	23.36±0.04	27.18±0.00
40	57.36±7.11	57.35±0.06	51.15±0.00	54.26±0.04
60	75.47±0.00	75.47±0.04	69.27±0.00	72.42±0.00
80	85.58±0.00	85.58±0.00	80.36±0.04	82.55±0.00
100	94.70±0.00	94.70±0.04	85.50±0.04	90.59±0.04

Value indicates the percentage of superoxide radical scavenging ability

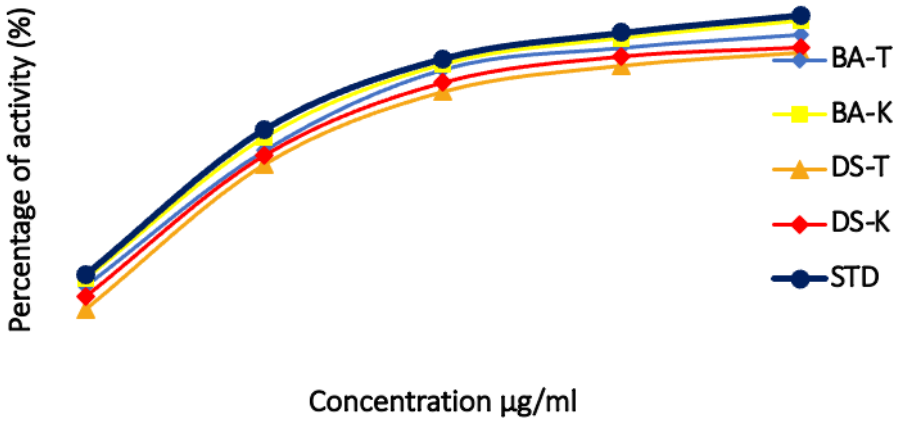

Fig. 6: Superoxide radicalscavenging activity in aqueous extract of Ba-T, Ba-k, Ds-T and Ds-K

The different concentration of aqueous extract of Ba-T (Y=0.785×+21.52), (Y=0.785×+21.52) and (Y=0.785×+21.52); Ba-K (Y=0.775×+22.32), (Y=0.775×+22.32) and (Y=0.775×+22.32);Ds-T (Y=0.767×+15.91), (Y=0.767×+15.91) and (Y=0.768×+15.83): Ds-K (Y=0.775x+18.86), (Y=0.775x+18.86) and (Y=0.775x+18.89) (Fig. 6) was linearly correlated with superoxide radical scavenging activity. The superoxide radical scavenging activity *Dentrocalamus strictus* variety was significantly ($p<0.05$) greater than *Bambusa arundinacea* variety. The superoxide radical scavenging activity of bamboo kernel flour was significantly ($p<0.05$) less than the ascorbic acid standard.

1.6.6. Metal Chelating Ability

Ferrozine can quantitatively form complexes with Fe^{2+} in the presence of chelating agents; the complex formation is disrupted resulting to decrease in red colour of the complex. Measurements of colour reduction make possible estimation of the metal chelating activity [59]. The result in Table 8 and Fig 7 f showed the interaction of bamboo kernel flour with iron. The different concentration of aqueous extract of Ba-T (Y=0.730×+13.17), (Y=0.730×+13.17) and (Y=0.730×+13.17); Ba-K (Y=0.704×+16.30), (Y=0.704×+16.30) and (Y=0.703×+16.33); Ds-T (Y=0.715×+8.956), (Y=0.715×+8.956) and (Y=0.715×+8.955); Ds-K (Y=0.723×+11.33), (Y=0.723×+11.33) and (Y=0.723×+11.33) was linearly correlated with metal ion chelating activity. The chelating ability of bamboo kernal flour was significantly ($p<0.05$) less than the ascorbic acid standard. The Ba-K (47.87±0.01 µg/ml) was found that significantly higher chelating activity with lowest concentration (Table 8).

TABLE 8: METAL CHELATING ACTIVITY OF BAMBOO KERNAL FLOUR AT VARIOUS CONCENTRATION

Concentration (µg/ml)	Ba-T	Ba-K	Ds-T	Ds-K
20	30.18±0.00	32.26±0.00	25.06±0.00	27.15±0.00
40	39.28±0.00	42.37±0.00	35.17±0.00	38.26±0.04
60	55.36±0.04	57.48±0.00	50.28±0.00	53.37±0.00
80	74.50±0.00	73.58±0.00	69.36±0.04	72.48±0.00
100	85.61±6.43	87.06±0.04	79.51±0.00	82.37±0.00

Value indicates the percentage of metal chelating ability

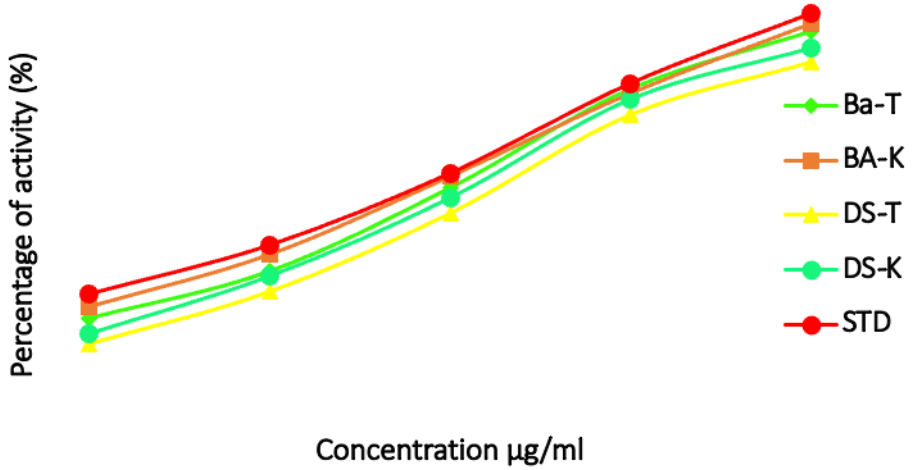

Fig. 7: Metal chelating ability in aqueous extract of Ba-T, Ba-K, Ds-T and Ds-K

CONCLUSION

In the current study, 24 compounds were identified in the ethanol extract of bamboo seed varieties through Gas Chromatography-Mass Spectrometry (GC-MS analysis). This analysis marks the initial step in comprehending the active principles present in these seeds and sets the stage for more comprehensive future studies. Further exploration into their pharmacological significance and diversity may expand our understanding of traditional medicinal practices. The DPPH activity's IC50 value revealed that Ba-T and Ba-K exhibited significantly higher activity ($p<0.05$) compared to Ds-T and Ds-K, while the scavenging activity of bamboo kernel flour was notably lower than that of the standard ascorbic acid. The FRAP values of Ba-T and Ba-K were significantly lower ($p<0.05$) than Ds-T and Ds-K kernel flour, possibly due to the higher phenolic levels in *Bambusa arundinacea* variety compared to *Dentrocalamus strictus* variety. In terms of the IC50 value for hydroxyl radical scavenging activity, Ba-K demonstrated the highest inhibition activity at the lowest concentration, followed by Ba-T, Ds-K, and Ds-T. Regarding the IC50 value for Nitric oxide radical scavenging activity, bamboo kernel flour of *Bambusa arundinacea* variety exhibited the strongest nitric oxide radical scavenging activity equivalent to the ascorbic acid standard, whereas *Dentrocalamus strictus* variety showed significantly lower inhibition activity than Ba-K, Ba-T, and ascorbic acid.

The superoxide radical scavenging activity of *Dentrocalamus strictus* variety was notably higher ($p<0.05$) than that of *Bambusa arundinacea* variety, while the superoxide radical scavenging activity of bamboo kernel flour was markedly lower ($p<0.05$) than the ascorbic acid standard. The chelating ability of bamboo kernel flour was significantly lower ($p<0.05$) than that of ascorbic acid standard, with Ba-K displaying the highest activity.

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مطالعه فعالیت آنتی اکسیدانی و پروفایل GC-MS واریته های مختلف دانه بامبو

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چکیده

تحقیقات گسترده ای در مورد خواص آنتی اکسیدانی عصاره دانه بامبو، به ویژه از گیاه بامبو انجام شده است. دانه‌های بامبو به دلیل محتوای آنتی‌اکسیدانی بالایی که دارند، برای مزایای سلامتی بالقوه‌شان در مبارزه با بیماری‌های مزمن مرتبط با افزایش سن مانند دیابت، سرطان، آلزایمر، پارکینسون و بیماری‌های قلبی عروقی مورد توجه قرار گرفته‌اند. مصرف محصولات مشتق شده از بامبو به صورت روزانه ممکن است در کاهش خطر ابتلا به این شرایط نقش داشته باشد. آنتی اکسیدان ها نقش مهمی در صنایع غذایی و دارویی ایفا می کنند، زیرا به مقابله با رادیکال های آزاد کمک می کنند که می توانند محصولات را در طول پردازش و ذخیره سازی تجزیه کنند. اجزای شیمیایی عصاره اتانولی دانه بامبو با استفاده از کروماتوگرافی گازی-طیف‌سنجی جرمی با شناسایی بیست و چهار ترکیب مورد تجزیه و تحلیل قرار گرفت. اینها شامل ترکیباتی مانند ۹،۱۲-اوکتادکادینوییک اسید (Z,Z)-، اتیل استر اسید هگزادکانوئیک و -۱۲-متیل متیل استر تترادکانوییک اسید بود که در *Bambusa arundinacea* و *Dentrocalamus strictus* یافت شد. علاوه بر این، اتیل استر اسید لینولئیک و اسید اکتادکانوئیک در *B. arundinacea* غالب بودند، در حالی که اتیل استر -9-(E)اوکتادکانوئیک اسید، n-هگزادکانوئیک اسید، اولئیک اسید، و ۱۲-E-متیل، -2،13-E اوکتادکا دی ان بودند. -ol فقط در *Dentrocalamus strictus* یافت شد. سایر مواد مغذی گیاهی نیز در انواع دانه های بامبو وجود داشت که نشان دهنده استفاده بالقوه آنها در درمان بیماری های مختلف به عنوان آنتی اکسیدان و به عنوان یک جایگزین مناسب برای سایر غلات خصوصا برنج است.

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Exploration Antioxidant Properties of Indonesian Local Single-Bulb Garlic Extract (Var. Temanggung) in a Mixed Solvent

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ABSTRACT

Single-bulb garlic is known for its high antioxidant activities. Single-bulb garlic (var. Temanggung) has a benefit potency, considering that Temanggung is one of Indonesia's most preeminent garlic cultivation areas. However, the quality of its antioxidant activities is yet to be explored widely. This research aims to carry out antioxidant characterization in single-bulb garlic (var. Temanggung) under the conditions of different solvent mixtures (ethanol and water). The Completely Randomized Design (CRD) was used with different ethanol-water mixture treatments (F1 = 100%:0%, F2 = 80%:20%, F3 = 60%;40%, F4 = 40%:60%, F5 = 20%:80%, and F6 = 0%:100% v/v). Extraction results were analyzed for total phenolic content, antioxidant activities using two methods (%RSA, IC_{50}, and FRAP), and chemical compound identification using GC-MS. The Results demonstrated that different solvent treatments produced antioxidant properties which also differed, either qualitatively or quantitatively. The best treatment was F2 (ethanol:water = 20%:80% v/v) at the highest antioxidant activity values (IC_{50} DPPH of 10.35 ppm and a FRAP value of 9.41 µM equivalent Fe(II)/g), and a TPC value of 61.02 mg GAE/g.

1. Intoduction

Four favorite synthetic antioxidants according to consumers, i.e., butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), propyl gallate (PG), and butylated hydroxytoluene (BHT), are also reportedly to come with adverse effects [1]. Flavonoids, polyphenols, and antioxidants are verified to exist in garlic [2]. Overall, studies have unveiled that we can find a high number of organic sulfur compounds in garlic, 33 in total, explaining where it gets its distinctive flavor and therapeutic properties [3]. Earlier research shows 1.3 g of ash and amino acid, 0.7 g of crude fiber, 4.4 g of protein, 0.2 g of fat, and 23 g of carbohydrates in 100 g of fresh garlic [4]; [5]; [6]. Currently, researchers have also found a substantial number of bioactive compounds in the essential oil of garlic, also adding that the oil has a high level of saponin ligands, phenolic compounds, essential amino acids, steroid saponins, and many different non-sulfur compounds, in addition to organic sulfides like diallyl disulfide (DADS) and DATS [7].

Additionally, garlic contains organosulfur components, two of which are S-allyl mercapto cysteine and S-allyl cysteine with a potent antioxidant activity [8]. Wilujeng & Anggarani [9] also report that imported garlic extract using ethanol contains lower antioxidant activity, total phenolics, and total flavonoid levels than local ones. Growing locations are closely associated with how secondary metabolites and antioxidant activity are produced as they impact plants' biochemical processes and environmental temperature, generating different levels of secondary metabolites, e.g., phenolic Growing environmental conditions influence the chemical compounds in garlic, including the quality and quantity of antioxidants. Temanggung, a district in Central Java, Indonesia, has a suitable agroclimatic requirement for garlic cultivation, giving the single-bulb garlic (var. Temanggung) distinguished antioxidant qualities. Temanggung is located in a highland area with mountains, these conditions affect the climate and soil. Temanggung has a cool climate, with volcanic soil and andosol. This type of soil is very suitable for growing garlic.

The garlic has also become a national superior garlic commodity with a productivity of 14 tons/hectare in 2023.

Isolating the garlic's antioxidant components is best carried out by extraction, the process of which requires the separation of the desired compound(s) from any interfering or undesired chemical mixture. We consider the step vital to obtain pure bioactive natural compounds for medical, scientific, and commercial applications. Meanwhile, while extracting, there are several factors which need to be emphasized in order to boost the efficiency, among them measuring the weight, measuring the volume, undertaking mixing, diluting, heating, cooling, fractionating, purifying, and preserving [10]. In addition, we also highlight another factor, namely the solvent used, when designing an extraction procedure. An effective solvent will contribute positively to the extracted compounds' selectivity, quantity, and quality.

Solvent extraction is the most common technique for the extract antioxidant compounds from plant [11]. Different polarities characterize different solvents and play a critical role in determining bioactive compounds' solubility and, in turn, the overall extraction efficiency [12]. In selecting an appropriate extraction solvent, we consider the chemical characteristics of the natural products extracted and the end product desired. There are two types of solvents typically used by people to extract natural products, which are polar solvents like acetone, ethanol, methanol, and water, and non-polar ones like ethyl acetate, chloroform, and hexane. They come with unique properties supportive of the extraction of compounds with a particular polarity [13].

The solvent mixture treatment was carried out to obtaining the best ethanol concentration ratio for extracting single-bulb garlic. The polarity of the solvent affects the type of antioxidant extracted. Water and ethanol both have polar properties, but ethanol has amphipathic properties (has polar and non-polar parts in its molecular structure). Water solvents are more relatively effective in extracting allicin than ethanol [14], but distilled water cannot extract antioxidant compounds which dissolve in polar solvents, e.g., DADS. Bajac et al. [15] convey that [16]ethanol provides better DADS extraction than distilled water. Accordingly, based on the background elaborated above, this research aims to describe antioxidant characterization in single-bulb garlic (var. Temanggung) under the conditions of different solvent mixtures (ethanol and water) .

The results of this research will provide information regarding the use of local Temanggung garlic either as a functional food, or as a natural antioxidant ingredient that can inhibit oxidative damage to food [17],[16], [18] . Garlic is widely used as an inhibitor of oxidative damage. The findings in this research will be important information for local farmers because they can provide the selling value of the single-bulb garlic var Temanggung.

2.Material and Method

2.1*Materials*

Tools employed was a blender (philips), sonicator (BRANSON 2510) , filter paper, UV-Vis spectrophotometry (Genesys 10S), GC-MS (Thermo Scientific Trace OQ301), and digital scale. Meanwhile, the materials included methanol, single-bulb garlic (var. Temanggung), distilled water, ethanol 96% , 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin, Folin Ciocalteu 50%, Na_2CO_3 20%, Gallic Acid, FeSO4.7H2O, 5% phenyl methylpolysiloxane, and Helium.

1.1 ***Research Population***

Single-bulb garlic (var. Temanggung) was obtained directly from local farmers in Temanggung, Central Java, Indonesia. Samples were uniform in size and age, but we only used garlic intact, uniform in size, not affected by diseases, smooth outer skin, not dry, not spotted, and not green in color. Samples were then tested for determination to decide the species of the samples used.

1.2 Experimental Design

A completely randomized design (CRD) with a mixture of distilled water and ethanol was applied, as demonstrated in **Table 1.** The results will be analyzed

using analysis of variance (ANOVA), if there are significant differences, Duncan's Multiple Range Test (DMRT) will be tested at a 95% confidence level.

1.3 Single-Bulb Garlic Extraction *[19]*

The garlic washed, crushed, and combined garlic cloves with the solvent in a 1:2 weight ratio according to the treatment. The garlic mixture into a sonicator for 15 minutes at room temperature and continued with the filtering process. The resulting supernatant was collected, decanted for 24 hours (refrigerator temperature), and kept in a sterile glass container as storage at 4°C for testing.

Table 1. Experimental Design Of Single-Bulb Garlic Extraction

Treatment	Mixed Solvent (%)	
	Water	Ethanol
F1	100	0
F2	20	80
F3	40	60
F4	60	40
F5	80	20
F6	0	100

1.4 Antioxidant Activity through DPPH and IC-50 Methods *[20]*

Blank antioxidant analysis was conducted by taking 4 mL of DPPH solution, vortexing it, and storing it in a place with minimum light at 37°C as the set temperature. Furthermore, absorbance was recorded at a wavelength of 517 nm. Meanwhile, 3.5 mL of DPPH was combined with 0.5 mL of the sample solution from each concentration to assess antioxidant activity. The combined solution was then vortexed and stored in a dark place at 37°C before measuring the absorbance at a wavelength of 517 nm.

$$\%SA = 1 - \frac{\text{sample absorbance}}{\text{DPPH absorbance}} \times 100\%$$

The percent inhibition was plotted on the y-axis. Meanwhile, the concentration of extract samples or the antioxidant quercetin was plotted on the x-axis to acquire the linear regression equation. This equation, represented as y = a + bx, was invoked to determine lC_{50} (50% inhibitor concentration) values for the respective samples by setting the y value to 50 as well as including the x value obtained from lC_{50}. lC_{50} values stated the concentrations of sample solutions which could reduce DPPH free radicals by 50%.

1.5 Total Phenolic Content (TPC) *[21]*

The Folin-Ciocalteu method was used to identify the total phenolic content (TPC) [21]. To begin with, a gallic acid solution was prepared to serve as a standard. The stock solution was prepared to create 100 ppm of a standard gallic acid solution, then diluted to 10, 20, 30, 40, and 50 ppm concentrations. 2 mL of each standard solution was combined with 5 mL of distilled water as well as 0.5 mL of 50% Folin Ciocalteu reagent and then vortexed. Next, they were incubated at 20-25°C for five minutes, followed by the addition of 1 mL of 20% Na2CO3 and incubation at room temperature for approximately 60 minutes. Absorbance was assessed at a

maximum wavelength of 784 nm. 0.5 mL of 50% Folin Ciocalteu reagent and 5 mL of distilled water were mixed with about 2 mL of solution, vortexed, and then incubated at 20-25°C for five minutes. The mixture was added 1 mL of 20% Na2CO3 and incubated for 60 minutes at room temperature. The extraction was performed three times. Absorbance was measured at 784 nm as the maximum wavelength. Total phenolic content (TPC) was reported as milligrams of Gallic Acid Equivalents (GAE)/g. The TPC data collected from the absorption values of the respective samples were plotted against a standard gallic acid curve and calculated with the following formula.

$$TPC = \frac{c \times V}{M}$$

TPC = total phenolic content

c = concentration from the calibration curve

V = volume of the extract

1.6 FRAP Analysis *[20]*

$FeSO_4.7H_2O$ stock solution of 10.000 µmol/L was made by dissolving 2.78 grams of $FeSO_4.7H_2O$ using 1.000 mL of distilled water and diluted to 1, 2, 3, 4, and 5 µmol/L concentrations. As much as 1 mL of the standard solutions was taken, combined with 3 mL of FRAP reagent, and measured at wavelengths between 588 and 598 nm with a UV-Vis spectrophotometer. 0.1 mL of the respective extract solutions was added 3 mL of FRAP reagent and placed into test tubes for absorbance measurement. Meanwhile, the solution absorption was recorded with a spectrophotometer at 596 nm.

1.7 Identifying Compounds in Optimum Garlic Extracts with Gas Chromatography-Mass Spectrometry (GC-MS) *[14]*

In identifying compounds in optimum garlic extracts, we used chromatography criteria (retention time) as well as spectrometry criteria (standard compounds, mass spectral interpretation, and comparison with library information). A gas chromatograph (Thermo Scientific Trace OQ301) was coupled with a DSQ mass spectrometer (electron impact ionization, eV 70; Thermo Scientific) to gather chromatographic and spectroscopic data. The DB-5MS (J & W Scientific) column was 30 meters in length and had a static phase of 5% phenyl methylpolysiloxane, a thickness of 0.25 µm, and an internal diameter of 0.52 mm. The GC-MS was programmed in the following modes: 50°C as the initial temperature for 1 minute, which was then increased by 10°C/minute until achieving a temperature of 280°C, with a hold at the latter for 15 minutes. The temperature of the injection port was regulated to 250°C, whereas transmission line's temperature was 280°C. The carrier gas was helium (99.99%), operating at 27 cm/min (constant current 1 ml/min) and a pressure of 155 KPa. Chromatograms and mass spectrometry were calibrated using mass scanning within 45-500 m/z at a rate of 5.1 scans per second.

3.Result and Discussion

1.8 Total Phenolic Content (TPC)

The organic molecules consisting of hydroxyl groups (-OH), phenolic compounds were attached to carbon atoms in aromatic rings. As antioxidants, phenolic compounds donated hydrogen atoms, reducing free radicals into a more stable form [22]. Determination of total phenolic content (TPC) was carried out with the Folin-Ciocalteu reagent using gallic acid as a standard. The principle of

the Folin-Ciocalteu method is that phenolic compounds are oxidized by the Folin-Ciocalteu reagent so that the test solution is blue which can be measured with a visible spectrophotometer at a wavelength of 750 nm. The total phenolic analysis method using the Folin-Ciocalteu reagent is a relatively reliable method and is often used in research and industrial applications, especially for the determination of total phenol in various types of food samples, but is less suitable when applied to pure samples [23] .

The single-bulb garlic extracts' total phenolic content (TPC) exhibited in **Table 2** was significantly influenced by different extraction treatments ($p > 0.05$). The highest TPC of 65.29 ± 0.4 GAE/g was obtained in the F5 treatment, while the lowest of 63.28 ± 0.2 (GAE/g) was acquired in the F1 treatment. Results indicated that TPC fluctuated in each treatment because the polarity of each different solvent produced different types of phenolic compounds extracted. Garlic had 20, and even above, phenolic compounds, and the number was exceeding that found in other vegetables [24]. It was defined that garlic from Tawangmangu had the highest TPC of 92.2 mg GAE/g. The primary phenolic compound was β-resorcylic acid, with other significant compounds including quercetin, protocatechuic acid, routine, gallic acid, and pyrogallol. Single-bulb garlic (var. Temanggung) had a relatively higher TPC level than that garlic grown in five regions in Korea had the highest TPC of 49.89 mg GAE/g [25].

The total phenol content in garlic extracts varied according to the cultivar, which could differ from one to another. Plants experiencing stress, also covering those which were exposed to various signal molecules or elicitors, would undergo the accumulation of secondary metabolites. Secondary metabolites were crucial for plant adaptation to their environment and coping with stressful conditions. Environmental conditions influenced antioxidant activity, flavonoids, and phenolics levels. A high-temperature stress in the environment would likely increase antioxidant activity, flavonoids, and phenolics levels generated [26].

A positive and highly significant relationship between total phenolics and antioxidant activity in plant products has been previously demonstrated. Miean & Mohamed [27]found relatively high concentrations of the myricetin, quercetin and apigenin flavonoids in garlic. The growing location and cultivation method influence the quality of garlic TPC. Garlic taken from 4 locations in Spain had a total phenolic content varying from 3.4 mg gallic acid equivalent (GAE)/g dry matter (dm) to 10.8 mg GAE/g dm [28] Meanwhile, local garlic from purbolinggo (Indonesia) has a total phenolic content of 28,756 mg GAE/g [29], This study had a higher TPC of 63.28 in the F2 treatment, higher than garlic from Spain, or from Purbalingga (Indonesia).

Local people consume single-bulb garlic var Temanggung to increase body endurance and reduce cholesterol levels. Functional food based on single-bulb garlic, apple vinegar, red ginger, honey and lemon can reduce triglyceride levels in hypercholesterolemic white mice ($p < 0.05$) [30]. However, the research did not explain in detail the variety of single-bulb garlic. Single-bulb garlic var Temangung has high antioxidants, so it is possible that the ability to reduce cholesterol levels in the body will also be higher.

3.2 **Antioxidant Activity (DPPH Method)**

The DPPH method could be applied for either liquid or solid samples but was not working specifically when deployed for certain antioxidant components. It measured the samples' total antioxidant capacity by identifying the hydrogen capture reaction by DPPH from antioxidant substances [31] [18], [32]. During an interaction between the free-radical DPPH and an odd electron, peak absorption was notable at 517 nm and indicated a purple color. A free-radical scavenger antioxidant reacted with DPPH to form DPPHH, which came with reduced absorbance compared to the first due to its less hydrogen content. If we compared it to the DPPH-H state, this radical form caused decolorization, engendering a yellow hue, when the electrons collected increased in number [33].

However, the DPPH method had some limitations. For instance, it could only dissolve in organic solvents, challenging us when we desired to analyze hydrophilic compounds. Metal ions, hydrogen, and water contributed to the mechanisms of the free radical process. The presence of these ions in samples with antioxidant potential was hence a crucial parameter of research. For instance, flavonoids had the capability of forming complexes with Cu (II) and Fe (III), which often demonstrated enhanced activity against free radicals and, therefore, had a reaction with DPPH stronger relative to compounds lacking metal ions [34] .

Table 2. Antioxidant And Total Phenol Levels Of Single-Bulb Garlic Extracts With Different Solvents

Treatment	TPC (GAE/g)	IC_{50}(ppm)	DPPH (%RSA)	FRAP (µMeq Fe(II)/g)
F1	63.28 ± 0.20 ^{b}	28.93 ± 7.54 ^{b}	91.39 ± 0.08 a	96.51 ± 2.49 c
F2	61.01 ± 0.17 ^{a}	10.3 ± 0.85 ^{a}	93.27 ± 0.01 b	94.14 ± 10.18 c
F3	63.98 ± 0.18 ^{c}	32.78 ± 0.01 ^{c}	93.96 ± 0.20 b	102.47 ± 2.99 c
F4	64.36 ± 0.26 ^{c}	107.78 ± 12.61 ^{c}	94.01 ± 1.21 b	79.40 ± 8.98 b
F5	65.29 ± 0.40 ^{d}	82.19 ±0.01 ^{d}	95.47 ± 0.47 c	44.14 ± 6.42 a
F6	62.88 ± 1.01 ^{b}	11.93 ± 2.12 ^{b}	96.21 ± 0.33 c	52.74 ± 1.39 a

*Different small letters in each row represent a significant difference at a 5% level.

$^{a, b, c, d}$Means with different superscripts within a row are significantly different ($p < 0.05$)

The highest antioxidant value namely 96.21 ± 0.33%RSA was obtained with the F6 treatment, while the lowest was 91.39 ± 0.08%RSA. The increase in TPC was directly proportional to increased antioxidant activity. Ethanol had groups of hydroxyls which were polar and groups of alkyls which were non-polar. It could dissolve all secondary metabolite compounds, in contrast to water, which had more polar properties. Extraction using water and extraction using ethanol resulted in different antioxidant results. Since DPPH was also dissolved in organic compounds, an ethanol solvent was considered more suitable. Various types of garlic and specifically addressing their antioxidant activity, the garlic extract with the highest antioxidant results was single-bulb garlic instead of imported [35]. The stability of antioxidants was not examined in this research, but in general antioxidants have sensitive properties and are easily damaged due to several things, including

oxygen, light, temperature and storage methods. Because of this, to increase antioxidant stability, a multilayer encapsulation will be made. Encapsulation will protect antioxidants from damage caused by environmental conditions.

3.3 ***IC_{50}***

IC_{50} values and antioxidant activity were correlated inversely, where lower IC_{50} values indicated a higher antioxidant activity [36]. A low IC_{50} value indicated a better antioxidant quality. The classification of a compound based on its IC_{50} value was as follows: weak (an IC_{50} value of above 150 ppm), moderate (an IC_{50} value of 101-150 ppm), strong (an IC_{50} value of 50-100 ppm), and very strong (an IC_{50}value of less than 50 ppm). The F2 treatment was the best as the IC_{50} value was the lowest, which was 10.3 ± 0.85 ppm. (**Table 2**). The treatment also had a strong antioxidant quality because its IC_{50} value was less than 50 ppm that was higher than the control quercetin, i.e., 3.9 ppm. Single-bulb garlic had an IC_{50} value of 10.61 mg/ml, while the local variety Ciwidey was 13.61 mg/ml [37].

Table 3 shows that the IC_{50} value in this study is better than the findings from garlic extracts from Porbolinggo (Indonesia) and West Sumatra (Indonesia), and close to the results obtained from garlic from Spain and Bangladesh. The IC50 value of garlic from Uganda and Portugal is smaller than in this study, namely less than 5 ppm, but it is still classified as a strong antioxidant.

Figure 1 shows that TPC was directly proportional to antioxidant levels tested using the DPPH method. It was because DPPH reacted in two ways, namely by hydrogen atom donor and electron donor mechanisms, where antioxidant compounds would provide hydrogen atoms or electron pairs to DPPH which was a radical. It would reduce the presence of free radicals in the sample [38].

The inhibitory potential of extracts was influenced by the position and number of the groups of hydroxyls, as well as the phenolic compounds' molecular weight. Phenolic compounds were identified as showing more efficient actions when being hydrogen donors. Accordingly, they functioned as antioxidants with effective characteristics. Besides, their inhibitory effect on DPPH increased when the concentration or degree of hydroxylation enhanced. Phenolic compounds as antioxidants could stabilize free radicals by releasing hydrogen atoms through electron transfer mechanisms, transforming phenol into phenoxyl radicals. Through resonance effects, phenoxyl radicals could undergo stabilization. This property made derivatives of phenol effective hydrogen donors in inhibiting reactions caused by radical compounds. The scavenging activity of phenolic compounds against free radicals was affected by the quantity of phenolic hydrogen positioned within their molecular structure. The higher the hydroxyl groups in phenolic compounds, the greater the antioxidant activity produced [39]. Using alcohols, including ethanol or methanol, contributed to the extracts' acquisition of strong antioxidant activity on account of the phenolic compounds' presence [40].

Table 3 IC_{50} values for various types of garlic in previous studies

Varieties	IC_{50}	Solvent	
Local Variety Garlic Of Bangladesh	7.8±0.8 µg/mL	ethanol	[41]
Local Garlic From Paimban, (Indonesia)	671.7395 µg/mL	ethanol	[42]

Local Variety Garlic Of Uganda	4.01 mg/mL	ethanol	[43]
Local Variety Garlic Of Uganda	5.64 mg/mL	water	[43]
Local Garlic From Probolinggo (Indonesia)	257,75 µg/mL	water	[29]
Local Variety Garlic Of Portugal	4.88 µg/mL	water	[44]
Garlic The Polish 'Harnaś' Cultivars (Spain)	6.52 µg/mL	water	[44]
Garlic 'Castano' (Spain)	7.59 µg/mL	water	[44]

3.4 Antioxidant Activity (FRAP Method)

A simple, fast, and efficient method for testing antioxidants, Ferric Reducing Antioxidant Power (FRAP) analysis required no special measurement equipment. However, the reagent in the FRAP test method was less stable, therefore it had to be used immediately. Additionally, the Fe^{3+}/Fe^{2+} reduction potential could be detected using the FRAP method despite no antioxidants contained [45] [31]. The FRAP method operated on the principle of reducing ferroin analogs, which were the Fe3+ complex from tripyridyl triazine $Fe(TPTZ)^{3+}$ to an intense blue $Fe(TPTZ)^{2+}$ complex by antioxidant compounds in an acidic environment. Another disadvantage of this method was the tendency for the reagent to settle, forming a suspension and contaminating the measurement equipment. Differences in antioxidant content between using the FRAP method and using the DPPH method were because of different testing principles. The highest antioxidant content of 102.47 ± 2.99 µMeq Fe(II)/g) was presented by the F3 treatment, while the lowest 44.14 ± 6.42 µMeq Fe(II)/g) was shown by the F5 treatment. The higher concentration of ethanol solvent produced lower antioxidants in the FRAP method.

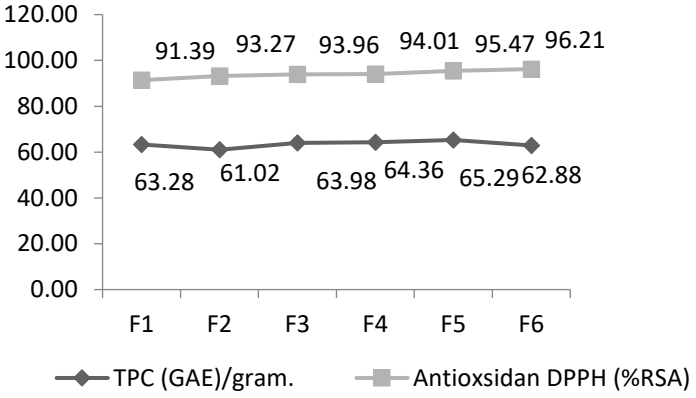

Figure 1. Comparison of TPC and Antioxidant (FRAP Methods)

Different solvent mixtures produced antioxidant content that was significantly different using the FRAP method since the mixture of solvents would provide different solubility properties. Flavonoids, alkaloids, carbohydrates, glycosides, and tannins were found exclusively in methanol extracts and water [46]. Meanwhile, water extracts included tannins, saponins, total protein, and carbohydrates yet demonstrated neither steroids nor alkaloids. Water has high polarity so it becomes one of the most suitable solvents for plant extraction [47],

[48]. Therefore, ethanol extracts had the most abundant secondary plant substances (steroids, alkaloids, saponins, tannins, total protein, and carbohydrates) if compared to garlic bulb extracts obtained with other solvents.

3.5 Chemical Compound Identification

Table 4 exhibits the chemical components in each extract. Different solvent mixtures had different effects on the extract content. In treatments F6, F5, and F4, the presence of the compound 5-(Hydroxymethyl) Furfural (5-HMF) was identified. 5-HMF could be developed as a novel natural antioxidant [49], Meanwhile, in treatments F3 and F2, the presence of oxalic acid was identified. Oxalic acid was an organic acid which functioned as an antioxidant in plants and vegetables. The synthesis of oxalate was documented from ascorbate or isocitrate. Several enzymes, e.g., oxalyl-CoA synthetase oxalate oxidase, and oxalate decarboxylase, were engaged in breaking down oxalate, varying with the tissue type, cell, or species of the plant concerned [50]. Plants from the same variety could be different in terms of the quantitative and qualitative compositions of their antioxidant compounds and the composition of their constituents, whose impact on the antioxidant characteristic assessment was often ignored or rarely considered. Some examples of the constituents were metal ions, natural acid, covering succinic acid, malic, ascorbic, oxalic, citric, and others, and water. As a result, some wonders arose concerning how varying concentrations of metal ions, hydrogen, and water affected the evaluation of these compounds' antioxidant characteristics [51]. Xanthosine was expressed in treatments F6 and F6. The lower the ethanol content, the lower the Xanthosine until it was no longer detected. Likewise, Guanosine was detected in treatments F6, F5, and F4 and disappeared in treatments F3 and F2.

Lestari et al. [37] also found Xanthosine and Guanosine in black garlic extract. Guanosine and Xanthosine were nucleosides, which were substrates for adenine deaminase and purine nucleoside phosphorylase. Purine nucleoside phosphorylase was an enzyme contributing to the pathways of purine catabolism. The enzyme catalyzed the reversible phosphorylation of N-riboside nucleotide bonds, hence resulting in the production of ribose 1-phosphate and purine bases. The purine bases created were hypoxanthine, which were substrates for xanthine oxidase and guanine deaminase, xanthine, and guanine. Subsequently, the guanine deaminase enzyme converted guanine into xanthine, while the xanthine oxidase enzyme catalyzed the conversion of hypoxanthine to xanthine, then ultimately to uric acid [52].

Table 4. Chemical Compound Identification By Gc-Ms

F6	F5	F4	F3	F2
5-(Hydroxymethyl) Furfural(HMF)	5-(Hydroxymethyl) Furfural(HMF)	5-(Hydroxymethyl) Furfural(HMF)	Ethanedioic Acid/Oxalic Acid	Ethanedioic Acid/Oxalic Acid
Xanthosine	Xanthosine	Xanthosine	1-Chloroisopropyl Alcohol	1-Chloroisopropyl Alcohol
Cytidine	Cytidine	Cytidine	3-Chloro-4-Methyl-2-Pentanol	Hexanol
Guanosine	Guanosine	Guanosine	4-(1'-Azepanyl)-2,6-Diphenylpyridine	5,5-Dimethylimidazolidin-2,4-Diimine
4,5-Dimethylhex-4-En-3-One	4,5-Dimethyl-4-Hexen-3-One	2-Amino-9-(3,4-Dihydroxy-5-Hydroxymethyl-Tetrahydro-Furan-2	1-Methoxy-2-Propanone	
Cis-5-Methyl-4-Hepten-3-One	Cis-5-Methyl-4-Hepten-3-One	Tris(Hydroxymethyl)Nitromethane		
4-Methyl-4-Hepten-3-One	4-Methyl-4-Hepten-3-One			
Tris(Hydroxymethyl) Nitromethane	Isobutyl Glycerol			
Guanosine Hydrate				
Triethanolamine Borate				
2,3,5,6-Tetrabromopyridine				

Aqueous and alcoholic garlic extract (GE) contain S-allyl-mercapto cysteine (SAMC), S-methyl-l-cysteine, Spropenyl-l-cysteine, and S-allyl-cysteine, all of which are derived from g-glutamyl-S-allyl-L-cysteines [53]. Table 4 indicates the absence of allicin or DADS as the test used GC-MS on a dilute sample with a ratio of garlic solvent (1:2 w/w). Garlic contains a number of antioxidants which play a role in fighting oxidative stress and protecting the body from damage caused by free radicals. Guanosine plays a role in biological mechanisms related to redox balance and protection against oxidative stress.

In the Future, the extraction results will then be encapsulated in order to maintain the quality of single-bubl garlic extract (var Temanggung). Encapsulated single-bulb garlic extract will protect the bioactive components of the garlic, besides that storage and transportation will also be easier. For this reason, it is very possible for encapsulated single-bulb garlic extract (var Temanggung) to be marketed not only locally but also internationally.

4. Conclusion

Single-bulb garlic var Temanggung contains a variety of antioxidants with strong antioxidant activity. The solvent mixture treatment gave significantly different results to the quality and quantity

of single-bulb garlic antioxidants. The best treatment was F2 (ethanol: water = 20%:80% v/v) that produced the highest antioxidant activity values (IC_{50} DPPH of 10.35 ppm and a FRAP value of 9.41 μM equivalent Fe(II)/g) and a TPC value of 61.02 GAE/g).

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The Impact of Ultrasonic Treatment on the Physiochemical and Microbial Properties of Iraqi Soft Cheese

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ABSTRACT

In recent decades, there has been a shift towards using alternative methods to enhance traditional milk processing techniques and their derivatives. One such method is the application of ultrasonic treatment as a substitute for conventional pasteurization. The chemical content of milk treated with ultrasonic waves (US) for durations of 5 and 10 minutes at a frequency of 20 kHz and a temperature of 50°C showed increases in protein, fat, and acidity levels after 5 minutes of US pasteurization, registering at 3.50%, 3.65%, and 0.19% respectively, while the ash content was 0.73%. The logarithm of total microbial counts in milk samples showed no growth post-US pasteurization. The active compounds in the milk samples were identified using GC-MS, revealing the highest concentrations after 5 and 10 minutes of US treatment were of Hydroxy-2,8-bis(trifluoromethyl)quinoline, 2-methylpropionate 4- and Succinic acid, 3-methylbut-2-yl 3-chlorophenyl ester, at concentrations of 31.826% and 35.318% respectively. Samples treated for 10 minutes exhibited superior firmness, cohesiveness, and elasticity in soft cheese, with values of 179.9, 0.66, and 4.38, respectively. The lowest pH observed in these samples was 4.60, with the highest acidity at 1.68%. Moreover, ultrasonic treatment enhanced the sensory characteristics of the cheese, demonstrating that ultrasound waves can improve the microbial, physical, chemical, and sensory properties of white cheese.

1-Introduction

For thousands of years, humans have relied on pasteurization to process milk intended for consumption or manufacturing, considering it a principal technology for reducing microbial contamination in the food industry, especially in milk, It extends the shelf life of derived products due to its ability to eliminate harmful microorganisms without compromising food safety [1]. Recent advancements in food sciences have led to the production of high-quality products using new techniques that save time, effort, and cost, including alternative methods to thermal pasteurization. Despite its effectiveness in eradicating pathogenic bacteria, thermal pasteurization can degrade essential nutrients and produce burnt flavor problems due to its reliance on heat [2]. An alternative technique is ultrasonic treatment, known for its high-frequency waves. This method is divided into two categories: low-intensity non-destructive ultrasound, which does not affect the chemical properties of milk but causes molecular vibrations, used at an intensity less than 1 watt/cm² and a frequency above 100 kHz with low energy, and high-intensity ultrasound, which is more than 10 watts/cm² at frequencies between 20 and 100 kHz [3]. High-energy ultrasonic waves contain sound energy bundles that induce physical and chemical changes in the material by providing high temperatures and pressure, They are used in the homogenization and quality control of dairy products and the pasteurization processes of milk for cheese making [4] . Recent research has shown that ultrasonic treatment of milk plays a role in enhancing fat production when manufacturing dairy products by improving the emulsification of fats in milk, homogenization, stability, and enhancing the activity of lactic acid bacteria by modifying the metabolic process, as well as improving quality characteristics such as water retention, texture, and structure [5]. Ultrasonic waves increase the temperature and pressure on the milk, reducing microbial contamination and causing physical changes, including cracking of the milk fat globule membrane and changes in casein formations and fat decomposition into triglycerides [4] . [6] High-intensity ultrasonic waves homogenize milk, reduce microbial growth, and form volatile flavor compounds, benefiting curd formation in treated dairy products thereby enhancing the quality characteristics and sensory properties of the final product [7]. Ultrasonic high-intensity waves cause changes in milk components, including reducing its viscosity, crystallizing lactose, homogenizing milk fat, and improving whey filtration, as well as the use of treated milk in cheese-making and increasing coagulation potential due to the waves' ability to break down fat and protein particles into smaller sizes, which accelerates milk coagulation and improves curd qualities [4]. To our knowledge, there are no published studies on the impact of ultrasound on spoilage microorganisms or the potential effects of ultrasound on the physical and chemical properties of white cheese during maturation. Therefore, this work aimed to study the effect of different ultrasonic frequencies on milk, compare it with thermal pasteurization and investigate the physicochemical, microbial, and sensory properties of Iraqi soft white cheese during the maturation period.

2-Materials and Methods

2-1 Experimental design

Raw cow's milk was prepared from the Agricultural Research Station at the University of Basra and divided into three

parts. One part was treated with ultrasonic waves (milk US) at a temperature of 50°C for 5 minutes and another for 10 minutes using Korean-made Ultra-Sonic device, with a frequency of 20 KH, power of 600 watts, and voltage of 220 volts. The third part was pasteurized at a high temperature of 72°C for 15 seconds and then cooled to 4°C. The fourth sample was a control sample, with three replicates for each sample. All samples were stored at 4°C for 4 hours post-milking, and physicochemical tests were conducted [8].

2-2 Cheese Manufacturing

Iraqi soft cheese was made using the microbial, fungal rennet Rhizomucor pusillus, produced by Meito Sengyo Co., LTD of Japan, within its shelf life and according to the company's recommendations. The process was repeated three times for each type of raw milk: US cheese, thermal pasteurized cheese, and control cheese, following the method described in [2].

2-3 Milk Testing

2-3-1 Physicochemical Tests

The percentages of protein, fat, lactose, ash, and moisture in the milk were estimated using the Eko milk analyzer. The pH of the milk was measured using a Sartorius pH meter made in Germany, and acidity was determined by titration with 0.1N NaOH [9].

2-3-2 Microbiological Tests

The total bacterial count in the milk was performed using Nutrient Agar prepared by Oxoid, following the manufacturer's recommendations using the pour plate method [10].

2-3-3 Estimation of Active Compounds by GC-MS Technique

Active compounds in the milk samples were identified using a GC-MS device, utilizing an HP-5ms column and helium gas at a flow rate of 1 ml/sec. The injection temperature was 290°C, and the GC oven program started at 40°C. It was raised to 300°C over 20 minutes at a rate of 10°C per minute. Separated peaks were matched with the spectral database from the NIST 2014 library [11].

2-4 Cheese Testing

2-4-1 Physical and Rheological Tests

The pH of the cheese was measured using a Sartorius pH meter made in Germany, and acidity was determined by titration with 0.1N NaOH. Texture properties such as elasticity, cohesiveness, and firmness were measured using a Texture Analyzer from CT3 4500 Brookfield, USA, according to the method followed [2].

2-4-2 Sensory Tests

The sensory properties of the Iraqi soft cheese samples processed from the treated milk, such as color, appearance, texture, odor, taste, and overall acceptance, were evaluated by 10 experts in the field of food [2].

2-5 Statistical Analysis

Statistical analysis was conducted using the statistical software SPSS (version 12, 2006). The data were analyzed using the CRD and LSD tests at a 0.05 probability level [12].

3- Results and discussion

3-1 Physicochemical Tests of Milk

Table (1) presents the percentage composition of milk derived from cow's milk, along with its pH and titratable acidity for raw milk samples (Control), milk treated with ultrasonic waves for 5 and 10 minutes, and milk pasteurized using thermal pasteurization. The statistical analysis ($P<0.05$) indicated significant differences in protein, fat, lactose, ash, moisture content, pH, and acidity. The 5-minute US-treated

milk samples showed superior protein and fat percentages compared to other treatments. Specifically, protein content increased from 3.20% in raw milk to 3.50% after 5 minutes of US treatment, decreased slightly to 3.47% after 10 minutes, and was 3.25% post-thermal pasteurization. Fat content decreased to 3.46% following thermal pasteurization from 3.50% in raw milk. However, it reached its highest at 3.65% after 5 minutes of US treatment. The highest lactose content was recorded at 4.87% after 10 minutes of US treatment. The acidity percentage for the milk treated with ultrasonic waves for 5 minutes was 0.19% with a pH of 6.21. Ash content for thermal pasteurization and 5 and 10-minute US treatments were 0.70%, 0.73%, and 0.78%, respectively, compared to 0.70% in raw milk. The highest moisture content was observed in milk post- thermal pasteurization at 87.96%, and the lowest was 87.28% after 10 minutes of US treatment.Ultrasonic waves enhance the enzymatic hydrolysis of whey proteins, producing biologically active peptides, transforming particulate casein into soluble casein, and reducing the size of these particles, unlike conventional pasteurization, which does not alter the structure of casein particles [13]. Additionally, ultrasonication improves lactose crystallization, reduces fermentation time, lowers pH through enhanced lactose hydrolysis, increases fat globule breakdown, improves emulsifying properties, raises the level of free fats, and thereby increases its susceptibility to oxidation [13,14]. Observations [15] indicated an increase in moisture and protein content and a decrease in carbohydrate and fat percentages in thermal pasteurized milk compared to raw milk (87.46%, 3.33%, 4.54%, and 3.94% vs. 87.40%, 3.16%, 4.74%, and 3.97%, respectively. The reduction in protein particle size due to structural disruption caused by ultrasonic waves enhances the gelation and foaming properties due to effects on hydrogen bonds and Van der Waals forces between the three-dimensional structures of proteins [16]. Ultrasonic waves break down fat globules in milk and homogenize them, reducing their diameter sizes due to repeated collisions [7]. Observe [17] an increase in protein content in milk treated with ultrasonic waves and its interaction with larger fat globules of unhomogenized milk, observed an increase in fat content post-treatment and homogenization, increased surface area of fat globules, and disrupted their membranes, and noted a slight increase in lactose content that does not affect acidity.

Table 1. Chemical composition of US ultrasound-treated and thermal pasteurized milk

Milk	Protein %	Fat%	Lactose %	Ash%	Moisture%	Ph	Acidity %
Control	3.20^{d}	3.50^{c}	4.64^{c}	0.70^{c}	87.96^{a}	6.23^{b}	0.18^{b}
Thermal pasteurization	3.25^{c}	3.46^{d}	4.63^{c}	0.70^{c}	87.96^{a}	6.17^{d}	0.20^{a}
US(5min)	3.50^{a}	3.65^{a}	4.82^{b}	0.73^{b}	87.30^{b}	6.21^{c}	0.19^{b}
US(10min)	3.47^{b}	3.60^{b}	4.87^{a}	0.78^{a}	87.28^{c}	6.25^{a}	0.17^{c}

*Different letters indicate the presence of significant differences, and similar letters indicate no significant differences between the treatments at the probability level (P<0.05).

3-2 Microbial Tests for Milk

Ultrasound treatment significantly reduces the microbial content in milk, surpassing the efficacy of conventional pasteurization. Table (2) illustrates the logarithmic total counts of bacteria present in milk processed using ultrasound for 5 and 10 minutes, pasteurized using the thermal pasteurization method, and in raw milk. Statistical analysis results, with a significance level of P<0.05, indicate significant differences in the logarithmic total bacterial counts. It was observed that the logarithm of the counts in thermal pasteurization pasteurized milk reached 2.34 cfu/ml, and no growth was noted post-ultrasound treatment of raw milk. [18] Illustrate that ultrasound treatment markedly inhibits the growth of microorganisms, with the extent of inhibition varying based on the amplitude, duration of exposure, and temperature; it was noted that this method resulted in the lowest logarithmic number of Enterobacteriaceae bacteria at 1.06151 cfu/ml. [17] noted that conventional pasteurization processes hinder the growth of some microorganisms, excluding pathogenic bacteria such as *E. coli*, which can proliferate in pasteurization equipment. However, ultrasound treatment results in lethal and inhibitory effects on pathogenic microorganisms due to the generated pressure in the milk, leading to the puncture and destruction of microbial cell membranes. While thermal treatments reduce the microbial counts of Psychrotrophs, ultrasound treatment significantly reduces microbial counts, including coliform bacteria, molds, and yeasts, thereby extending the shelf life of products manufactured from ultrasound-treated milk [19].

Table 2. Logarithm of the total numbers of bacteria present in milk processed using the US method and thermal pasteurized milk

Milk	Total plate count (cfu/ml)
Control	7.29^{a}
Thermal pasteurization	2.34^{b}
US(5min)	$< 1^{c}$
US(10min)	$< 1^{c}$

*Different letters indicate the presence of significant differences, and similar letters indicate no significant differences between the treatments at the probability level (P<0.05).

3-3 Analysis of Active Compounds in Milk by GC-MS Technique

Numerous active compounds were detected in milk treated with ultrasound, as indicated in Table (3) and Figure (1), which illustrates the chromatogram of the active compounds, their retention times, and concentrations in milk processed by ultrasound for 5 minutes. This includes volatile compounds as well as fatty acids and active peptides with health benefits. It was observed that the highest

concentration was of Hydroxy-2,8-bis(trifluoromethyl)quinoline, 2-methylpropionate 4-, at 31.826% after 34 minutes from the start of separation. Conversely, results from Table (4) and Figure (2) for milk treated with ultrasound for 10 minutes show a decrease in the concentrations of active compounds compared to the 5-minute treatment, with the highest concentration being Succinic acid, 3-methylbut-2-yl 3-chlorophenyl ester at 35.318% after 49 minutes. Additionally, various compounds appeared at different ratios, such as maltol, catechol, alcohols, organic acids, phenolic compounds, and benzene, among others, contributing to the flavor-enhancing volatile compounds. . [20] pointed out that raw milk contains many desirable volatile compounds like ethyl butanoate, 1-octen-3-ol, and phenylethanol, which impart a distinct aroma to milk but are lost after thermal or ultrasound processing due to physical and chemical changes that adversely affect the flavor of milk by producing undesirable compounds from increased treatment impact. Ultrasound treatment leads to an increase in volatile compounds compared to thermally pasteurized milk, with notable appearances of compounds such as aldehydes from the oxidation of unsaturated fatty acids by ultrasound, and maltol, an active aromatic compound, along with organic acids such as acetic, hexanoic, and butyric, formed by the rupture of fat globule membranes, along with benzene and toluene, and some aliphatic compounds [19]. The formation of volatile compounds is linked to the degree of lipid and protein decomposition by enzymes and by ultrasound, which promotes the formation of free fatty acids, active peptides, and amino acids, as well as free radicals that cause oxidation, leading to the production of undesirable compounds like methyl ketones, aldehydes, esters, secondary alcohols, or sulfur compounds [20].

Table 3. Active compounds, retention time, and concentration of milk treated with ultrasound for 5 minutes

Peak	RT.	Area%	Library/ID
1	10.333	0.468	1,2-Dimethyl-3-isopropyldiaziridine
2	11.29	0.452	[(2-Amino-3-hydroxypropanoyl)amino]acetic acid
3	11.548	0.505	6-Amino-1,3,5-triazine-2,4(1H,3H)-dione
4	11.847	1.055	1H-Imidazole-4-carboxylic acid, methyl ester
5	12.105	1.094	Allyl 2-methylbutyrate
6	12.335	0.382	Maltol
7	12.519	0.491	Cyclopentasiloxane, decamethyl-
8	12.722	0.366	2-Propanamine, N-methyl-N-nitroso-
9	12.899	1.527	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
10	13.218	0.876	2-Methylheptanoic acid
11	13.713	1.09	Catechol
12	14.155	11.529	5-Hydroxymethylfurfural
13	15.058	0.646	Cyclohexasiloxane, dodecamethyl-
14	15.35	1.165	1,3-Butadiene-1-carboxylic acid
15	16.022	1.371	2,3-Pentadienoic acid-, ethyl ester
16	16.476	3.866	3,4-Altrosan

17	17.284	0.37	Cycloheptasiloxane, tetradecamethyl-
18	17.902	4.009	2,3-Pentadienoic acid-, ethyl ester
19	19.423	1.265	Furan, 3-(4,8-dimethyl-3,7-nonadienyl)-, (E)-
20	21.541	0.516	Cyclo(L-prolyl-L-valine)
21	22.525	0.357	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione
22	22.905	1.555	n-Hexadecanoic acid
23	23.224	0.338	Hexadecanoic acid, ethyl ester
24	24.596	1.358	9-Octadecenoic acid, (E)-
25	24.806	0.616	Octadecanoic acid
26	27.922	0.81	Bis(2-ethylhexyl) phthalate
27	29.164	0.344	4-tert-Butylphenol, TMS derivative
28	29.816	0.593	Tris(tert-butyldimethylsilyloxy)arsane
29	30.814	0.329	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
30	31.167	1.465	4-(7-Methyloctyl)phenol, TMS derivative
31	32.498	1.757	Cholest-5-en-3-ol (3.beta.)-, tetradecanoate
32	34.032	31.826	4-Hydroxy-2,8-bis(trifluoromethyl)quinoline, 2-methylpropionate
33	36.014	17.363	2-Ethylbutyric acid, 2,7-dimethyloct-5-yn-7-en-4-yl ester
34	42.606	2.05	4-tert-Octylphenol, TMS derivative
35	49.503	6.195	Cyclotrisiloxane, hexamethyl-

Table 4. Active compounds, detention time, and concentration of milk treated with ultrasound for 10 minutes

Peak	RT.	Area%	Library/ID
1	10.333	0.562	Cyclohexanol, 4-methyl-
2	11.84	0.425	3-Furancarboxylic acid, methyl ester
3	12.132	3.198	5-tert-Butyl-1,2,3,4,5,6,7-[1,2,3]triazolo[4,5-e][1,2,3,4]tetrazine-1,3,7-trione
4	12.899	0.785	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
5	13.306	1.368	L-Serine, N-methyl-, methyl ester
6	13.761	1.706	Catechol
7	14.162	2.756	5-Hydroxymethylfurfural
8	14.657	0.45	Butyrolactone, 3-cyano-4,4-dimethyl-
9	15.058	0.676	Cyclohexasiloxane, dodecamethyl-
10	15.424	0.454	3-Octadecene, (E)-
11	16.551	1.72	Pyrazole-5-carboxylic acid, 3-methyl-
12	19.253	0.361	Cyclooctasiloxane, hexadecamethyl-
13	20.828	0.655	Tetradecanoic acid
14	21.554	0.823	Cyclo(L-prolyl-L-valine)
15	21.873	0.376	1,3-Cyclohexanedione, 2-propyl-

16	22.532	0.603	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione
17	22.702	0.469	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone
18	22.837	0.529	Octahydrodipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione-, (5aR,10aR) (isomer 1)
19	22.919	3.403	n-Hexadecanoic acid
20	24.603	2.509	9-Octadecenoic acid, (E)-
21	24.813	1.09	Octadecanoic acid
22	26.008	0.356	1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one
23	27.922	1.182	Bis(2-ethylhexyl) phthalate
24	29.164	0.392	2-tert-Butylphenol, tert-butyldimethylsilyl ether
25	29.823	0.443	Vanadium, (.eta.7-cycloheptatrienylium)(.eta.5-2,4-cyclopentadien-1-yl)-
26	32.504	7.198	Cholesta-3,5-diene
27	32.796	0.528	Cyclotrisiloxane, hexamethyl-
28	34.819	1.485	1-(3-Chlorophenyl)-3-methyl-1H-pyrazol-5-amine
29	34.935	1.481	Glutaric acid, 3,4-difluorobenzyl hexyl ester
30	37.82	0.925	1,2-Benzisothiazol-3-amine, TBDMS derivative
31	38.051	3.311	4-Amino-2-butyl-N-(tert-butylimino)-1-oxo-1$l^{5},2,3-triazol-5-imine oxide
32	42.205	2.547	Cyclotrisiloxane, hexamethyl
33	42.667	11.082	2-Methyl-pentanoic acid [4-(2-methyl-pentanoylsulfamoyl)
34	43.774	1.86	1,4-Bis(trimethylsilyl)benzene
35	48.75	6.976	Tetrahydrofuran-2-carboxylic acid, dibenzofuran-3-ylamide
36	49.693	35.318	Succinic acid, 3-methylbut-2-yl 3-chlorophenyl ester

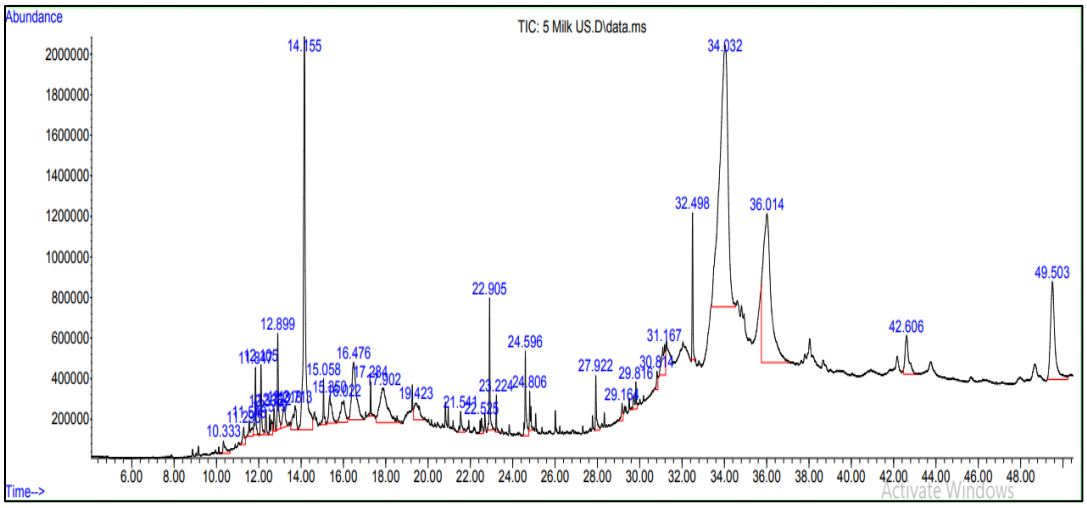

Figure 1. Chromatograms of active compounds, retention time, and concentration of milk treated with ultrasound for 5 minutes

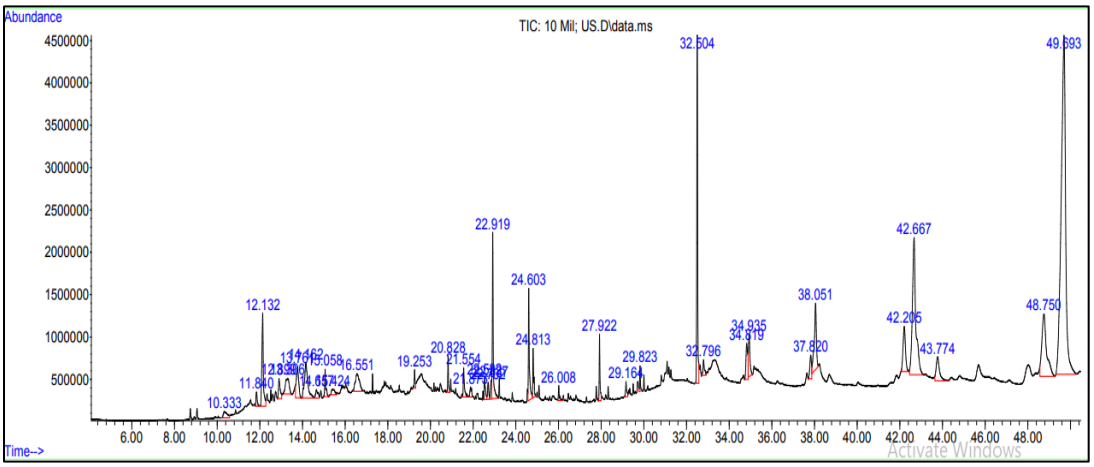

Figure 2. Chromatograms of active compounds, retention time, and concentration of milk treated with ultrasound for 10 minutes

3-4 Physical and Rheological Examinations of Cheese

Table 5 delineates the physical characteristics of Iraqi soft cheese produced from both raw and pasteurized milk using thermal pasteurization and ultrasonic (US) methods for durations of 5 and 10 minutes. Statistical analysis results ($P<0.05$) indicate significant differences in the properties of hardness, cohesion, elasticity, acidity, and pH levels of the cheese. It was observed that samples treated using the US method outperformed other samples. Specifically, those treated for 10 minutes showed superior hardness, cohesion, and elasticity, with respective values of 179.9, 0.66, and 4.38. Additionally, the lowest pH value was recorded in cheese samples from milk treated with the US method for 10 minutes, at 4.60. The highest acidity reached 1.68%, compared to 4.72 and 1.60% for thermal pasteurization, respectively. The pH values decrease, and acidity increases post-cheese production due to the action of coagulating microorganisms, and ultrasonic treatment further reduces the pH values and increases acidity, thus decreasing the time required for coagulation. This effect is attributed to the fact that ultrasonic waves enhance the hydrolytic breakdown of fats, thereby impacting the characteristics of hardness, cohesion, and elasticity that vary with the frequency used in the US treatment. The increase in these values is due to reduced moisture content resulting from protein hydrolysis; ultrasonic waves increase the cheese's hardness by promoting coagulation processes [2]. Ultrasonic waves also alter the structure of proteins and stimulate hydrophobic groups on their surface, which enhances gel formation and increases its hardness [21]. The change in the size of milk components post-ultrasonic treatment improves the gel structure by increasing the gel strength and curd firmness and accelerating the coagulation process. The reduction in the size of milk fat globules due to ultrasonic vibrations enhances emulsifying properties, which is attributed to the rupture of milk fat globule membranes and the interaction of casein particles with these membranes, as well as the division of larger fat globules [13].

Table 5. Physical properties of Iraqi soft cheese made from milk treated with US ultrasound for 5 and 10 minutes and thermal pasteurization

Milk	Hardness/gm	Cohesiveness	Springiness/mm	Ph	Acidity%
Control	175.3[c]	0.78[a]	4.55[a]	4.73[a]	1.59[c]
Thermal pasteurization	170.4[d]	0.62[b]	3.99[c]	4.72[a]	1.60[c]
US(5 min)	177.5[b]	0.65[b]	4.36[b]	4.70[b]	1.65[b]
US(10 min)	179.9[a]	0.66[b]	4.38[b]	4.60[c]	1.68[a]

*Different letters indicate the presence of significant differences, and similar letters indicate no significant differences between the treatments at the probability level (P<0.05).

3-5 Sensory Tests for Cheese

Table (6) presents the sensory characteristics of soft Iraqi cheese made from raw, thermal pasteurized, and ultrasonically treated (US) milk for 5 and 10 minutes, focusing on color, texture, aroma, taste, and overall acceptance. Statistical analysis results, with a significance level of P<0.05, indicate significant differences in the mentioned attributes. Cheese samples processed with ultrasound for 10 minutes excelled with the best overall score of 22.49, showing superior results in color, taste, and overall acceptance with scores of 4.78, 4.36, and 4.48, respectively. In contrast, samples made from thermal pasteurized milk exhibited the best aroma, scoring 4.50, possibly attributed to the increased formation of aromatic volatile compounds in US-treated milk compared to thermal pasteurization. Ultrasonic waves enhance the sensory quality of cheese due to their impact on fat emulsification and protein breakdown in milk, influenced by the formation of volatile compounds from enzymatic reactions of milk components, as well as the formation of complex compounds from the degradation of hydroxides in secondary oxidation processes [13]. [2] observed that ultrasonics positively affect the sensory properties of cheese by improving its color, appearance, and texture and enhancing flavor and taste due to the breakdown of fats and proteins.

Table 6. Sensory properties of Iraqi soft cheese made from milk treated with US ultrasound for 5 and 10 minutes and thermal pasteurization

Milk	Color /5	Texture/5	odor/5	Flavor/5	Overall acceptability/5	Total/25 mark
Control	4.00^{d}	4.75^{a}	3.05^{d}	3.30^{c}	3.39^{d}	18.49^{d}
Thermal pasteurization	4.30^{c}	4.15^{d}	4.50^{a}	4.05^{b}	4.30^{c}	21.30^{c}
US(5 min)	4.75 ^{b}	4.70^{c}	4.12^{c}	4.35^{a}	4.45^{b}	22.37^{b}
US(10 min)	4.78^{a}	4.72^{b}	4.15^{b}	4.36^{a}	4.48^{a}	22.49^{a}

*Different letters indicate the presence of significant differences, and similar letters indicate no significant differences between the treatments at the probability level (P<0.05).

4- Conclusion

This study highlights the potential of utilizing ultrasonic treatment for pasteurization and modifying the properties of milk prior to its conversion into dairy products. This technique provides non-destructive, non-oxidative heat to the milk. It facilitates homogenization, which enhances the sensory and physical characteristics of the resultant dairy products. Moreover, it improves the physicochemical and sensory properties, as well as the texture and consistency of these products. Additionally, ultrasonic treatment helps reduce microbial contamination. It leads to the formation of active and volatile compounds with functional health benefits, resulting from the hydrolysis of proteins and fats in the milk and its products. Consequently, this enhances the functional and health properties of these products. This technology can be widely adopted in dairy plants to reduce time, effort, and costs while achieving superior results compared to traditional pasteurization methods.

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Scientific Research

Enhancing functional characteristics and antioxidant activity of *Prosopis juliflora* pods' protein isolate through pH adjustment, while detecting the physicochemical properties and antibacterial inhibition activity

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ABSTRACT

This study evaluated the physicochemical properties of *Prosopis juliflora* pods and the preparation of its protein isolate. Changes in functional properties were observed when pH levels were adjusted to 5, 7, 8, and 10, with significant differences ($P \leq 0.05$) in functional characteristics and antioxidant and antibacterial activities. A high solubility value was achieved at pH 5.10 and lipid binding at pH 10, while the lowest value for both properties was found at pH 7. The highest foaming capacity was observed at pH 5 after 5 minutes of whipping, but decreased after 1 minute at pH 8. The emulsifying capacity was greatest at pH 10 and lowest at pH 7. The DPPH radical scavenging activity was high at pH 5, but decreased at pH 8 and also protein isolate showed inhibition activity against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. However, the highest inhibition was against Bacillus bacteria at 0.5% protein isolate concentration, while the protein digestibility was higher. The isolated protein had no noticeable cytotoxic effect on human blood cells. Besides, FTIR test was used to determine the structure of the protein.

1-Introduction

The utilization of plants is experiencing growing and engaging accomplishments. Many plants remain largely unstudied yet hold significant economic, nutritional, and health value and potential. Several secondary source plants have been examined to create innovative eco-friendly products and alternative protein-rich feed stocks, as well as bioactive compounds (de Melo *et al*., 2021). The food industry can greatly benefit from food by-products, as stated by Cavalcante *et al*. (2022). Using fully processed foods can help enhance products and prevent waste while meeting basic nutritional needs and promoting good health. Minimizing the chances of illness (Iglesias and Alejandre, 2010). Numerous food compounds with high protein content, beneficial bioactive properties, and health-friendly characteristics from unconventional sources have been researched for the purpose of creating innovative products (de Melo *et al*., 2021). The Fabaceae or Legume family consists of the genus Prosopis, which comprises around 45 species of spiny trees and shrubs. This type of species is present in tropical and subtropical areas worldwide. Studies have looked into the feasibility of using whole mesquite grains as an ingredient because mesquite is mainly used in animal diets as an alternative food source. According to Gusmão *et al*. (2018) and Gonzalez Baron *et al*. (2020), mesquite flour is used in the making of cakes and bread for human consumption. It has antioxidant and antimicrobial properties to treat a variety of conditions such as asthma, labour/ postpartum pain, corns, conjunctivitis, diabetes, diarrhea, phlegm, fever, influenza, lactation, liver infection, malaria, and otitis. media, pain, lice, and rheumatism. Scabies infestation on the skin. Additional uses have been discovered (references 3-5). Cavalcante *et al*., 2019; Battista *et al*., 2018) demonstrate the technological possibilities of mesquite. In this instance, utilizing this product for human use is crucial (Cavalcante *et al*., 2020). Proteins play a crucial role in the development and overall well-being of the human body. Individuals require sufficient protein for regular body functions, development, maturation, pregnancy, breastfeeding, and healing from injuries and illnesses (Khan and Varshney, 2018). The need for alternative protein sources is driven by a growing global population and diminishing resources to meet protein demands. Plant proteins are significantly essential in the diet and more cost-effective to manufacture compared to animal proteins. Traditional legumes are important for food and feed in many countries but are not grown enough to keep up with increasing populations and demand from animal feed industries (Siddhuraju and Becker, 2003). The pH level can impact the structure and chemical characteristics of proteins, impacting how proteins interact in food systems throughout various stages like processing, storage, consumption, and preparation, ultimately affecting the overall quality and sensory aspects of food systems. Furthermore, protein can impact food characteristics like hydration, fat retention, emulsification, and foam formation (Feyzi *et al*., 2017). Nevertheless, limited scientific data exists on the process of preparing a protein isolate from musk pods. Therefore, this research focused on creating isolates from musk pods and analyzing the physical and chemical aspects of the pods, including protein amino acid quality, digestibility, cytotoxicity, and active groups. In addition, the effect of pH variations on functional characteristics and antioxidant activity was evaluated.

2- Materials and methods

2.1 Preparation of the sample

The pods of *P. juliflora* were cleaned and ground into powder and stored in a two-layer package of aluminum and

polyethylene with obstacles against light and oxygen in the refrigerator until use.

2.2 Chemical composition

The total pH value measured by using a digital pH meter calibrated with buffers at pH 4.0 and 7.0. The protein content was evaluated using the Kjeldahl method, the total lipid content using hexane extraction in a Soxhlet, and the ash content using a muffle furnace at 550 C°. (Association of Official Agricultural Chemists, AOAC, 2000). The difference between the total amount of the other constituents (% moisture + % ash + % fat + % protein) and 100% was used to calculate the total amount of carbohydrates (Barbi *et al*., 2020). The measurements were performed with three replications.

2.3 protein isolate

With minor modifications, extraction procedure was performed in accordance with Mariod *et al*. (2010). The material was mixed mechanically for one hour after being diluted in distilled water at a ratio of 1:20 (w/v), and pH 9.0 was achieved by adding a 1.0 M NaOH aqueous solution. The resulting filtrate was centrifuged at 10,000 rpm for 20 minutes at room temperature using white cheesecloth. In order to enhance protein precipitation, the supernatant was placed into a beaker and agitated once more for 20 minutes while the pH was adjusted to 4.5 with 0.1 M HCl. The sediment protein slurry was dialyzed overnight against water at 4°C after being centrifuged at 10,000 rpm for 10 min at room temperature. While changing pH, after bringing the protein precipitate's pH level to 7.0, it was collected and freeze-dried (Delta 2 24/LSC plus, Germany). For a subsequent experiment, the protein powder was vacuum-packed and kept at 18 °C.

2.4- Functional characteristics

2.4.1 Protein solubility

With slight adjustments, Kumar *et al*. (2014) approach was used to determine the proteins solubility. Accordingly, 100 mg of the material was dissolved in 5ml of distilled water. Using 0.1M HCl or 1M NaOH the pH of the mixture was changed to 5,7,8 and 10. The mixture was centrifuged at 4000g for 20 minutes after being agitated for an hour at room temperature. The Biuret was used to measure the protein levels in the supernatants. The protein content in isolated samples was measured with Kjeldahl method. The solubility of the protein was determined using the below equation:

$$\text{Solubility (\%)} = \frac{A}{B} \times 100$$

A represents the protein content found in the supernatant, while B corresponds to the total protein content present in the sample.

2.4.2 Foaming capacity (FC)

FC was measured according to the method described by Liu *et al*. (2021) with some modifications. The sample (500 mg) was added into 50 mL of distilled water, and the pH was adjusted to 5, 7, 8, and10 with either 0.1 M NaOH nor 0.1 M HCl. The solution was whipped using the maximum speed of a homogenizer (Sartorius Japan) for 2 min foaming ability It was expressed out as follows:

$$\text{FA (\%)} = \frac{V1-V0}{V0} \times 100$$

where V0 is the volume of liquid before homogenization (in ml), and V1 is the volume of foam after homogenization (in ml).

2.4.3 Emulsifying capacity (EC)

EC were calculated using a slightly modified version of the Lamsal *et al*. (2007) method. After being homogenized for 1

minute in 20 mL of distilled water with the sample (300 mg), the pH was changed to 5, 7, 8, 10 and 15 mL of soybean oil were added to the protein solution, which was then homogenized for 1 minute. It was then centrifuged for three minutes at 5,000 rpm. EC was determined via the following formula.

$$EC\ (\%) = \frac{Hieght\ of\ emulsified\ layer}{Height\ of\ total\ \ content} \times 100$$

2.4.4 Oil holding capacity (OHC)

In a centrifuge tube, 10 ml of soybean oil and 0.5 g of the sample were combined, and the tubes were shaken vigorously (at 100 rpm) for 30 seconds each. At 3000 rpm for 30 minutes, the oil dispersion was centrifuged. Oil-holding capacity was determined as the amount of oil that was absorbed per gram of protein sample, which was assessed (Wasswa *et al.*, 2007).

2.5 Biochemical properties

2.5.1 DPPH assay:

The plant extracts' ability to scavenge DPPH radicals was assessed by mixing different concentrations of test extracts into 2.9 mL. of a DPPH solution with a 0.004% ethanol concentration. Following a 30-minute incubation period at ambient temperature, the absorption was evaluated at 517 nanometers. opposed to an empty [24]. IC50 values were determined based on the concentration of sample needed to eliminate 50% of free radicals. equation for regression. BHA was utilized as a positive control, with each test being conducted three times. DPPH's radical scavenging activity. The calculation of free radical inhibition (I%) was done using equation (1).

$$Inhibilation\ percentage = \frac{A\ blank - A\ sample}{A\ blank} \times 100$$

where A blank and A sample are respectively absorbance of control sample and absorbance of tested sample at the end of reaction.

2. 5.1 The *in vitro* protein digestibility:

Method was established based on Algadi and Yousif's (2017) explanation. 200 mg of the protein from the aliquot was suspended in 15 mL of 0.1 N HCl that also contained 1.5 mg of pepsin. For up to three hours, the mixture was submerged in water heated to 37 ^{o} C. In addition, the hydrolysate from the pepsin digestion was neutralized with 7.5 mL of 0.2 N NaOH, and then 4 mg of pancreatin was added to 7.5 mL of phosphate buffer (pH 8.0) before the samples were incubated (MIR154, Sanyo, Japan) at 37 °C for an additional 24 hours. After incubation, the sample was treated with 10 mL of a 10% trichloroacetic acid solution, centrifuged for 20 min. at 5,000g at room temperature, and the collected supernatant was used to calculate the total nitrogen content using the Kjeldahl method (Nielsen, 2010). Casein from isolated bovine milk was employed as a standard reference for comparison purposes. The following equation was used to determine the IVPD values:

$$\text{Protein digestibility (\%)} = \frac{Nitrogen\ supernatat}{Nitrogen\ in\ sample} \times 100$$

2.5.2 Toxicity of the isolated protein

The toxicity of the isolated protein was determined by placing 1 ml of fresh human blood in 20 ml of normal saline. To detect the toxicity of the isolated protein, a range of concentrations were prepared, ranging from 100 to 1000 micrograms/ml, and 100 microliters of each concentration were mixed with 2 ml of human blood. A control sample was prepared by adding 100 µL of distilled water. The mixture was heated to a temperature of 37 °C, and the turbidity was

checked after 10, 30, and 60 minutes. (Nair *et al*., 1989)

2.6 Antibacterial assay:

Different bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*) were collected from the Department of Food Science at the College of Agriculture, University of Basrah. Experiment with the excitatory impact of the protein isolate made at various concentrations (0.2, 0.3, and 0.5%) through the co-etching technique as described in the study by De Oliveira *et al.* (2011). The traditional McFarland solutions with a turbidity level of 0.5 and a cell integrity of 1.5 x 108 cells were utilized. The level of antibacterial activity was assessed with creating a 6 mm-diameter hole on the Mueller-Hinton medium's surface including a 50-microliter protein bolus for each concentration. Treatment involved the use of distilled water. The plates were incubated at 37 °C for 24 hours. The measurement of the diaphoresis diameter was taken in mm.

2.7 Characterization of proteins

2.7.1. Amino acid analysis

The amino acids of protein isolation were identified using the Levin and Grushka (1985) technique through High-Performance Liquid Chromatographic Analysis.

2.7.2. Fourier-transform infrared spectroscopy (FTIR) test

After mixing KBr with freeze-dried samples, tablets were made. The band 400–4000 cm^{-1} was scanned using an FTIR (Thermo Scientific Nicolet iS5, USA) (Lin *et al*., 2021).

2.8 Statistical Analysis:

Statistical methods were utilized to examine the variation in the data from Gen Stat Release 12.1. All comparisons were performed using the least significant difference (LSD) to evaluate specific differences, with a significance threshold of 0.05.

3. Result and discussion

3.1 Physicochemical tests

The physico-chemical characteristics of the *P. juliflora* pods were determined (Table 1). The moisture 8.37%, protein 11.93%, fat 3.65%, ash 5.41%, and carbohydrate 70.64% content, while pH value was 6.43. It was observed that the protein and fat were higher, found at 9.7% protein and 1.9% fat, according to Choge *et al*. (2007). Silva *et al*. (2007) examined *P. juliflora* pods as well and discovered average values of 2.1 and 7.2% for crude protein and fat. The protein content indicated in other studies can be seen to be lower than the levels obtained in this study.

Table 1: Physico-chemical properties of the *P. juliflora* pods.

Component	Percent (%)
Moisture	**8.37**
Protein	**11.93**
fat	**3.65**
Ash	**5.41**
Carbohydrate	**70.64**
pH	**6.43**

The presence of proteins indicates its potential as a food source. According to Summo *et al.* (2019), mesquite seed values are comparable to those of black chickpea (18,2%), a commercial source of vegetative protein. A food product can be regarded as a source of proteins if it contains at least 6 g of this component in a 100-gram serving, according to the Technical Regulation on Nutritional Information of Foods (ANVISA, 2012).

3.2 Functional characteristics:

3.2.1 Solubility:

From the (1) figure, it can be observed that the pH value change influences the solubility of the protein isolate. Solubility increases as we move further from neutrality towards either acidity or basicity. The solubility was observed to rise at pH 5 and 10, reaching 40.21% and 40.22% each, while it decreased at pH 7, reaching 35.45%. It is nearing the neutralization point of the protein isolate. The solubility varies depending on the pH level. Mune *et al*. (2016) reported a decrease in protein solubility near the protein's isoelectric point, with higher protein solubility observed outside this point. There was a statistically significant difference ($p \leq 0.05$) observed between the pH groups.

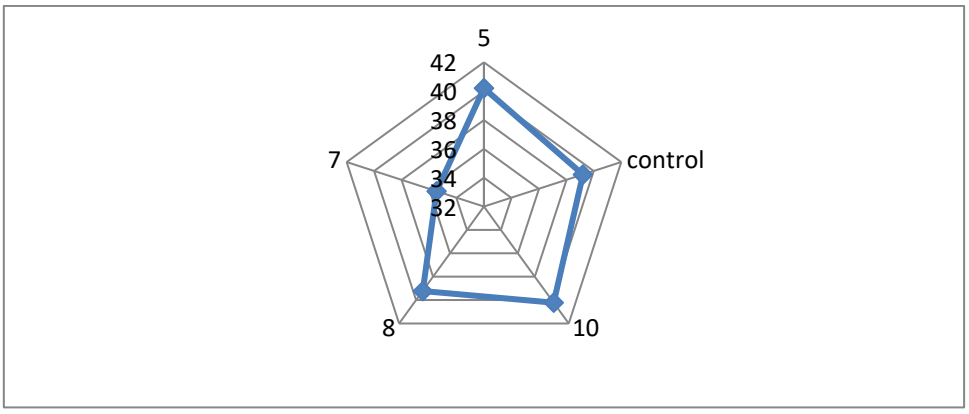


Figure 1: Effect of deferent pH on protein isolate solubility

3.2.2 Foaming capacity:

The foaming capacity of the isolated protein was measured as shown in the figure 2 at a time of 1 min as a starting point. It was highly dependent on different levels of pH. The lowest foaming capacity was recorded at pH 5 at 1 minute of whipping 66.67% followed by pH 4 (71.43) while the highest foaming capacity was obtained at pH 5 at 5 minutes of whipping 140% followed by pH 7 (130.77%) with a significant difference ($p \leq 0.05$). The higher foaming capacity at pH 5 may be due to increases in the net charge of the protein molecules, which weakens hydrophobic interactions and also increases protein flexibility. In this case, it is allowed to diffuse to the air-water interface quickly, thus encapsulating air molecules and increasing foam formation (Lawal, 2004).

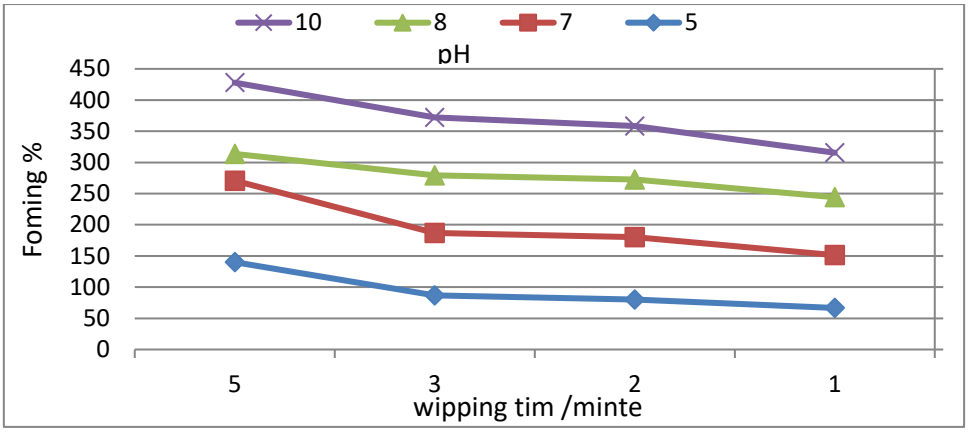


Figure (2) Effect of deferent pH on protein isolate foaming capacity.

3.2.3 Oil holding capability:

It can be observed in figure (3) that the oil holding capacity depended on the pH. When the pH nears the pH 10, there is an increase in oil holding of 2.3 g/ml, and the lowest value occurred at pH 7 (1.7g/ml). Also observed was that the oil holding capacity of the protein isolated was significantly ($P<0.05$) . The presence of several non-polar side chains many bind the hydrocarbon chain of fat, thereby resulting in higher absorption of oil (Sathe *et al*., 1982).

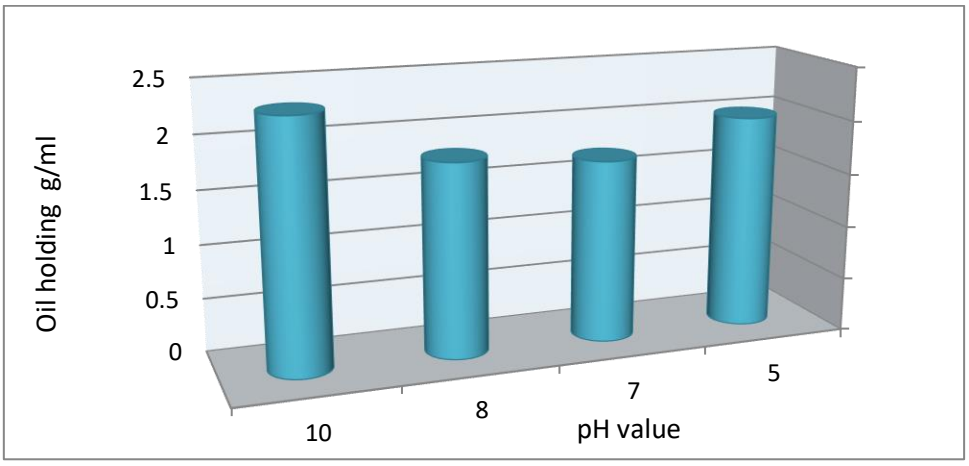


Figure 3: Effect of deferent pH on protein isolate oil holding capacity

3.2.4 Emulation capacity:

Emulation capacity evaluates how well protein-based emulsifiers work by assessing the absorption of protein at the interface. Emulation capacity is a characteristic of protein molecules that is dependent on pH and affects the balance between hydrophobic and hydrophilic properties (Figure 4). In this outcome, significant levels ($p\leq 0.05$) of emulation capacity were detected at both low and high pH levels, with the highest emulation capacity value recorded at pH 5 and 10. Therefore, the hydrophobic force decreased due to the increased net charge and flexibility. Therefore, proteins can quickly spread to the air-water interface, leading to low emulation capacity, according to Yuliana *et al.* (2014). The discovery is comparable to the outcome documented by Feyzi *et al*. (2015) concerning the protein isolate of Fenugreek (*Trigonella foencem graecum*) seed and Lupinus luteus (Burgos

Diaz *et al*., 2016), wherein the greatest EC was detected in extremely acidic and alkaline environments.

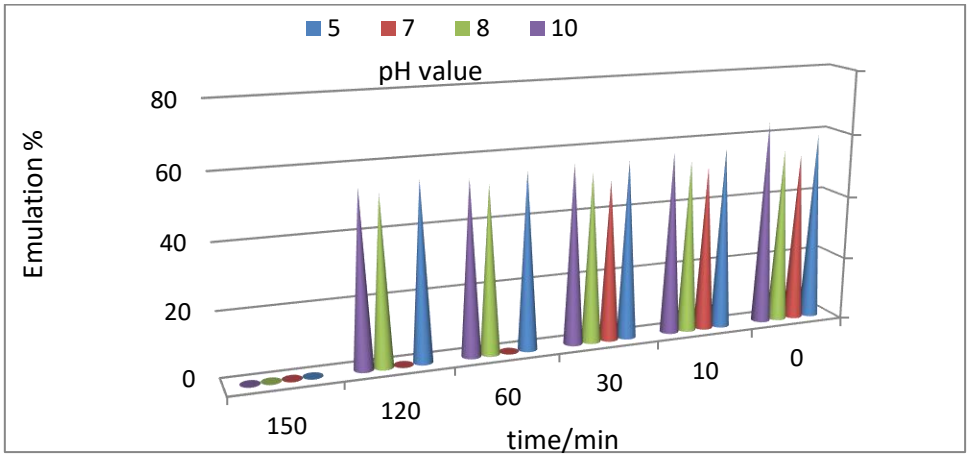


Figure 4: Effect of deferent pH on protein isolate emulation capacity.

3.3 Biochemical properties:

3.3.1 Antioxidant activity:

Antioxidant activity of protein isolation was determined through the analysis of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals for free radical scavenging activity. The percentage of DPPH radical inhibition represented the radical scavenging activity (Figure 5). The results showed that the free radical scavenging activity was most effective at pH 5 (31.44%), followed by pH 7, with the lowest inhibition of DPPH radical at pH 8 (8.03%) with a significant difference ($p \leq 0.05$). This demonstrates the pH variation effect of antioxidant activity. showing that these protein isolates have a higher number of amino acid residues exposed, leading to an increased amount of active sites for scavenging DPPH radicals. The analysis of amino acid composition in Figure also reinforces these findings, with the protein isolate having the highest levels of antioxidant amino acids (Trp, Met, His, Tyr, and Lys). These amino acids stabilize free radicals by providing protons or electrons (You *et al*., 2009). Variables that may impact antioxidant effectiveness could be the type of protein, the arrangement of amino acids, and the protein's level of hydrophobic amino acids. Proteins rich in hydrophobic amino acids like phenylalanine, tryptophan, and methionine exhibit antioxidant properties. The act of attaching amino acids to fats increases, leading to a decrease in the presence of free radicals and an increase in antioxidant activity at a pH of 4.5-7.5-10. The protein has significant amounts of hydrophobic amino acids like leucine, iso-leucine, cysteine, methionine, and basic amino acids including histidine, arginine, and lysine. that antioxidant activity depends upon the oxidation rate of antioxidant compounds, and this oxidation rate was influenced by the surrounding pH (Jovanovic *et al*. 1994).

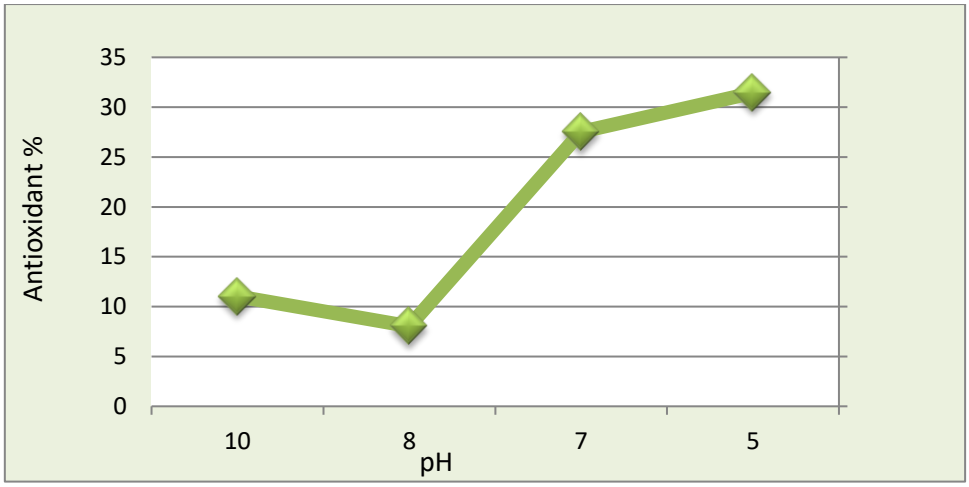


Figure (5) Effect of deferent pH on protein isolate antioxidant activity

3.3.2 Protein digestibility:

Bioavailability is the fraction of consumed food components that can be used for various physiological functions at their intended site of action (Guerra *et al.*,2012). Bioavailability involves the readiness of a substance to be absorbed and used by the body. The entire journey of a nutrient after it is consumed, which includes its breakdown and absorption in the digestive system. In this study, the digestibility of the isolated protein of *P. juliflora* sprouts was determined, and the digested protein of *P. juliflora* sprouts was 80.18%, and this value was much higher to that of soy protein isolate (71.04%) (Wang *et al.*, 2010).

3.3.3 Cytotoxic protein detection

Figure 6 displays the findings of cytotoxicity detection in *P. juliflora* protein isolated. The findings indicated that the protein isolated at concentrations ranging from 100 to 1000 micrograms/ml did not induce any noticeable alterations in human blood cells, such as sedimentation or degradation, when exposed to 37°C for 10, 30, and 60 minutes, in comparison to the control group.

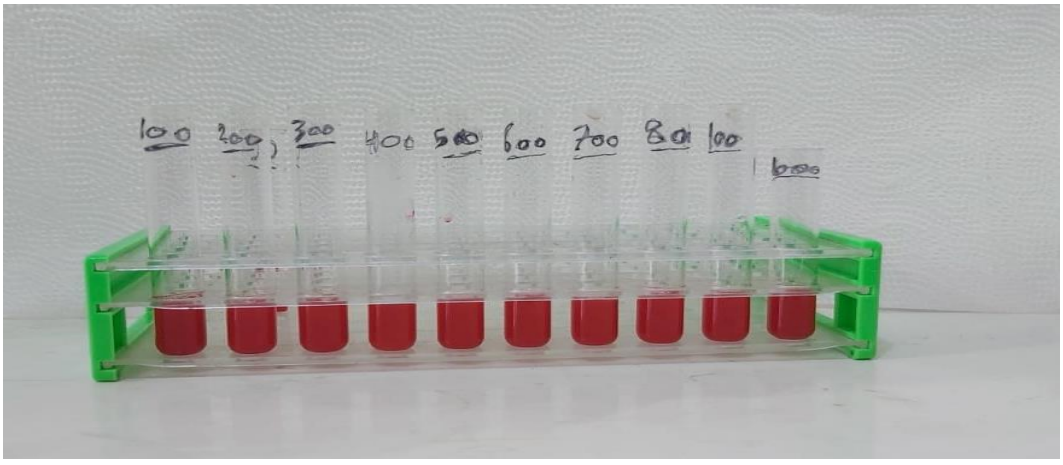


Figure 6: Cytotoxic activity of protein isolate at different concentrations

3.4 Inhibitory activity of the protein isolates against different bacteria

The table 7 displays the protein isolate's ability to stimulate activity against three different bacteria strains. The protein

isolate had varying stimulating effects depending on the bacteria type, with a significant difference ($p \leq 0.05$) observed and the largest halo diameters of 2.5, 2, and 2 mm observed with concentrations of 0.5% for *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, respectively, in pigeons. A concentration of 0.2% resulted in a minimum areola diameter of 0.5 mm. The protein's ability to inhibit bacteria could be attributed to its high levels of amino acids, particularly lysine, arginine, and histidine, which are positively charged. The positive charge network boosts the interaction between the protein and the bacteria's surface negative charge, resulting in an initial bond via electrical interaction with the positive protein. Lipopolysaccharides lead to membrane disruption, creating an unstable area that results in membrane destruction and cell death.

Table 2: Antibacterial activity of protein isolate at different concentrations

Inhibitory zone (mm)			**Concentration**
Staphylococcus aureus	*Escherichia coli*	*Bacillus subtilis*	**%**
0.5	1	2	0.2
1	1.5	2	0.3
2	2	2.5	0.5
-	-	-	0

(-) Indicates no inhibition

3.5 Protein characterization

3.5.1 Amino acids profile:

The amino acid composition (µg/g protein) of *P. juliflora* pods protein isolate is shown in the table 3 and figure 8. Proline was predominant among the nonessential amino acid (97.47µ/g protein),while the essential amino acid are isoleucine and histidine (97.44 and 96. 66 µ/g protein) and the presence of hydrophobic amino acid ,such as leaucine, methionine ,proline and alanine ,also showed significant performance in the functional properties of food proteins ,amino acid composition .Hashim *et al*.(2024) observed that the amino acids found in the trypsin –hydrolyzed pod of *P. juliflora* include aspartic acid 11.4%,glutamic acid 2.8% serine 5% histidine 2.6% tyrosine 4.9% , 27.1% cysteine ,39.9%valine,5.7%methionine ,0.1% isoleucine and 0.1%leucine. The hydrolyzed papain pod of *P. juliflora* contains 30% aspartic acid ,2.1% glutamic acid, 5.8% serine ,5% histidine ,7.7% cysteine ,9.2% phenylalanine ,18.1% isoleucine ,12.8% leucine and 3.1% lysine.

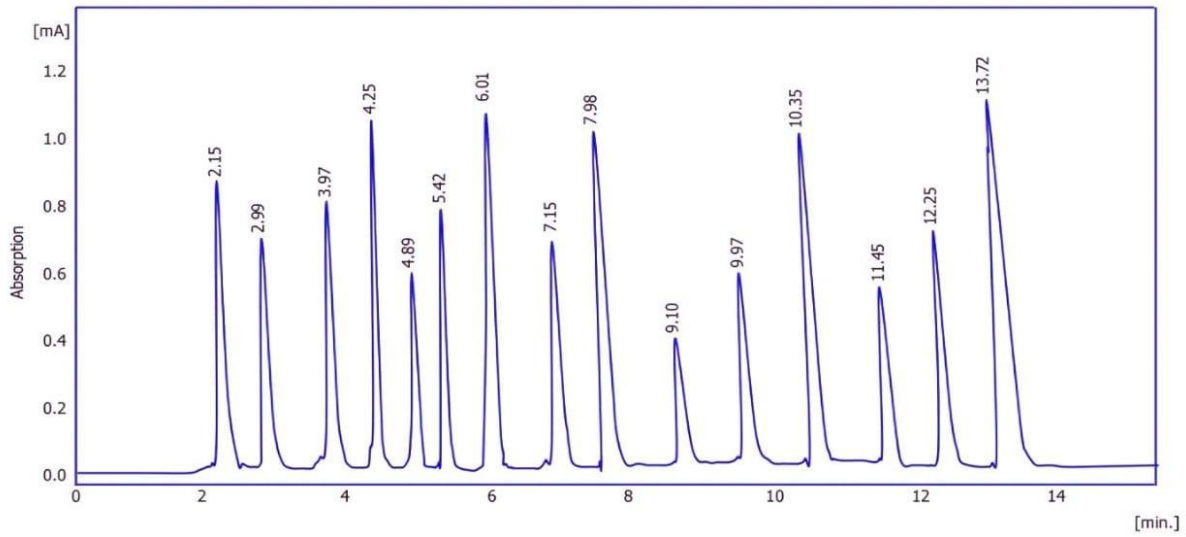


Figure 8: Content of protein isolate of amino acid

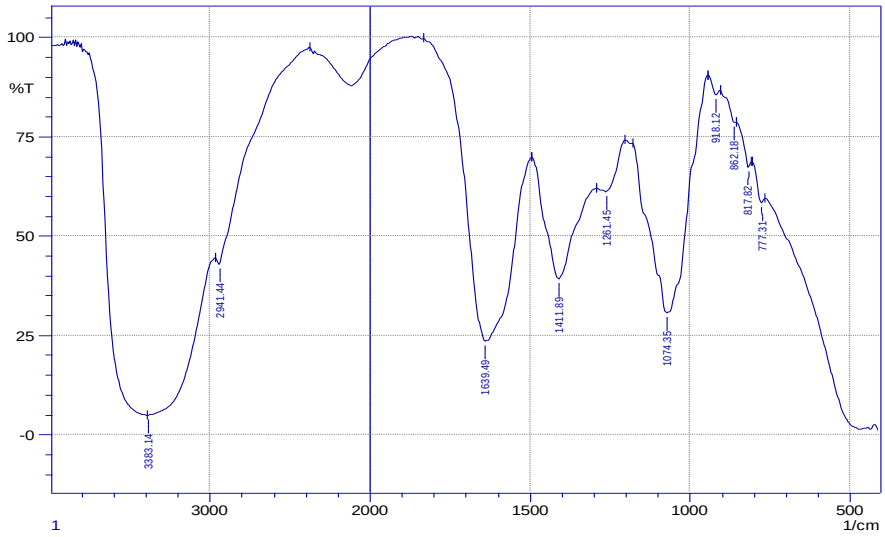
Table 3. Content of *P. juliflora* pods protein isolate of amino acid

No.	Reten. Time (min)	Area(mAU.s)	Compound name	Amount (µg/gm)
1	2.15	1256.89	Lysine	90.25
2	2.99	2541.00	Methionine	85.46
3	3.97	1985.48	Trptophan	75.99
4	4.25	3526.44	Arginine	94.28
5	4.89	4256.98	Threonine	82.13
6	5.42	5214.89	Valine	94.56
7	6.01	1623.25	Isoleucine	97.44
8	7.12	4265.28	Lucin	90.25
9	7.98	24513.65	Pheynlalanine	48.97
10	9.10	2013.65	Glutamic acid	62.58
11	9.97	4215.98	Aspartic acid	84.15
12	10.35	3201.46	Histidine	96.66
13	11.45	2155.98	Serine	42.58
14	12.25	1954.78	Proline	97.47
15	13.72	2013.65	Cysteine	88.05

3.5. 2 FTIR test

The arrangement of atoms in a biological compound can be identified. Use FTIR spectroscopy to analyze the infrared radiation absorbed by the sample based on wavelength and intensity. This method is commonly used to analyze the secondary structure of proteins or polypeptides and their hydrolases. The FT-IR spectra of the isolated protein are shown in Figure 8. Characteristic IR signals of amides A, B, and I–VI were identified. The two most important vibrational bands in the protein backbone are the amide I and II bands. The Amide I region, located in the 1700-1600 cm spectral range, is the most sensitive region for analysis of secondary structural elements of polypeptides due to its association with C=O stretching vibrations. Protein functional groups (N-H and C=O)

were analyzed in isolated *P. juliflora* pods protein through FT-MIR spectroscopy. The associated IR bands were identified at 1411.89 cm -1 (amide II bands), 1639.49 cm -1 (amide I band), between 3333.14 and 2941.44 cm -1 (amide A and B bands), and 1261.45-777.31 cm -1 -1 (Tiwari and Singh, 2012). Food components such as carbohydrates and fats have distinct intramolecular bonds. Carbohydrates generate a peak around 1200-900 cm-1, indicating stretching of CO-C and CO-OH (Nugrahani *et al*., 2020).



4. Conclusion

P. juliflora pods can serve as a source of protein due to their high protein content. The solubility, foaming, oil binding and emulsifying properties and also antioxidant activity of proteins are greatly affected by the pH level. Besides, the protein isolates showed the ability to inactivate three types of bacteria, excellent digestibility, and a high level of essential amino acids necessary for human body growth, without any cytotoxic effects.

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مقاله علمی_پژوهشی

افزایش خواص عملکردی و فعالیت آنتی اکسیدانی ایزوله پروتئین غلاف *Prosopis juliflora* از طریق تنظیم pH، همزمان با ارزیابی ویژگی‌های فیزیکوشیمیایی و فعالیت باکتریواستاتیک.

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چکیده

این مطالعه به بررسی خواص فیزیکوشیمیایی غلاف *Prosopis juliflora* و تهیه ایزوله پروتئینی آن پرداخته است. هنگامی که سطوح pH در ۵، ۷، ۸ و ۱۰ تنظیم شد، تغییرات در خواص عملکردی با تفاوت معنی دار ($P \leq ۰.۰۵$) در ویژگی‌های عملکردی و فعالیت های آنتی اکسیدانی و ضد باکتریایی مشاهده گردید. مقدار حلالیت بالا در pH 5 و10 و اتصال لیپید در pH 10 به دست آمد، در حالی که کمترین مقدار برای هر دو ویژگی در pH 7 یافت شد. بالا ترین ظرفیت کف کردن در pH 5 پس از ۵ دقیقه مشاهده اما پس از ۱ دقیقه در pH 8 مقدار آن کاهش یافت. ظرفیت امولسیون کنندگی در pH 10 و فعالیت مهار رادیکال DPPH در pH 5 بالا بدست آمد، اما در pH 8 مقدار آن کاهش یافت و همچنین ایزوله پروتئین فعالیت مهارکنندگی را در برابر اشریشیا کلی، باسیلوس سوبتیلیس و استافیلوکوکوس اورئوس از خود نشان داد. با این حال، بیشترین بازدارندگی در برابر باکتری باسیلوس در غلظت پروتئین ایزوله ۰.۵ در صد مشاهده شد، در حالی که قابلیت هضم پروتئین بالاتر بود. پروتئین جدا شده هیچ اثر سیتوتوکسیک قابل توجهی بر سلول های خونی انسان نداشت. همچنین برای تعیین ساختار پروتئین از آزمون FTIR استفاده گردید.

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Scientific Research

Isolation and Identification of Lactic Acid Bacteria from Different Sources and Testing their Ability to Produce Cellulases Enzyme

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ABSTRACT

The current study aims to enhance work in the field of isolating microorganisms that produce enzymes with practical uses, sustainability and food industries, by isolating and identifying lactic acid bacteria strains from different sources, some of which contain cellulose, which microorganisms depend on, primarily for their nutrition and reproduction, and which is present in the environment in which they are found, and the possibility of producing the enzyme Cellulase, which is considered one of the important enzymes in the analysis of polysaccharides (cellulose) and the production of monosaccharides and simple sugars. This study included the isolation and identification of Lactobacillus bacteria from different sources, purification, screening, identification and determination of their efficiency in producing cellulase enzymes. The results showed that fifteen isolates were obtained from various sources including soil, fruits, vegetables, pickles, dairy products and live fish entrails. Agar MRS medium with 0.5% (w/v) CaCl2 granules was used to isolate lactic acid bacteria, which were identified by phenotypic, biochemical and Gram staining tests. A preliminary screening was performed by estimating the enzyme activity on the solid medium by measuring the diameter of the transparent halo formed around the colonies in the medium. The results were enhanced by measuring the activity of the enzyme produced on the liquid medium by measuring the light absorption using a spectrophotometer. The results obtained showed that the best isolates in enzyme production are the isolates that produced the highest enzyme efficiency on the solid medium, which is the one isolated from the guts of live fish (fish 3), which formed a transparent halo with a diameter of 3 cm on the solid medium and the highest efficiency in the liquid medium, which obtained the highest absorption in the spectrophotometer, highest enzymatic activity, reaching ٤,٥٤٦ and Specific activity ١٠,٣٣١. After selecting the best isolate from among the isolates, the optimum conditions for enzyme production were studied in different situations, including [temperature, pH, vibrating incubator speed, inoculum quantity, different carbon and nitrogen sources and the period required for fermentation], and the following results were obtained: The optimum temperature for production is 35 °C with an enzymatic activity reached (3.425) and Specific activity (4.502), the pH value was 6 with the enzymatic activity reached (3.437) and Specific activity (4.399), the enzymatic activity was (3.419), and specific activity was (4.804), a fermentation time of 72 hours with an efficiency of (3.065) and Specific activity (4.305),the best carbon source cellulose with an efficiency of (5.44), and the best Nitrogen source wheat bran with an efficiency of (3.634) and a inoculum content of 5% with an efficiency of (3.399).

1-Introduction

Lactic acid bacteria (LAB) are important microorganisms that produce lactic acid primarily as a by-product during metabolic activities and play multifaceted roles in the agricultural, food and clinical sectors. Lactic acid bacteria are used in many fermentation processes, as the use of these bacteria is considered one of the most traditional and used sciences in food processing and preservation. Due to the importance of lactic acid bacteria in many food applications and because they have therapeutic properties to enhance human health, research is ongoing to obtain strains with properties that enhance the quality of food products. [1]. These bacteria are associated with humans through fermented foods, dairy products and other sciences and applications [2]. They are heterotrophic, Gram-positive, single rods or short chains [*3*], non-spore-forming, anaerobic but aerobic, and produce lactic acid as one of the main fermentation products by using carbohydrates during fermentation. It produces organic materials that contribute to flavor, taste and smell, which give the products in which it is used unique sensory properties [4]. Lactobacillus plantarum is one of the most widely used genera in the food industry, whether as microbial starters or as probiotic microorganisms. Several strains of L. plantarum have been shown to produce different antimicrobial compounds such as organic acids, hydrogen peroxide, as well as bacteriocins and antimicrobial peptides, each of which has a variable spectrum of action [*5*]. These receptors are thought to be a defense mechanism deployed by bacteria to prevent microorganisms from colonizing their natural environment by inhibiting their proliferation and survival. Other important biological activities such as biofilm formation and the production of biosurfactants play a vital role in suppressing the attachment of pathogens [6,7]. Lactic acid bacteria produce organic acids and other metabolites that enhance the development of flavor in food, prevent spoilage, and are therefore very useful in many applications, especially in the food and dairy industries. The dairy sector in particular greatly benefits from lactic acid bacteria, and therefore the potential of lactic acid bacteria as starters should be investigated as product quality and sensory appeal are greatly influenced by the role of starters in dairy [8]. Lactic acid bacteria have been classified into different genera and species based on their acid-production properties through the fermentation of sugars and their growth at specific temperatures [9]. In addition, lactic acid bacteria can be classified as homozygous or heterozygous organisms based on their ability to ferment carbohydrates [10]. Homogeneous lactic acid bacteria such as Lactococcus and Streptococcus produce two molecules of lactate from one molecule of glucose while heterogeneous lactic acid bacteria such as Leuconostoc and some lactobacilli generate lactate, ethanol and carbon dioxide from one molecule of glucose [11]. There are more than 260 species of lactic acid bacteria that exhibit a wide range of physical, ecological and genetic traits. The genus Lactobacillus was recently reclassified by scientists into 25 genera. This reclassification was necessary because of the diversity of the original genus, which made it extremely difficult to classify, name and differentiate between different lactobacilli. The new genera are Lactobacillus, Paralactobacillus and 23 new genera. The twenty-three new genera include [23] [12] (*Amylolactobacillus*, *Acetilactobacillus*, *Agrilactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioa*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, Lentilactobacillus, *Levilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Loigolactobacilus*, *Paucilactobacillus*, *Schleiferilactobacillus*, and *Secundilactobacillus*) [13].

The term “cellulases” refers to a group of enzymes that catalyze the hydrolysis of cellulose into polysaccharides and is an important enzyme in industrial biotechnology today. All living systems regulate their biological activity through enzymes. An enzyme is a protein molecule that acts as a biological catalyst that increases the speed and rate of a reaction, and most cellular reactions occur much faster than they would in the absence of an enzyme. [14]. Cellulolytic enzymes produced by microorganisms have

many biotechnological and industrial applications. Due to the use of cellulose in many industries such as textiles, detergents, food, animal feed, biofuel, paper and pulp, pharmaceuticals, and waste management, they are required in large quantities. The first step in developing a process for producing cellulases is the isolation and characterization of bacterial strains capable of producing them. This is a very important step due to the demand for enzymes with many applications in biotechnology. [15] and [16]. The cellulases group consists of three types of enzymes secreted outside the cell: β-glucosidase, 1,4-β-endoglucanase, and 1,4-β- exoglucanase. [17] The most important organisms that produce cellulases are bacteria and fungi, and these microorganisms are usually found in the soil. The potential bacteria to produce cellulases are Cellulomonas, Pseudomonas, Thermoactinomycetes, and Bacillus spp. [18]

2.Materials and methods:

2.1 Sample Collection: 15 samples were collected from different sources to isolate lactic acid bacteria. These samples included fruits [bananas, watermelons, grapes, tomatoes], vegetables [cucumbers, watercress], yogurt, 3 pickle samples, and 4 fish samples. They were brought to the laboratory in sterile, tightly sealed plastic bags, except for fish samples that were brought to the laboratory live, dissected, and the digestive system was extracted. Microorganisms were isolated from the viscera of live fish.

2.2 Isolation of Lactic Acid Bacteria: Twenty-five grams of each sample were added to 250 ml of 0.85% saline solution [NSS] and decimal dilutions of the samples were made by taking one ml of the sample to nine ml of physiological solution and dilution continued to obtain concentration 7-10. After that, 0.1 ml of each dilution was spread on (MRS Agar) with CaCl2 granules at a rate of 0.5% [w/v] and in two replicates for each dilution. The plates were incubated at 37 °C for 48-72 hours under anaerobic conditions. The formation of a transparent halo was observed on MRS agar with CaCl2. The isolates that formed halos were grown individually on MRS agar [the process was repeated three times] to obtain pure isolates. [19, 20]

2.3 Bacterial Diagnosis

- *Microscopic examinations:*

1- Staining bacteria with Gram stain: Colonies growing on a solid MRS medium were selected and cells were stained with Gram stain to identify cell morphology and clustering [21].
2- Motility assay: A drop of 24-hour-old bacteria from the tubes was placed on a motility slide. [21].

- *Biochemical tests:*

1- Catalase test: This test was performed by mixing a bacterial colony aged [24] hours with a drop of water, then adding a drop of 3% hydrogen peroxide prepared on a clean glass slide. Note the formation of bubbles on the slide as evidence of a positive test. [22].
2- Oxidase test: A bacterial colony of 18-24 hours of age was transferred using a sterile wooden stick to a filter paper and a drop of oxidase indicator was added to it. The colony turned dark purple, indicating a positive test [22].
3- SCA: Isolates were cultured at 24 h on the prepared SCA medium and then incubated at 37°C for 7 days. A Change in the color of the medium from green to blue is a positive result [23].
4- Indole test: The tubes containing indole medium (Tryptone Broth were prepared by dissolving 2% g of tryptone and 0.5% NaCl, then the pH was adjusted to 7, then distributed into test tubes at a rate of 10 ml for each tube, then sterilized) were inoculated with a bacterial culture aged [24] hours. It was incubated at a temperature of 37°C for [48-24] hours. After completing the incubation period, a few drops of Kovacs reagent were added [the reagent was obtained from the Al-Basheer Scientific Office in Baghdad Governorate. [24].
5- Growth at temperatures above 45°C: All isolates were grown on MRS agar medium in two replicates at a temperature above 45°C and incubated inverted for 48-24 hours to observe their ability to grow. [21].
6- Growth at temperatures below 5°C: All isolates were grown on MRS agar medium in two replicates at a temperature below 5°C

and incubated inverted for 48-24 hours to observe their ability to grow [21].

7- Gas production test from glucose: MRS liquid culture tubes and Durham tubes were inoculated inverted with a drop of 24-hour-old bacterial cultures and incubated at 37°C for 24-48 hours to observe their ability to produce gas from glucose [21].
8- Sugar fermentation test: The medium was prepared using the components of liquid MRS without adding glucose and meat extract, and added to its chlorophenol red converter at a concentration of 0.004%. Then, sugars were added (each separately) at a concentration of 2% to the aforementioned medium, which included (fructose, mannose, glucose, galactose, sucrose, maltose, menthol, radiculose, salicin, xylose, melibiose, raffinose, arabinose, and arabinose) after adjusting the pH (6.2-6.5]. The medium was sterilized, while the sugars (mannose-maltose-xylose) were filtered before adding them to the previously sterilized medium. [21].

2.4 Production of Cellulases Enzyme on Solid Medium: The special solid medium was used for the production of Cellulases, which were prepared from the following components g/l (CaCl2, 0.3 and FeSO4,7H2O, 5 and MnSo4. H2O, 1.6 and CMC, 8 and Tween80, 2 and Peptone, 0.8 and KH2PO4, 2 and Urea, 0.3 and MgSO4,7H2O, 0.5 and ZNSo4, 1.4 and $(NH4)_2So4$, 1.4 and Agar, 20 and distilled water, 1 litre). The solid medium was inoculated with active bacteria aged 24 hours and the plates were incubated at 37°C for 48 hours [25].

2.5 Detection of the Enzyme Product: The production of the enzyme Cellulases is indicated by using the iodine-hydrochloric acid (HCl)-I reagent. This reagent is prepared by taking 100 ml of 0.1 N HCl (+ 500 ml of) + ½ l KI 2% (this reagent is added to the dish containing the pure bacterial colony and left for 5 minutes, then the solution is poured out and the dish is left for 10 minutes. Then a light-colored halo is observed around the colony, indicating the conversion of cellulose into simple sugars] [26]

2.6 Production of Cellulase Enzyme in Liquid Medium

The liquid nutrient medium for the production of Cellulases enzyme was used and prepared from the following materials (FeSO4.7H2O, 0.0004%, CACl2.2H2O, 0.0001%, MgSO4.7H2O, 0.02%, Tryptone, 0.20%, KH2PO4, 0.4%, Na2HPO4, 0.04%, and CMC, 1.0%). The pH was adjusted to 7 and sterilized at 121°C for a quarter of an hour]. 50 ml of the medium was placed in a 250 ml flask for each isolate. The medium was inoculated with 2.5 ml (5%) of the bacterial isolate and incubated in a shaking incubator at 37°C for 48 hours at a speed of 150 rpm. After incubation, the medium was transferred into test tubes and the medium was centrifuged to remove unwanted materials at a speed of 6000 rpm for 10 minutes. The filtrate was collected after centrifugation, which is considered the source of the raw enzyme and is used to determine the enzyme activity. [27]

2.7 Optimal Conditions for the Production of Cellulase Enzyme from the Obtained Isolate

The optimum conditions for the production of the cellulase enzyme from the local isolate were studied by studying the production and enzymatic activity in the cell-free medium and at different temperatures.

1. The temperatures (45, 40, 35, 30, 25) °C were used, and the enzyme production was then estimated by Enzymatic activity through measuring optical absorption at 550 nm.
2. The effect of the PH was studied, the values (10, 9, 8, 6, 5, 4) were used and the enzyme production was then estimated by Enzymatic activity through measuring optical absorption at 550 nm,
3. The effect of the speed of the incubator Shaker was studied, and the following speeds (180, 160, 140, 120, 100) rpm were used, after which the enzyme production was estimated.
4. The effect of the fermentation time to produce enzymes using the local isolate was also tested at (144, 120, 96, 72, 24) minutes and the enzyme production was estimated.
5. Studying the effect of different carbon sources on enzyme production from the local isolate was done, as the following carbon sources were used (glucose, molasses, sunflower oil, cellulose, glycerol, mantol),

6. and the enzyme production was then estimated.
7. The effect of the nitrogen source was also studied using the components of the previous medium with a change in the nitrogen source used in production. The following nitrogen sources were used (wheat bran, urea, glutamic acid, whey, ammonium nitrate), and the enzyme production was then estimated.
8. The effect of the inoculum ratio on the enzyme production from the local isolate was studied, as the following inoculum ratios were used (7, 6, 4, 3, 2, 1).

2.8 Statistical analysis

All measurements were done in triplicate and data presented are mean values ± standard deviation. A one-way analysis of variance (ANOVA) was applied to assess the diffusion diameters of cellulase activity, however, a two-way analysis was performed to analyze the screening of cellulase producing lactic acid bacteria, enzymatic activity and Specific activity and Optimal Conditions for the Production of Cellulase Enzyme from the Obtained Isolate. Mean comparisons were carried out using the L.S. D test at a level of significance $pp \leq 0.05$. SPSS program ver. 25 was applied to analyze data

3.Results and Discussion

The results of the isolation of Lactobacillus bacteria resulted in obtaining 15 isolates belonging to this genus. The isolates obtained varied according to the sources of isolation used in this study, as the sources varied to include (milk, cucumber, banana, watermelon, soil, grapes, fish four isolates, pickles three isolates, watercress and tomatoes). Figure [1] shows the results of the morphological examinations of the bacteria.

The results showed that the colonies of Lactobacillus bacteria growing on the solid MRS medium were circular in shape and small in size, some were convex and others were flat, smooth, soft and shiny. As for their color, some were white while others were creamy in color. Microscopic examination showed that they were cells of different shapes, as some were rod-shaped while others were spherical rod-shaped, some were single and some were double, while some were in the form of long or short chains, according to what was stated in [28]. It is a Gram-positive, non-spore-forming, non-motile bacterium, and the results were consistent with what was mentioned by [29].

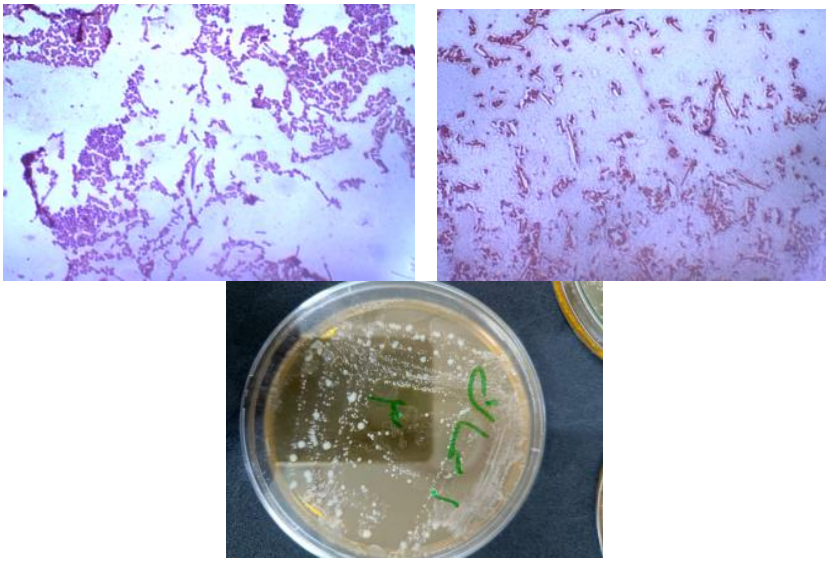

Figure [1] Phenotypic tests of lactic acid bacteria

3.1 Biochemical characteristics

When conducting biochemical tests on Lactobacillus isolates, it was found that all isolates were positive for Gram stain, did not produce catalase except for isolate [Fish 2] and oxidase, did not consume citrate, did not produce indole, could ferment glucose, and grew at 45°C but did not grow at 5°C. Table [1]

Table [1] Biochemical tests of bacterial isolates

Indole	Growth at 5	Growth ٤٥	Gram stain	Gas production	consume citrate	Catalase	Oxidase	
-	-	+	+	+	-	-	-	**Yogurt**
-	-	+	+	+	-	-	-	**cucumber**
-	-	+	+	+	-	-	-	**banana**
-	-	+	+	-	-	-	-	**Watermelon**
-	-	+	+	+	-	-	-	**soil**
-	-	+	+	-	-	-	-	**Grapes**
-	-	+	+	+	-	-	-	**Fish 1**
-	-	+	+	+	-	+	-	**Fish 2**
-	-	+	+	+	-	-	-	**Fish 3**
-	-	+	+	+	-	-	-	**Fish 4**
-	-	+	+	-	-	-	-	**Pickle 1**
-	-	+	+	+	-	-	-	**Pickle 2**
-	-	+	+	+	-	-	-	**Pickle 3**
-	-	+	+	+	-	-	-	**Watercress**
-	-	+	+	-	-	-	-	**tomatoes**

3.2 Sugar Fermentation

The ability of Lactobacillus bacteria to ferment some types of sugars was tested, which included [galactose, mannitol, sucrose, melibiose, sorbiose, lactose, raffinose, glucose, fructose, sorbitol, and xylose]. The results in Table [2] showed that Lactobacillus isolates had different abilities to ferment sugars, as there were [4] isolates that could ferment galactose sugar, [4] isolates that could ferment mannitol sugar, [6] isolates that could ferment sucrose sugar, two isolates that could ferment melibiose sugar, none of the isolates could ferment sorbose sugar, three isolates could ferment lactose sugar, and there were [5] isolates that could ferment raffinose sugar, [5] isolates that could ferment glucose sugar, [4] isolates that could ferment fructose, and [6] isolates that could On fermentation of sorbitol and all isolates have the ability to ferment xylose.

Table [2] Fermentation of sugars by the isolates under study

Sugar Fermentation											
Xylose	Sorbitol	Fructose	Glucose	Raffinose	Lactose	Sorbiose	Melibiose	Sucrose	Manthol	Galactos	
+	+	+	+	-	+	-	-	+	+	+	Yogurt
+	+-	+	+	+	+	-	-	+	+	+	cucumber
+	-	-	-	-	+	-	-	-	-	-	banana
+	-	-	-	-	-	-	-	-	-	-	Watermelon
+	+	-	-	-	-	-	-	-	-	-	soil
+	-	-	-	-	-	-	-	-	+	-	Grapes
+	-	-	-	+	-	-	-	-	-	-	Fish 1
+	-	-	-	+	-	-	-	-	-	-	Fish 2
+	+	+	+	+	-	-	-	+	-	+	Fish 3
+	-	-	+	-	-	-	∓	-	-	-	Fish 4
+	-	-	-	-	-	-	+	-	+	-	Pickle 1
+	-	+	+	-	-	-	-	+	-	-	Pickle 2
+	-	-	-	+	-	-	-	-	-	+	Pickle 3

+	+	-	-	-	-	-	-	+	-	-	Watercress
+	+	-	-	-	-	-	-	+	-	-	tomatoes

3.3 Screening of Isolates for the Production of Cellulases

A preliminary screening of the isolates obtained through the study was conducted Table [3] by measuring the diameter of the transparent halo formed around the colonies growing on the solid medium containing CMC as a carbon source. The results showed that the isolates isolated from live fish aggregations were superior to the other isolates from different sources. If the isolate Fish 3 obtained the highest halo diameter, which reached 3 cm, then the isolate Fish 1 had a halo diameter of [2.6] and Fish 4, which was equal to it, the isolate Pickle 2, which had a halo diameter of 1.4 cm. These results were close to the results of the isolates isolated from pickles, which ranged between 1.4 and 1.2 cm. It was followed by watercress, which was equal to pickle 1 with a result of 1.3 cm, then tomatoes, then watermelon, soil, grapes, cucumbers, yogurt, and finally the isolates isolated from bananas, which had a diameter of 0.4 cm.

Table [3] Diameter of the transparent halo around the colonies on the solid medium for the production of Cellulases

isolation	Yogurt	cucumber	banana	Watermelon	soil	Grapes	Fish 1	Fish 2	Fish 3	Fish 4	Pickle 1	Pickle 2	Pickle 3	Watercress	tomatoes
zone diameter	٠,٥	٠,٦	٠,٤	٠,٩	٠,٨	٠,٧	٢,٦	0	٣	١,٤	١,٣	١,٤	١,٢	١,٣	١,١

The isolates that excelled in production were then selected and a secondary screening was conducted for them by estimating the activity of the enzyme produced in the liquid medium containing CMC as a carbon source. After preparing and sterilizing the medium and inoculating it with the active isolates aged 24-48 hours, and through three replicates for each isolate, the results in Table [4] were obtained, which showed the superiority of Isolate (Fish 3), which obtained the highest absorption in the spectrophotometer, which represents the highest enzymatic activity, reaching ٤,٥٤٦ and Specific activity ١٠,٣٣١. It was followed by Fish Isolate 4 and Pickle 2, which were ٢,٦٩٣ and ٢,٦٣٩, respectively and a Specific activity ٤,٩٠٥, ٤,٨٥١, respectively. The results showed a decrease in the enzymatic activity and Specific activity of the enzyme produced by the other isolates, which included Fish 1, Pickle 1 and Pickle 3, which were equal to ٢,١٦٩, ١,٧٧٥ and ٢,٠٤٩, with Specific activity ٣,٨٠٩, ٣,٧٦٠ and ٣,٦١٢. as in Figure [2]. Fish Isolate 3 was chosen as the best isolate for enzyme production.

Table 4 Secondary screening of some lactic acid bacteria isolates producing the enzyme Cellulases

Isolate	Specific activity	Enzymatic activity
Fish 1	٣,٨٠٩ ± 0.002	٢,١٦٩ ± 0.003
Fish 3	١٠,٣٣١ ± 0.003	٤,٥٤٦ ± 0.004
Fish 4	٤,٩٠٥ ± 0.002	٢,٦٩٣ ± 0.002
Pickle 1	٣,٧٦٠ ± 0.002	١,٧٧٥ ± 0.003
Pickle 2	٤,٨٥١ ± 0.004	٢,٦٣٩ ± 0.002
Pickle 3	٣,٦١٢ ± 0.004	٢,٠٤٩ ± 0.003

3.4 Study of the optimum conditions for the production of the enzyme Cellulases

The study of the optimum conditions for the production of the enzyme from the selected isolate [Fish 3] included all of the temperatures, acidity function, incubator speed, storage period, carbon and nitrogen sources, and the percentage of vaccine used.

4.Temperature

The results in Figure [2] showed that the effectiveness increased with the increase in the fermentation temperature and that the best temperature for enzyme production from local fish isolates [3] was 35 degrees Celsius, as the enzymatic activity reached (3.425) and Specific activity (4.502) compared to temperatures (25, 30, 37, 40, 45) in which the enzymatic activity was (2.١٦٩, 2.497, 2.214, 1.645, 0.٥20) and Specific activity (3.309, 3.504, 3.66, 3.051, 1.015) respectively. The reason for the high effectiveness at temperatures of 35 degrees and temperatures close to it is due to the high ability of the bacterial isolate under study to produce the enzyme, as the temperature is ideal for the growth of these bacteria. The results of the statistical analysis showed significant differences in both enzyme activity and specific activity during the temperature experiment. The results agreed with the results obtained by [30], who confirmed that the best temperature for production is 35 degrees Celsius and agreed with what was reached by [31] and the results agreed with what was stated by [32], who stated that the best temperature was 35°C, while it differed from what [33] reached, who stated that the best temperature is 30°C. The reason for the best temperature for production may be due to the source of the isolation and the conditions in which the isolation lives, as its source is fish that live in waters that are often moderate to low temperatures.

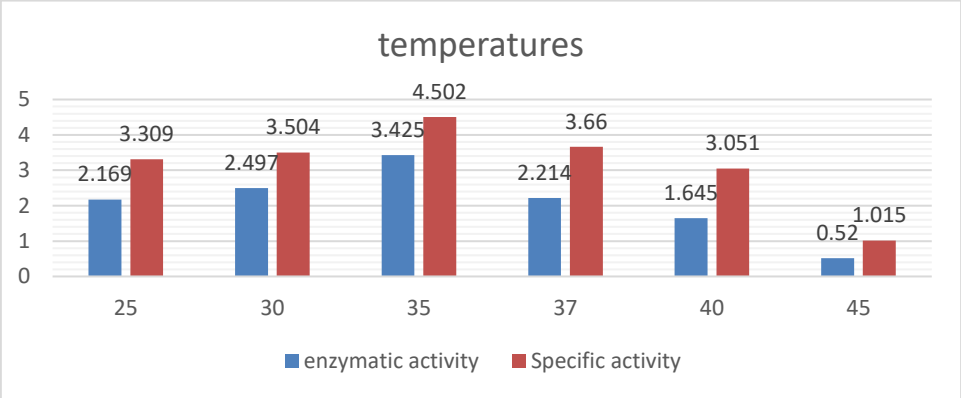

Figure [2] The effect of temperature on the production of the enzyme Cellulases.

L.S.D.= 0.831 for the interference between enzymatic activity and temperature.
L.S.D.= 1.021 for the interference between specific activity and temperature.

1- The PH

The results of the study confirmed that the best production of the cellulase enzyme and the highest enzymatic activity of the enzyme produced from the local isolate [fish 3] were at (PH 6) where the enzymatic activity reached its highest levels and reached (3.437) with Specific activity (4.399) and while the results for the rest of the acid functions were 0.278 at (PH 10) , 1.010 at (PH 9) , 1.989 at (PH 8) and 2.125 at PH 7 while the activity decreased from its highest value at the acid function (PH 6) to 1.295 at PH 4 and 2 at PH 5 and Specific activity (1.201, 2.02, 2.505, 3.101, 2.333, 3.534) respectively as shown in Figure [3] The results of the pH study showed highly significant differences in both enzyme activity and specific activity at the ($p \leq 0.05$). and the results agreed with what was reached by [30,34] who confirmed that the best enzyme production and activity were at (PH 6) and did not agree with the results of [35] and [31]] who found that the best degree of production was (PH 6) and

(PH 8) and [36] found that the optimal pH was [7].

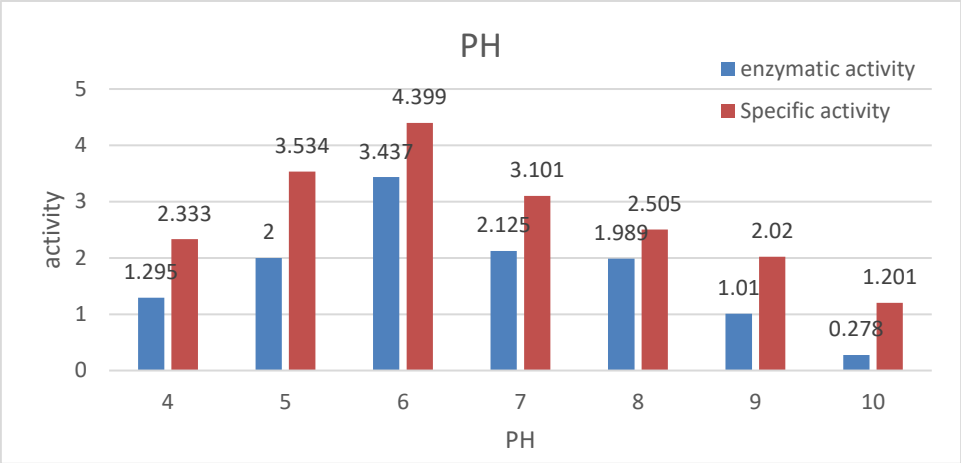

Figure [3] Effect of pH on Cellulase production

L.S.D.= 1.121 for the interference between enzymatic activity and Effect of pH.
L.S.D.= 0.8.02 for the interference between specific activity and Effect of pH.

2- Incubator Speed

The results of the study confirmed that the enzyme activity began to increase with an increase in the speed of the vibrating incubator, as it reached the highest activity (3.419) and with Specific activity (4.804) at a speed of 150 rpm, and the activity decreased when the speed increased or decreased from this level. The enzymatic activity ranged between 2.879 at a speed of 160 rpm and 2.459 at a speed of 100 rpm, Also, the Specific activity reached its highest levels at a speed of 150 rpm, reaching 4.804. After that, Specific activity decrease with an increase or decrease in the speed of the incubator, reaching its lowest level of 3.694 at a speed of 100 rpm. as shown in Figure [4]. The results of the research were consistent with the results obtained by researchers (37, 38, 33, 31) who proved that the best speed of the vibrating incubator for the production of the cellulase enzyme was 150 rpm. The results differed from (35, 30, and 39), who showed that the best production speed was 120 cycles/minute.

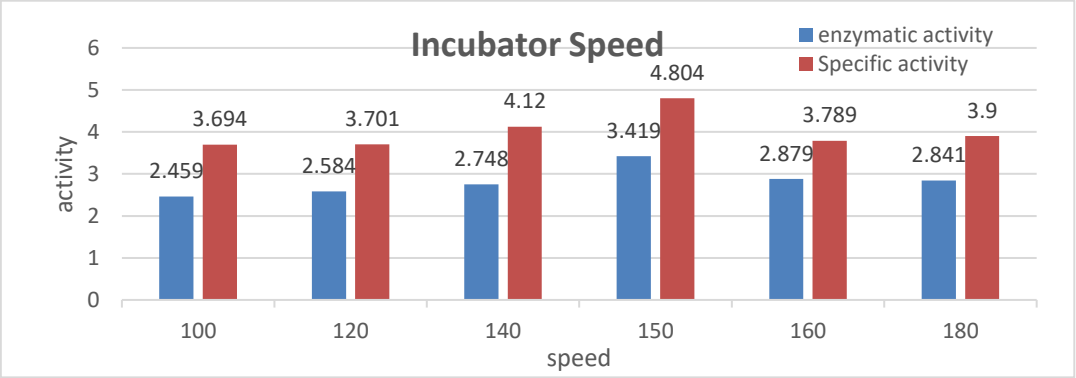

Figure [4] Effect of the vibrating incubator speed on Cellulase production

L.S.D.= 0.560 for the interference between enzymatic activity and Effect of the vibrating incubator speed.
L.S.D.= 0.587 for the interference between specific activity and Effect of the vibrating incubator speed.

3- Fermentation Time

Figure [5] shows the results of estimating the enzymatic activity of the cellulase enzyme produced by the local isolate Fish 3. It was found that the activity began to increase from the beginning of fermentation and after 24 hours from the start of the experiment, the

enzymatic activity reached 2.672 and began to increase after 48 hours and reached 2.722.

The enzymatic activity reached its highest levels after 72 hours from the start of the experiment and reached the highest value of the activity (3.065) and a Specific activity of (4.305). Then the activity began to decrease with time until it became (2.551, 2.377, 2.246, and 1.939 after 96, 120, 144 and 150) hours, respectively.

The reason for this increase in enzyme production and enzyme activity is due to the availability of suitable conditions and nutrients for the bacterial isolate, which reached its highest levels after 72 hours, after which the concentration of nutrients began to gradually decrease, which was accompanied by a decrease in the concentration of the produced enzyme as well as the inhibitory effect of fermentation products, which include the enzyme and other metabolic products.

The results were consistent with the findings of [40], [35], and [36], who found that the optimum time for enzyme production from lactic acid bacteria was 72 hours. The results differed from [14], [41], and [42], who found that the optimum period for fermentation was [24], [48], and [96], respectively.

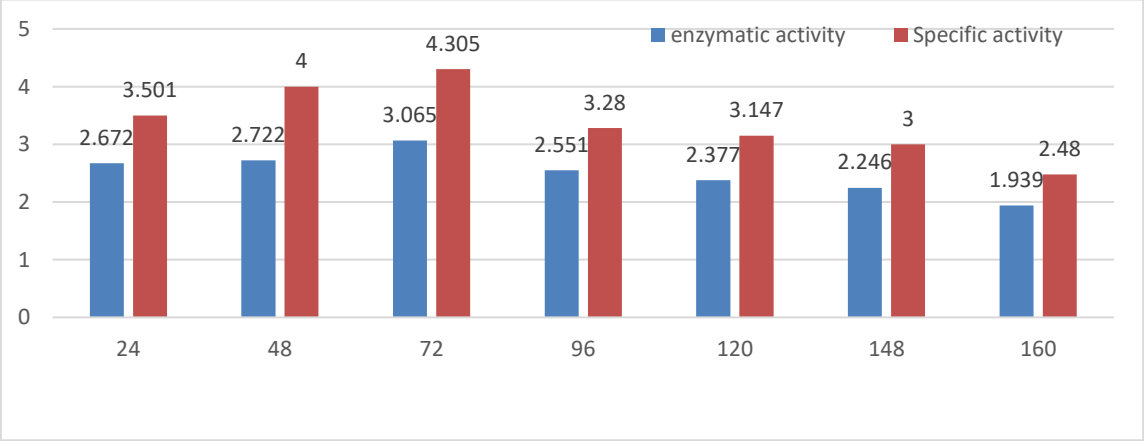

Figure [5] Effect of fermentation time on **Cellulase** production.

L.S.D.= 0.340 for the interference between enzymatic activity and Effect of fermentation time**.**
L.S.D.= 0.301for the interference between specific activity and Effect of fermentation time**.**

4- Carbon Source

The results of the study showed as in Figure [6] that the best carbon source for the production of the Cellulases enzyme by the local isolation of fish 3 was cellulose, where the enzyme activity reached its highest values when used and was (5.44) and a Specific activity of (6.8), followed by CMC which reached an activity of (5.229), then glucose, which reached an activity of 3.169 when used, then molasses, then mannitol, sunflower oil, and finally glycerol (2.896, 1.868, 1.786, 1.617) respectively. This work is similar to the work of [43], [30] and [33].

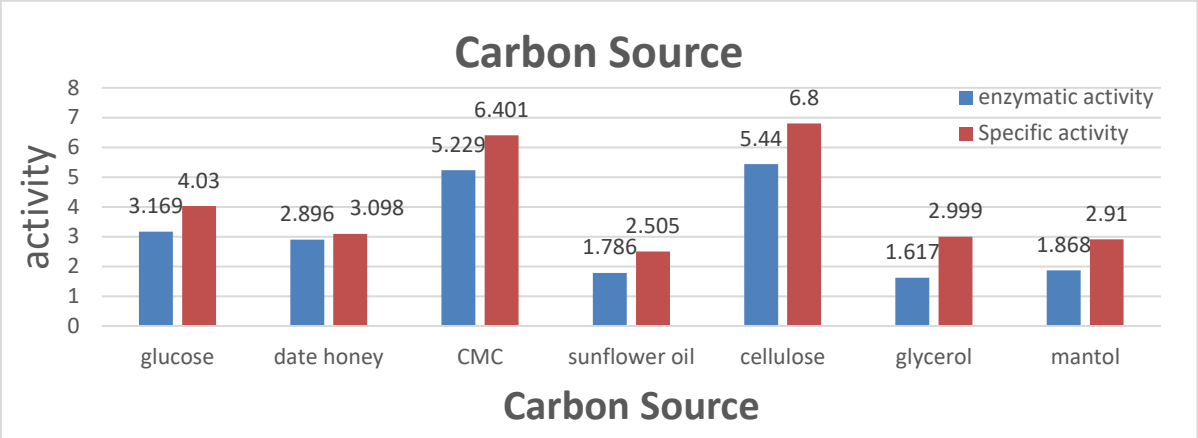

Figure [6] The effect of the carbon source on the production of the **Cellulase.**

L.S.D.= 0.380 for the interference between enzymatic activity and Effect of the carbon source**.**
L.S.D.= 0.410 for the interference between specific activity and Effect of the carbon source**.**

5- Nitrogen Source

Figure (7) shows the results of the effect of the carbon source used in the production of the Cellulases enzyme by the isolate under study, which is Fish 3. Wheat bran outperformed all the nitrogen sources used, as the effectiveness reached its highest value (3.634) and a Specific activity of (4.402), followed by tryptone as the second-best nitrogen source with effectiveness of 3.185, while the results were close when using urea and whey, whose effectiveness reached 2.786 and 2.535, respectively, then came glutamic acid with an effectiveness of 2.076, and finally came ammonium nitrate with effectiveness of 1.863, as in Figure [7]. This work is similar to the work of

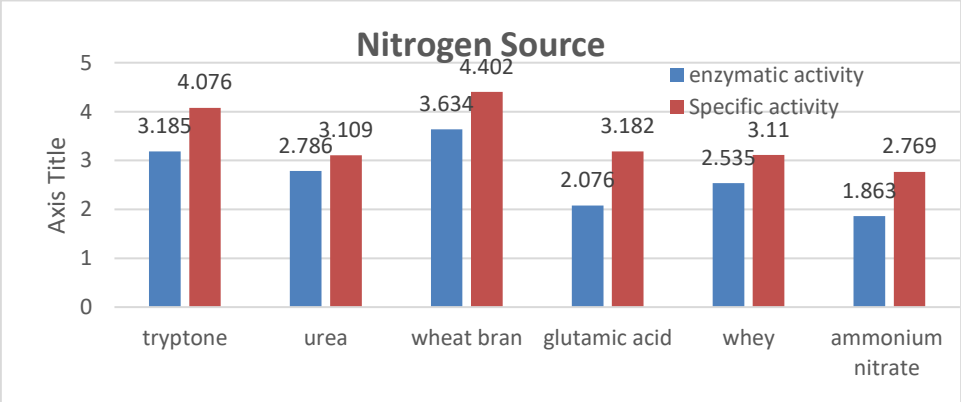

Figure [7] Effect of the nitrogen source on **Cellulase** production.

L.S.D.= 0.567 for the interference between enzymatic activity and Effect of the nitrogen source**.**
L.S.D.= 0.398 for the interference between specific activity and Effect of the nitrogen source**.**

6- Inoculum Size

Figure [8] shows a noticeable increase in the enzymatic activity of the enzyme produced with the increase in the percentage of vaccine from the active culture at the age of 24-48 hours. The figure shows that the activity increased from its lowest level of 0.169 when using a vaccine percentage of 1% to the highest level (3.399) at a vaccine percentage of 5% and the Specific activity was (5.002). It then began to decrease at a vaccine percentage of 6% and 7%, and the activity reached 2.786 and 2.628.

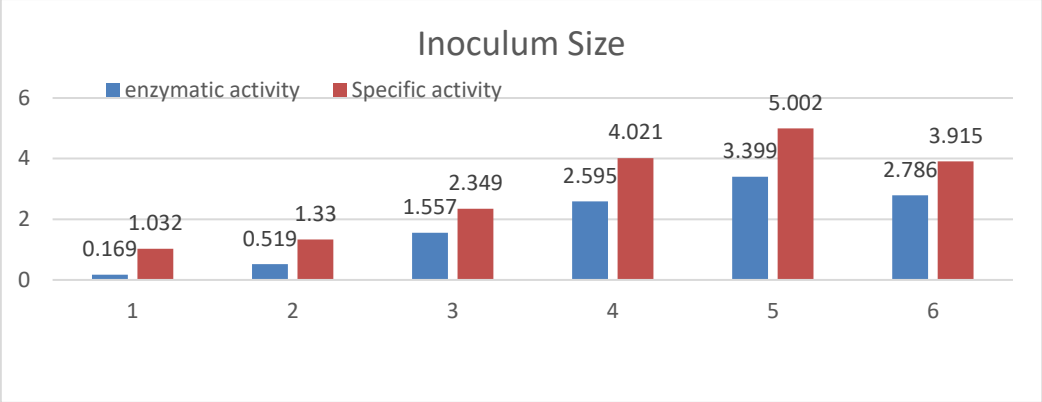

Figure [8] Effect of the vaccine percentage on Cellulase production

L.S.D.= 0.586 for the interference between enzymatic activity and Effect of the vaccine percentage**.**
L.S.D.= 0.102 for the interference between specific activity and Effect of the vaccine percentage**.**

Conclusion: The current study showed that 15 isolates of lactic acid bacteria were acquired and diagnosed by morphological and biochemical tests. the ability of lactic acid bacteria to produce cellulase enzyme was tested as well as the screening by estimating the enzyme activity and Specific activity.

It was found that there are 14 isolates capable of producing the enzymes by measuring the diameter of the clear zone around the colonies as a result of the enzyme activity, as well as estimating the enzyme activity and Specific activity by measuring the light absorption, which proved that the isolate (fishes 3) is the best isolate.

The optimum conditions for enzyme production were studied using the same light absorption method, which included (temperature, acidity function, incubator speed, fermentation time, inoculum size, carbon source and nitrogen source).

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Scientific Research

A Comparative Analysis of the Impact of Incorporating Modified Potato and Corn Starch on the Textural, Rheological, and Sensory Attributes of Low-Fat Mayonnaise

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ABSTRACT

In response to the detrimental effects of dietary fat on consumer health, particularly its association with cardiovascular diseases, obesity, and various other health-related issues, there has been a concerted effort in contemporary food production to develop low-fat food products. The objective of this study is to evaluate the production of low-fat mayonnaise with oil content levels of 20%, 25%, and 30%. This investigation employs modified potato and corn starches at concentrations of 2. 5%, 3%, and 3. 5% The study aims to systematically assess the relative impact of these formulations on key parameters, including viscosity, pH, acidity, stability, and physical properties, throughout a storage period of six months. Additionally, sensory properties will be evaluated to provide a comprehensive analysis of the product's quality and consumer acceptability. The findings indicated that the incorporation of both starches and elevated fat levels resulted in significant increases in pH values, viscosity, emulsion stability, tissue stiffness, and the adhesion properties of mayonnaise, concurrently leading to a reduction in the acidity of the samples. During the storage period at refrigeration temperatures, a significant reduction in viscosity, pH, firmness, and tissue adhesion was observed in the mayonnaise samples, accompanied by an increase in acidity and creaminess ($p<0.05$) The findings from the sensory evaluation demonstrated that elevated levels of starches and fats significantly improved the scores of sensory parameters ($p < 0.05$) The sensory evaluation revealed that the formulation of low-fat mayonnaise, which comprised 30% fat and 3. 5% modified potato starch, exhibited the highest level of sensory acceptance among the tested samples. The present study demonstrated that low-fat treatments utilizing modified potato starch exhibited superior quality characteristics in comparison to samples incorporating corn starch. In conclusion, the incorporation of 30% fat and 3. 5% modified potato starch as a fat substitute yields beneficial physicochemical and sensory properties in mayonnaise.

1- Introduction

Mayonnaise is widely recognized as one of the most prevalent salad dressings globally. This formulation represents a semi-solid oil-in-water (O/W) emulsion, composed of approximately 70-80% vegetable oil, along with vinegar, salt, egg yolk, and a thickening agent. Nonetheless, the elevated oil content of this product categorizes it as an unhealthy food option, as excessive consumption may result in elevated lipid levels within consumers' bodies. Increased consumption of dietary fats is associated with elevated cholesterol levels, contributes to the development of obesity, and markedly heightens the risk of cardiovascular disease [1]. The growing apprehension regarding the detrimental effects of excessive fat consumption on human health has prompted considerable initiatives within the industry to formulate products with lower fat content. Fat is integral to the structural and organoleptic properties of food products, significantly influencing their aroma, flavor, and mouthfeel. This reliance on fat presents considerable challenges in the formulation of low-fat food products [2]. In the formulation of low-fat products, it is essential to incorporate additives that are capable of replicating the critical organoleptic characteristics of fats. These additives serve as viable alternatives, facilitating the reduction of fat content in these products while maintaining desirable sensory attributes [3] .Starches represent a notable alternative to fats in food formulations, and prior research has examined the influence of various starch types on the quality characteristics of low-fat mayonnaise [2] [4].

Starches serve as significant additives in the food processing industry, contributing to enhancements in both the aesthetic qualities and sensory attributes of food products. These substances function as bulking or thickening agents, improving the visual appeal and texture of food products without contributing additional nutritional value. In the industry, a diverse range of starch sources, including potato, corn, cassava, and sweet potato, is utilized for this objective [5]. Approximately 80% of the total global starch production is ascribed to corn starch [6]. This starch serves a dual purpose; it is employed in the production of corn syrup and functions as a thickening agent in the formulation of a diverse range of food products [7]. Potato starch is a significant and extensively utilized polysaccharide in the food industry, functioning as a stabilizing, gelling, and thickening agent in the formulation and processing of various food products. This particular starch exhibits elevated transparency and viscosity, along with a relatively low gelatinization temperature. Nevertheless, the applicability of native and natural starches in the food industry is constrained by factors such as the variability of pH levels, low temperature conditions, and the mechanical processes involved in food preparation[8].

Modified starch is a derivative of starch that is produced through various physical and chemical treatments applied to raw starch. Through these modifications, the processed

starch develops novel properties that fulfill distinct requirements across multiple industries, in contrast to raw starch, which does not possess such functionalities. It demonstrates functionality [9] . Modified starch can be synthesized through various techniques, including chemical, physical, enzymatic, and genetic methods, or through a combination of these approaches. Chemical methods represent the oldest and most prevalent approaches to starch modification. In this methodology, the incorporation of specialized chemical moieties, including ester functional groups, distinct ions, and acetyl groups, onto starch molecules facilitates the emergence of novel properties. Acetate starch, octenyl succinyl starch, and monophosphate starches are highly regarded in food applications due to their remarkable stability during the defrosting process [10]. Acetylated starch has been recognized for approximately one century and is utilized to enhance concentration, stability, and texture in various applications. Acetylated starch is synthesized through the process of esterification, wherein natural starch is reacted with anhydrous acetic acid in the presence of an alkaline catalyst. Acetylation enhances various physicochemical properties of starch, notably increasing solubility, swelling capacity, viscosity, and stability in relation to thermal processing and prolonged storage. Furthermore, it contributes to a reduction in gelatinization temperature and mitigates detrimental alterations in food products that may arise from starch retrogradation during processing and storage. The acetylation of starch is influenced by various parameters, including the concentrations of the reactants, the duration of the reaction, the pH of the reaction medium, and the presence of a catalyst. These factors collectively determine the extent of substitution of the target functional groups within the starch polymer [11]. The objective of this research was to examine the impacts of utilizing acetylated corn and potato starch as fat replacers on the qualitative and organoleptic characteristics of low-fat mayonnaise.

2- Materials and Methods

2-1-Materials

Oil, sugar, salt, eggs, and vinegar were procured from a local retail establishment in Tehran. Mustard powder and sodium benzoate were sourced from Adonis Goldaro in Iran, citric acid was procured from Merck in Germany, while acetylated corn starch was obtained from Roquette in France, and acetylated potato starch was acquired from Lyckeby in Sweden. The chemicals utilized in the experiments were procured from Merck (Germany) [3].

2-2- Preparation of Mayonnaise Treatments

The formulations of the low-fat mayonnaise treatments examined in this study are presented in Table 1. In the preparation of mayonnaise utilizing an oil substitute, xanthan gum and guar gum were combined with modified starches in a single container. Concurrently, other powdered ingredients were placed in a separate container. Initially, water was introduced into the tank, following which the spatula and homogenizer were activated. Subsequently, the powder materials excluding starch, comprising sugar, salt, citric acid, mustard, potassium sorbate, and sodium benzoate, were introduced into the tank via the funnel valve designated for powder materials.

Following a duration of two minutes, the egg yolk was subsequently introduced. Less than fifty percent of the oil was combined with the starch powders, specifically xanthan, guar, and modified starch. Subsequently, this mixture was gradually introduced to the other ingredients through a funnel while being stirred for a duration of three minutes. After a duration of three minutes, the homogenizer was calibrated to a setting of 53, at which point vinegar was introduced through the funnel valve into the liquid material. The valve was gradually opened to facilitate the incremental addition of vinegar, while oil was simultaneously introduced through the adjacent funnel valve. The homogenizer was set to a speed of 59, adhering to the protocol that vinegar should be added prior to the incorporation of oil. Upon the completion of the oil extraction, the valves were systematically closed. Subsequently, after a duration of five minutes, the homogenizer was adjusted to an operational setting of 70. Subsequent to an additional five minutes of operation, the homogenizer was deactivated, and the vacuum pump was engaged for a duration of one minute to establish a vacuum. After a duration of one minute, the valve for the liquid material was activated, and the homogenizer was calibrated to a setting of 7/33 in order to facilitate the filling of the mayonnaise jars [12] .

Table 1. Formulations of low-fat mayonnaise

Ingredients	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	**T_7**	**T_8**	**T_9**
Sugar	150	150	150	150	150	150	150	150	150
Salt	51	51	51	51	51	51	51	51	51
Mustard	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5
Xanthan	9	9	9	9	9	9	9	9	9
Citric acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Guar	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Potassium sorbate	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Sodium benzoate	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Egg yolk	68.1	68.1	68.1	68.1	68.1	68.1	68.1	68.1	68.1
Vinegar	165.0	165.0	165.0	165.0	165.0	165.0	165.0	165.0	165.0
Water	1940.4	1790.4	1640.4	1865.4	1850.4	1835.4	1865.4	1850.4	1835.4
Oil	600	750	900	600	600	600	600	600	600
Starch L	0	0	0	75	90	105	0	0	0

Starch R	0	0	0	0	0	0	75	90	105

Ingredients	**T_{10}**	**T_{11}**	**T_{12}**	**T_{13}**	**T_{14}**	**T_{15}**	**T_{16}**	**T_{17}**	**T_{18}**
Sugar	150	150	150	150	150	150	150	150	150
Salt	51	51	51	51	51	51	51	51	51
Mustard	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5
Xanthan	9	9	9	9	9	9	9	9	9
Citric acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Guar	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Potassium sorbate	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Sodium benzoate	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Egg yolk	68.1	68.1	68.1	68.1	68.1	68.1	68.1	68.1	68.1
Vinegar	165.0	165.0	165.0	165.0	165.0	165.0	165.0	165.0	165.0
Water	1715.4	1700.4	1685.4	1715.4	1700.4	1685.4	1565.4	1550.4	1535.4
Oil	750	750	750	750	750	750	900	900	900
Starch L	0	0	0	75	90	105	0	0	0
Starch R	75	90	105	0	0	0	75	90	105

Ingredients	**T_{19}**	**T_{20}**	**T_{21}**
Sugar	150	150	150
Salt	51	51	51
Mustard	10.5	10.5	10.5
Xanthan	9	9	9
Citric acid	0.9	0.9	0.9
Guar	2.7	2.7	2.7

Potassium sorbate	1.2	1.2	1.2
Sodium benzoate	1.2	1.2	1.2
Egg yolk	68.1	68.1	68.1
Vinegar	165.0	165.0	165.0
Water	1565.4	1550.4	1535.4
Oil	75	90	105
Starch L	0	0	0
Starch R			

2-3-Viscosity Measurement

The viscosity of mayonnaise was assessed utilizing the DV-π+ Pro viscometer manufactured by Brookfield Engineering Laboratories. To achieve this objective, a total of 500 grams of the sample were transferred into a 600 mL beaker. Subsequently, the viscosity of the samples was measured in pascal-seconds (centipoise) utilizing spindle number 7 at a rotational speed of 100 revolutions per minute (rpm) [13].

2-4- Measurement of pH and Acidity

The pH levels of the mayonnaise treatments were determined utilizing a pH meter, in accordance with the procedure outlined by the Iranian National Standard No. The temperature was recorded at 2454 Kelvin under ambient conditions. To assess the acidity of the treatments, the methodology delineated in Standard Number 2454 was utilized, and titration was conducted utilizing 0. 1 N sodium hydroxide.

2-4- Measurement of Emulsion Stability

To assess the physical stability of the mayonnaise emulsion, a sample weighing 15 grams was placed in a centrifuge tube with an inner diameter of 15 mm and a height of 125 mm. Subsequently, the tubes were subjected to centrifugation for a duration of 30 minutes at a rotational speed of 5000 revolutions per minute (rpm). The percentage of emulsion stability was determined utilizing the following equation [14]:

$$\text{Emulsion Stability } (\%) = \frac{\textit{The initial weight of the sample}}{\textit{Sample weight after oil separation:}} \times ۱۰۰$$

(۱

2-5- Texture Analysis

The textural characteristics of the mayonnaise samples were assessed using a CT3 texture analysis system manufactured by Brookfield (USA). A cylindrical probe with a diameter of 57 mm and a load cell capacity of 4500 grams was employed in this study. It is essential to acknowledge that the instructions supplied by the

manufacturing entity were adhered to in order to select the appropriate type of probe and to determine the other relevant parameters employed in the process. The probe exhibited a penetration speed of 1 mm/s, achieving a penetration depth of 30 mm into the sample. The present study examined tissue parameters, specifically focusing on tissue stiffness and adhesion [15].

2-6- Sensory Evaluation

A sensory evaluation of mayonnaise samples was performed utilizing a hedonic testing method based on a 5-point hedonic scale. In this scale, a score of 1 was designated as the lowest evaluation, while a score of 5 represented the highest assessment for each sensory attribute. The sensory attributes examined in this study encompassed taste, texture, coloration, and overall acceptability [16] .

2-7- Data Analysis

The experiments were executed utilizing a completely randomized design, comprising three repetitions for each condition. The results were subjected to a two-way analysis of variance (ANOVA) using SPSS software version 22 to assess the presence of significant differences among the data. To conduct a comparison of the mean values associated with the various treatments, Duncan's multiple range test was utilized, with a significance threshold set at 5% ($p < 0.05$) The graphs presented herein were generated utilizing Microsoft Excel software.

3- Results and Discussion

3-1- Viscosity of Mayonnaise

The findings regarding the variations in viscosity observed across various mayonnaise treatments throughout a six-month storage duration under refrigerated conditions are presented in Table 2. The viscosity of mayonnaise exhibited a significant dependence on both the formulation of the sauce, specifically the type of starch employed and the proportion of fat utilized, as well as the duration of storage ($p < 0.05$) The data presented in the table indicates a significant decrease in viscosity over the storage period, specifically from the initial day to the conclusion of the sixth month ($p < 0.05$) It was observed that an increase in fat content from 20% to 30% resulted in a significant enhancement in the viscosity of all tested samples. A comparative analysis of the produced samples infused with modified potato and corn starch demonstrated that, at equivalent concentrations, the formulations incorporating potato starch exhibited superior viscosity. Increasing the fat content in mayonnaise formulation from 20% to 30% contributes to the attainment of the requisite fat necessary for the formation of a stable oil-in-water emulsion. Moreover, the escalation in the quantity of fat and the proliferation of oil droplets resulting from agitation contribute to the viscosity of the emulsion. This phenomenon occurs through the establishment of a continuous structural framework and the formation of spatial barriers [17]. Furthermore, an increase in the concentration of modified starch resulted in elevated viscosity levels. This phenomenon can be attributed to the hydrophilic characteristics and water absorption capacity of these compounds. Upon the immersion of modified starches in water, a notable absorption of water occurs, resulting in an increase in their hydrodynamic volume. Hydrodynamic

volume refers to the spatial extent occupied by an individual polymer molecule following its immersion in water and subsequent absorption of water. An increase in the volume occupied by the polymer corresponds to a pronounced influence on the rheological properties, as it is associated with a greater capacity for water retention [18], Consequently, an increase in the quantity of potato starch is expected to promote a greater hydrodynamic volume in comparison to corn starch. This enhancement is likely to result in an elevated viscosity of emulsions utilizing potato starch when contrasted with those employing corn starch. Conversely, the incorporation of higher quantities of modified starches into mayonnaise formulations enhances viscosity in the aqueous phase. This phenomenon can be attributed to the hydrophilic characteristics of modified starches, which facilitate the formation of hydrogen bonds with water molecules via their hydroxyl groups. This subsequently results in an enhancement of both the viscosity and the stability of the overall emulsion system [19]. The observed reduction in viscosity across various mayonnaise samples during the storage period is likely attributable to the concomitant decrease in pH and the increase in acidity levels. As the pH level decreases, the stability of the emulsion tends to decline, resulting in a concomitant reduction in viscosity [20]. Pishan et al., 2019 revealed that the incorporation of starch into a low-fat and low-calorie mayonnaise formulation resulted in an augmentation of the viscosity of the resulting samples [21]. Consistent with these findings, Wang et al., 2022 research indicated that the apparent viscosity of the samples exhibited an increase following the incorporation of modified corn starch (hydroxypropylated starch) into the formulation of low-fat mayonnaise [22] . The research conducted by Agyei-Amponsah et al., 2021 revealed an increase in the viscosity of low-fat mayonnaise following the incorporation of starch-based fat substitutes [23].

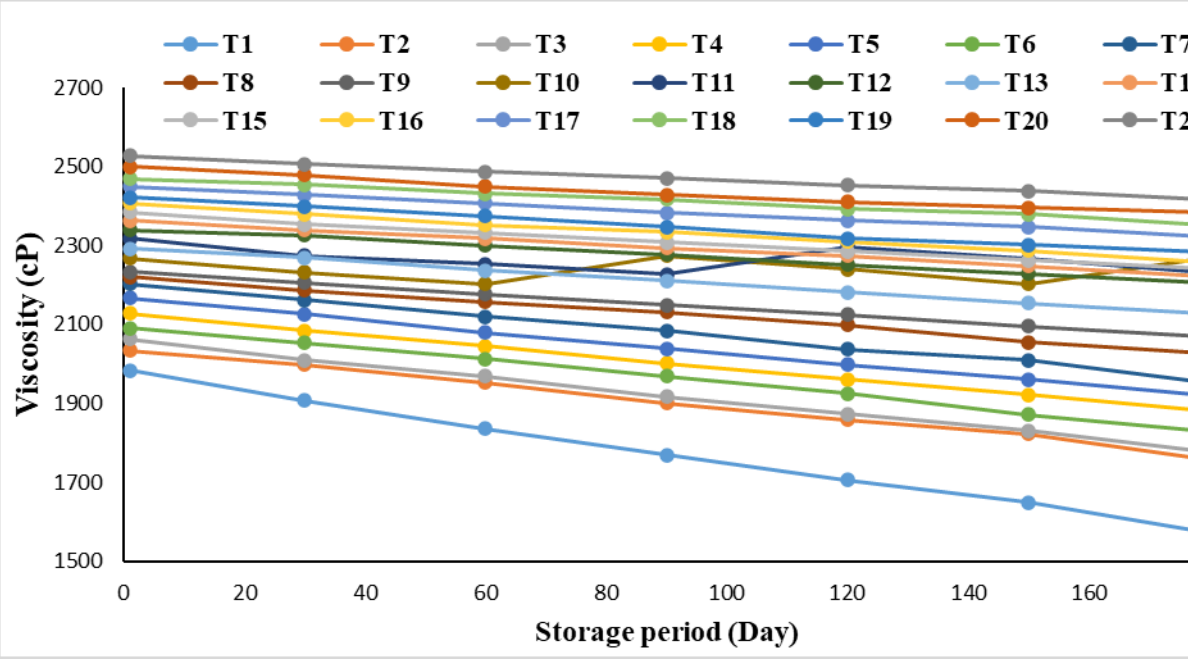

Fig 1. Changes in viscosity (cP) values of low-fat mayonnaise treatments during storage period

3-2- pH and Acidity of Mayonnaise

The alterations in the mean values of pH and acidity across various mayonnaise treatments throughout the storage duration are presented in Tables 3 and 4, respectively. The findings indicate that on the initial day of observation, there were no statistically significant differences in pH and acidity among the samples ($p < 0.05$). The incorporation of higher proportions of both modified starches in the formulation of low-fat mayonnaise resulted in a significant increase in pH, accompanied by a notable decrease in acidity ($p < 0.05$). Nevertheless, the data indicated a significant decrease in pH levels and a corresponding increase in acidity with prolonged storage duration ($p < 0.05$). Furthermore, an analysis comparing the treatments that incorporated modified corn and potato starch throughout the storage period revealed that the formulations containing potato starch exhibited a statistically significant increase in pH and a corresponding decrease in acidity. The observed increase in total acidity and the concomitant decrease in pH during storage are likely attributable to the metabolic activity of lactic acid bacteria present in the aqueous phase of mayonnaise [24]. The observed elevation in acidity and concomitant reduction in pH may also result from the hydrolytic and oxidative activities of the enzymes present in the eggs utilized in the formulation of mayonnaise sauce [25]. In accordance with Iranian Standard No. According to the established standards, the minimum acidity level of mayonnaise must be 0.6% when measured in terms of acetic acid percentage. The findings of this research indicated that the acidity levels of all treatments analyzed fell within the parameters established by the national standards of Iran, specifically ranging from 0.61% to 01% It is probable that an increase in the concentration of starches will result in a decrease in the water activity of mayonnaise. Furthermore, the utilization of potato starch may prove to be more efficacious in diminishing water activity. As the water activity diminishes, the quantity of water accessible for the metabolic activities of lactic acid microorganisms, along with other microbial entities present in the sauce, correspondingly decreases. As a result of the diminished activity of these microorganisms, there will be a reduced production of acid, leading to a lesser alteration in acidity and pH levels when compared to treatments characterized by lower starch content [26]. Amiri *et al*., 2019 conducted a study to assess the impact of incorporating uncoated barley beta-glucan as a fat substitute on the physicochemical properties of low-fat mayonnaise. The findings of this study indicated that the incorporation of beta-glucan, along with an increase in its concentration, led to an elevation in pH levels and a reduction in acidity when compared to treatments involving lower quantities of beta-glucan. Conversely, extending the storage duration of various mayonnaise treatments resulted in an elevation of acidity levels, which subsequently led to a reduction in the pH of the products [13]. An increased pH in low-fat mayonnaise resulting from the incorporation of native sago, tapioca, and corn starches was evidenced in the research conducted by Paramitasari *et al*., 2024, aligning with the findings of the current study. Hakimian et al., 2022 observed a reduction in pH and an increase in acidity of mayonnaise sauce over the course of the storage period [28].

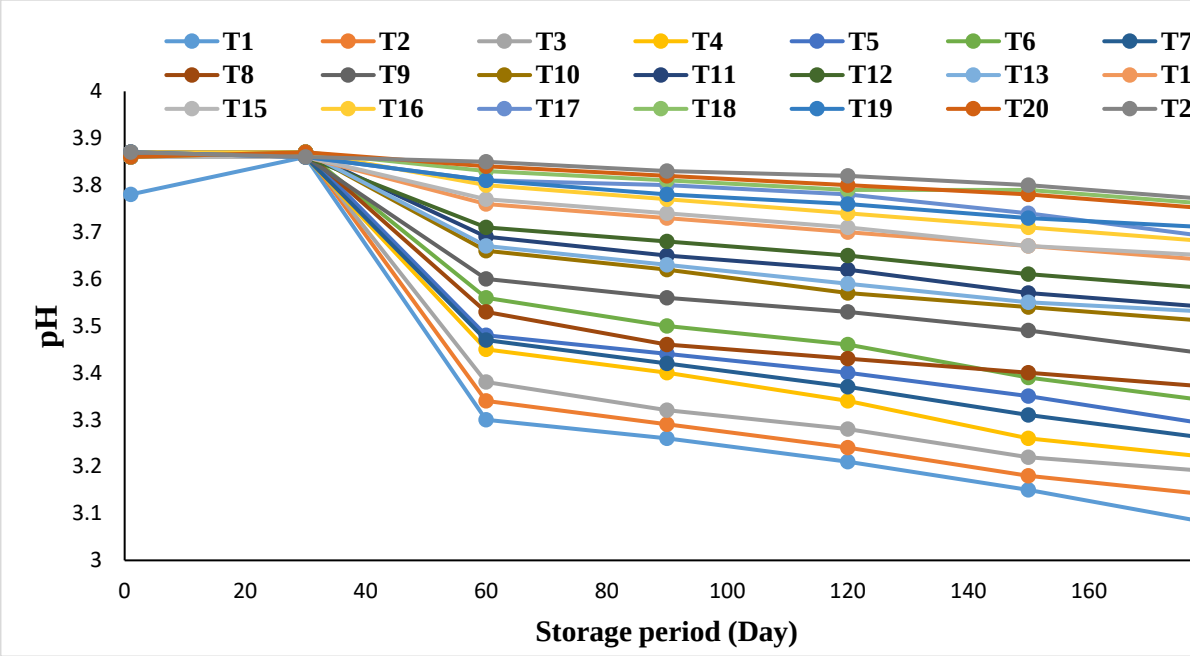

Fig 2. Changes in pH values of low-fat mayonnaise treatments during storage period

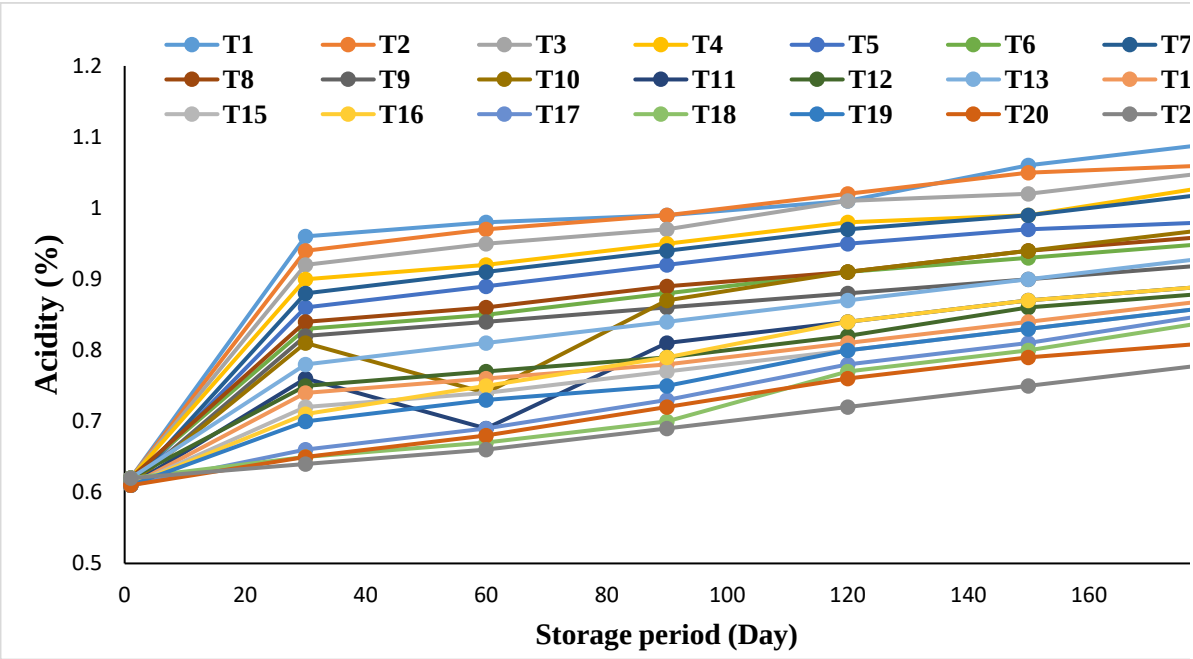

Fig 3. Changes in acidity (%) values of low-fat mayonnaise treatments during storage period

3-3- Emulsion Stability of Mayonnaise

A stable emulsion is typically characterized by the absence of coalescence, flocculation, and creaming processes. The phenomenon of creaming is observed less frequently in high-fat mayonnaise samples characterized by elevated oil content (80%). This reduced incidence can be attributed to the close interactions between oil droplets, which generate significant friction. Such friction

serves as a deterrent to the creaming process. This phenomenon is predominantly observed in low-fat formulations, such as low-fat mayonnaise. However, it can be mitigated through the incorporation of thickening agents and appropriate fat substitutes, including xanthan gum and modified starch [13]. The evaluation of the stability of emulsion-treated mayonnaise sauce (refer to Table 5) indicates that variations in stability across different treatments are significantly influenced by the sauce formulation, specifically the type of starch utilized and the quantity of fat incorporated, as well as the duration of storage ($p < 0.05$) The findings of this research indicate a significant reduction in stability correlated with increased storage time, as evidenced by a p-value of less than 0.05 Furthermore, the augmentation of fat and modified starch content within the formulation of mayonnaise sauces resulted in a statistically significant enhancement of sample stability ($p < 0.05$). A comparative analysis of treatments incorporating corn and potato starch indicates that mayonnaise samples formulated with corn starch exhibit significantly lower stability than those formulated with potato starch, with a statistical significance of $p < 0.05$ The stability results exhibited a direct correlation with the viscosity findings, and these results demonstrated internal consistency. The enhanced stability observed in treatments containing elevated concentrations of fats, modified starches, and, in particular, modified potato starch can likely be attributed to the increased viscosity associated with these formulations. The incorporation of fat substitutes in the formulation of mayonnaise may mitigate the occurrences of coalescence, flocculation, and creaming. This phenomenon may be ascribed to the elevated viscosity of this sample relative to others, which can be attributed to the incorporation of a stabilizer [29]. The findings of this study were consistent with the results reported by other researchers in the field. Nikzade et al., 2012 conducted an investigation into the physical properties of low-fat mayonnaise formulated with soy milk and a variety of stabilizers. The findings of the researchers indicated that the incorporation of various stabilizers significantly enhanced the stability, consistency, and viscosity of the final product. This fortification effectively inhibited the phase separation and preserved the creaminess characteristic of low-fat mayonnaise 15. In the study conducted by Werlang et al., 2021 , it was demonstrated that low-fat mayonnaise formulated with corn starches displayed superior emulsion stability in comparison to the control mayonnaise [30]. Park et al., 2020 demonstrated that low-fat mayonnaises formulated with arrowroot starches exhibited greater emulsion stability in comparison to the control group. These results are also in alignment with the findings reported by Pishan et al., 2019.

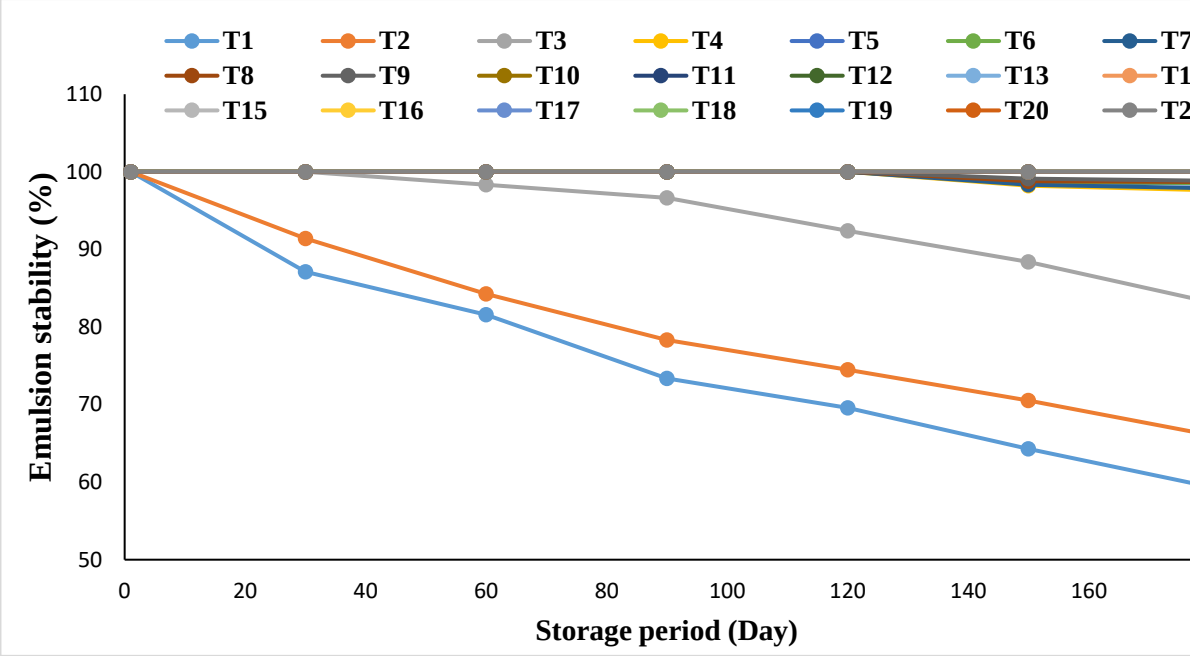

Fig 4. Changes in emulsion stability (%) values of low-fat mayonnaise treatments during storage period

3-4- Textural Properties of Mayonnaise.

Mayonnaise is classified as a food emulsion, a characteristic that leads consumers to anticipate a comparatively viscous texture. The observed thickness of the mayonnaise is attributable to the significant concentration of oil present in its formulation. In the event that a portion of the oil is extracted, it is imperative to give careful consideration to the appropriate textural qualities of this low-calorie product when selecting a fat substitute [16]. Figure 6 illustrates the variations in the average values of stiffness and adhesion of mayonnaise treatments throughout the storage period. The findings suggest that the incorporation of modified fats and starches in mayonnaise formulations resulted in a statistically significant enhancement of both stiffness and adhesion indices ($p < 0.05$) Conversely, an increase in storage duration was associated with a significant reduction in both stiffness and adhesion ($p < 0.05$). The stiffness and adhesion indices exhibit a direct correlation [32]. Wang et al., 2022 research indicated a notable decrease in both the stiffness and stickiness of control and starch-based mayonnaises over the course of the storage period [33]. The augmentation of fat content and the incorporation of modified starch resulted in an increase in textural indices. This phenomenon can be attributed to the elevated viscosity associated with these treatments, which is likely induced by the addition of modified starches. The presence of hydrocolloid compounds appears to facilitate the formation of a gel-like structure, which effectively entraps oil droplets and diminishes their mobility. This phenomenon subsequently results in an increase in viscosity, thereby enhancing the textural characteristics of the mixture [34]. Notably, as previously documented, an extension of the storage duration resulted in a reduction in the viscosity of the mayonnaise samples. This reduction is likely to contribute to a decrease in the quantitative measures of textural properties as storage time increases. The results obtained in this study align with the findings reported by other researchers in the field. Agyei-Amponsah et al., 2021

documented an increase in both the viscosity and adhesive properties of low-fat mayonnaise following the incorporation of starch-based fat substitutes, this finding corroborates the results obtained in the current study.

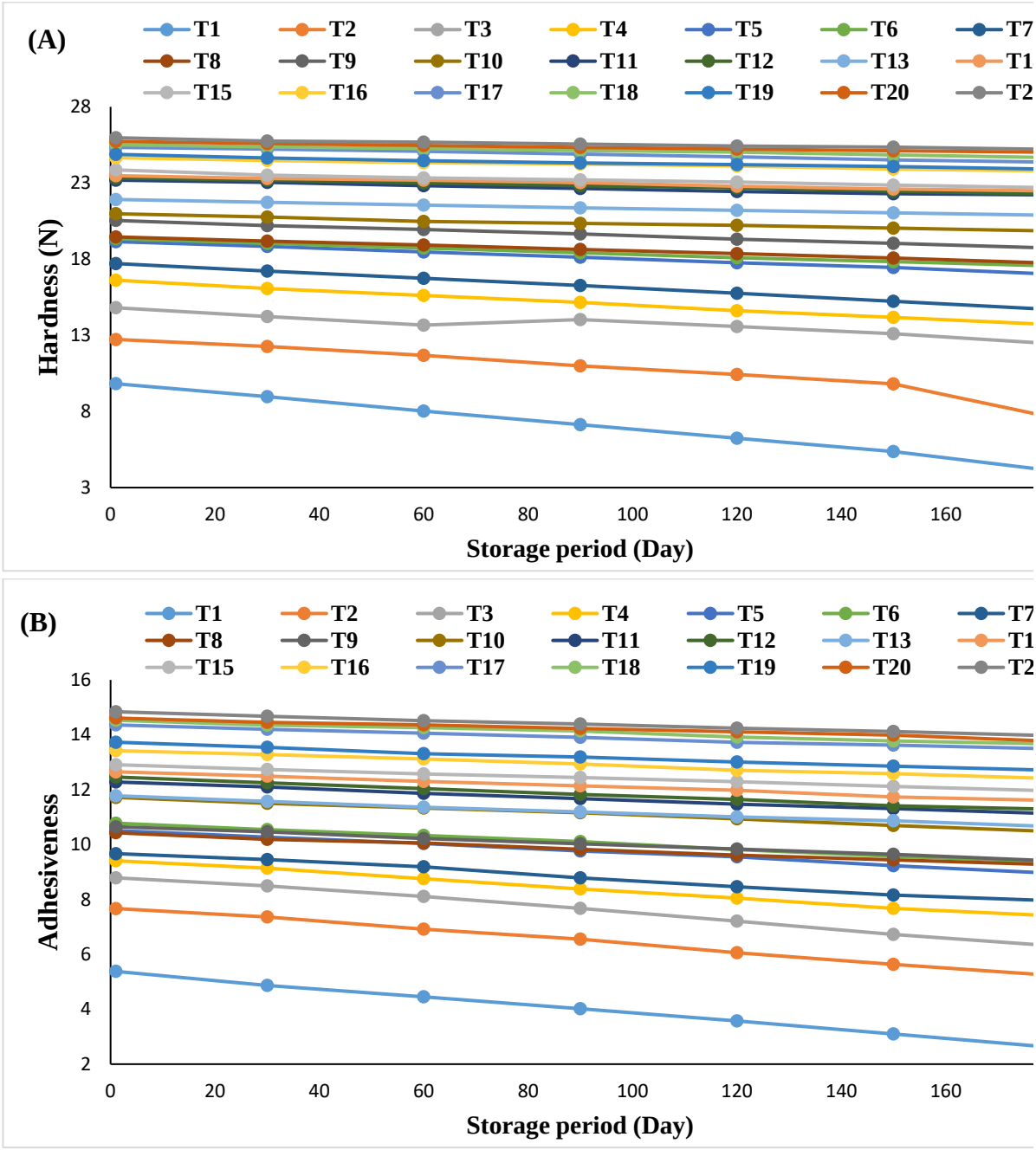

Fig 5. Changes in (A) Hardness (N) and (B) adhesiveness values of low-fat mayonnaise treatments during storage period

3-5- Sensory Evaluation of Mayonnaise

Figure 6 presents a comparative analysis of the sensory scores associated with various mayonnaise treatments. The findings demonstrated that treatments possessing elevated fat content and incorporating modified starches exhibited significantly higher sensory scores in comparison to alternative treatments ($p < 0.05$) The treatment designated as 21 exhibited the

highest overall acceptance rate, with its scores for all sensory characteristics significantly surpassing those of other treatments ($p < 0.05$) Furthermore, the sensory evaluation of the treatments incorporating modified potato starch demonstrated superior performance relative to those utilizing modified corn starch. This phenomenon can be attributed to the observed enhancement in sensory scores, which correlates with the increased levels of fat and modified starches that influence the physical and textural characteristics of the products. Consequently, as articulated, an increase in the fat content of low-fat mayonnaise sauces, in conjunction with a rise in the concentration of modified starches, significantly contributes to improvements in viscosity, stability, and textural firmness. The augmentation of these factors consequently enhances the sensory indicator scores, which, in turn, elevates the overall acceptance score. Moreover, an analysis of the textural and physical properties of the formulations prepared with modified potato starch revealed these characteristics to be markedly superior to those of the formulations utilizing corn starch. Consequently, the sensory evaluations of the treatments incorporating modified potato starch yielded higher scores compared to those composed of modified corn starch.

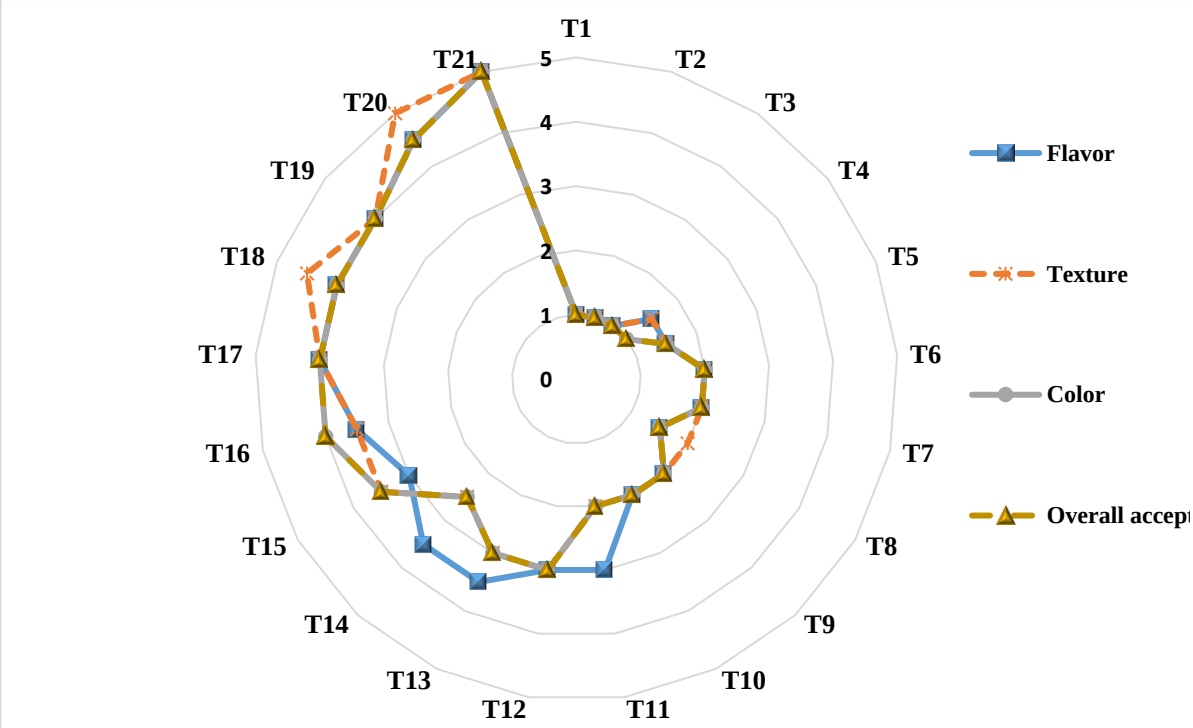

Fig.6. Sensory properties of low-fat mayonnaise treatments

4- Conclusion

The findings of this study indicate that the incorporation of modified starches, along with an increased fat content in the formulation of low-fat mayonnaise sauces, significantly improved the viscosity of the final product. The incorporation of modified starches demonstrated efficacy in mitigating drastic fluctuations in pH and acidity, while concurrently preserving the creaminess of mayonnaise over an extended duration. The incorporation of modified starches in the formulation of low-fat mayonnaise sauces yielded products

characterized by enhanced firmness and increased stickiness in texture. The incorporation of modified starches in the formulation of low-fat mayonnaise sauces significantly enhanced the sensory attributes across all parameters assessed. Notably, the low-fat formulation comprising 30% fat and 3. 5% modified potato starch demonstrated the highest overall acceptability when compared to the alternative treatments. The findings of this investigation suggest that the incorporation of modified potato starch, specifically at a concentration of 3. 5% alongside 30% oil, can be employed to formulate low-fat mayonnaise that exhibits superior quality and sensory attributes.

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چکیده

به دلیل تأثیر نامطلوب چربی بر سلامت مصرف‌کنندگان و ارتباط آن با بیماری‌های قلبی-عروقی، چاقی و غیره، امروزه تلاش‌های بسیاری برای تولید محصولات غذایی کم‌چرب در حال انجام است. هدف از این مطالعه ارزیابی تولید سس مایونز کم چرب (۲۰، ۲۵ و ۳۰ درصد روغن) به وسیله نشاسته‌های اصلاح شده سیب‌زمینی و ذرت با سطوح ۲/۵، ۳ و ۳/۵ درصد و تأثیر نسبی آن‌ها بر ویسکوزیته، pH، اسیدیته، پایداری و خصوصیات فیزیکی به مدت ۶ ماه نگهداری و همچنین خصوصیات حسی بود. نتایج نشان داد که بکارگیری هر دو نشاسته و افزایش سطح چربی به‌طور معنی‌داری مقادیر pH، ویسکوزیته، پایداری امولسیون، سفتی بافت و چسبندگی مایونزها را افزایش داد، ولی موجب کاهش اسیدیته نمونه‌ها گردید. طی دوره انبارمانی در دمای یخچال، کاهش در میزان ویسکوزیته، pH، سفتی و چسبندگی بافت و افزایش در اسیدیته و خامه‌ای شدن تیمارهای سس مایونز مشاهده گردید ($p<۰/۰۵$). نتایج ارزیابی حسی نشان داد که افزایش سطح نشاسته‌ها و چربی به‌طور معنی‌داری منجر به افزایش امتیاز پارامترهای حسی شد ($p<۰/۰۵$). بالاترین پذیرش حسی مربوط به مایونز کم‌چرب با ۳۰ درصد چربی و حاوی ۳/۵ درصد نشاسته اصلاح شده سیب‌زمینی بود. نتایج این مطالعه نشان داد که تیمارهای کم‌چرب با نشاسته اصلاح شده سیب‌زمینی دارای خصوصیات کیفی بهتری در مقایسه با نمونه‌های حاوی نشاسته ذرت بودند. به‌طور کلی می‌توان نتیجه گرفت که استفاده از ۳۰ درصد چربی و ۳/۵ درصد نشاسته اصلاح شده سیب زمینی به عنوان جایگزین چربی منجر به خصوصیات فیزیکوشیمیایی و حسی مطلوب در سس مایونز می‌شوند.

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Scientific Research

Lallemantia royleana seed mucilage-based active edible films: The effects of zinc oxide nanoparticles and zoulang plant's essential oil

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ABSTRACT

In this research, *Lallemantia royleana* seed mucilage was used as the base of the film, while essential oil derived from the zoulang plant's leaves and stems, along with zinc oxide nanoparticles (ZnO), were incorporated as additives to develop active edible films. Findings indicated that incorporating ZEO enhanced the films' antioxidant capabilities, permeability, thermal properties, tensile strength, and elongation of the film. FT-IR analysis confirmed the creation of new hydrogen-oxygen bonds between ZEO and the polysaccharide chains, contributing to a reinforced film structure. Conversely, the addition of ZnO nanoparticles was found to decrease the antioxidant properties, permeability, crystallinity, thermal resistance, moisture content, and tensile strength of the films. Additionally, ZnO nanoparticles contributed to enhanced plasticity, which increased the films' stretchability and resulted in greater thickness. SEM imaging verified the interaction between ZEO and ZnO nanoparticles, aligning with the findings from the FT-IR analysis. Overall, the study suggests that films developed using *Lallemantia royleana* mucilage, in combination with ZEO and ZnO nanoparticles, demonstrate strong potential for applications in food packaging and active films or coatings, particularly for materials vulnerable to oxidation.

1- Introduction

Food products can be compromised by pests, insects, and microorganisms at various stages of production. To address this, food packaging technology has been developed to extend the shelf life of foods. Being ensure about food availability and maintaining product quality until consumption are also crucial factors [1].

Active packaging involves the inclusion of specific components designed to improve the functionality and effectiveness of packaging systems. These systems are typically categorized into two types. In the first category, the active elements are enclosed in separate sachets or pads placed within the package, and in the other, the active components are embedded directly into the packaging material itself [2].

In this study, the second type of packaging was employed, where active components were integrated into the matrix of an edible film. Driven by consumer demand for more sustainable and cost-effective solutions, researchers have focused on developing biodegradable and edible materials that enhance efficiency, food quality, freshness, and overall food safety [3]. The Production of edible films and coatings represents an innovative packaging approach aimed at preserving or even improving food quality. These covering materials must meet essential food preservation needs. For instance, coatings with low oxygen permeability are necessary to protect oxidation-sensitive products, while films with selective mass transfer capabilities help reduce moisture loss in fruits and vegetables during storage [4].

Nowadays, hydrocolloids employing has been increased in the food products as a thickening agent, food preservative, texturizer, gelling agents, film-forming agents, and foam stabilizer [5]. Hydrocolloids are extracted from various sources such as herbal seeds. *Lallemantia royleana* (LR) is an annual herb of the *Lamiaceae* family. This ilk is one of the largest and most distinctive plants in the world, growing widely in different parts of the Europe and the Middle East such as Iran. The antimicrobial and antioxidant properties of LR have been presented in some studies [6]. *Lallemantia royleana* seeds (LRS) are consist of carbohydrates (45.25%), crude fiber (30.67%), ash (3.63%), oil (18.27%), protein (25.60%) and it also contains linoleic, oleic, palmitic, stearic and beta-Sitosterol fatty acids [6]. LRS gum contains arabinose (37.88%), galactose (33.54%), rhamnose (18.44%), xylose (6.02%), and glucose (4.11%) [7].

Essential oils are one of the natural compounds in plants. They are aromatic substances that are naturally produced by the plant as secondary metabolites and their constituent ingredients are mainly terpenoids and phenolic compounds [8]. Essential oils would reduce the rate of oxidation in different ways. For example, it can reduce the initial reactions of fatty acid oxidation, absorb free radicals, and decrease metal ion binding [9]. Incorporating essential oils into the formulation of edible films can positively influence their mechanical characteristics, vapor barrier properties, surface texture, roughness, and color of the film [10-12]. Zoulang has healing and soothing properties. It is used to stimulate fertility, improve hemorrhoids and treat rheumatic diseases. This plant also participates in hematopoiesis due to its folic acid and iron content [13]. Terpenoids and phenolic compounds such as monoterpene, sesquiterpene, triterpenoids, flavonoids, coumarin, steroids, and acetylene are the main component of this plant [14].

Nanotechnology is employed increasingly in food industry. One of its important applications is in the field of edible films. Nanoparticles are used to enhance the

performance and increase the bioavailability of biopolymer-based films [15]. Also, they are used to enhance the performance of films based on biopolymers [16]. Nanoparticles like titanium oxide, silica, zinc oxide and magnesium oxide are commonly used in food industry.

Considering tendency to use natural components instead of synthetic materials that are made by chemically changing, the purpose of the current research was to investigate the effects of zoulang essential oil (ZEO) addition on physicochemical and antioxidant characteristics of an edible films based on *Lallemantia royleana* seeds mucilage (LRSM) and feasibility of its application as a natural preservative.

2.Materials and methods

Materials

The Lallemantia royleana seeds and zoulang were bought from a traditional market in Babol, Mazandaran, Iran. Other materials, such as glycerol, Tween 80, calcium sulfate, calcium nitrate, and potassium sulfate, were procured from Merck Co. (Darmstadt, Germany). ZnO nanoparticles were supplied by US Research Nanomaterials, Inc., and 2,2-diphenyl-1-picrylhydrazyl was obtained from Sigma-Aldrich (MO, USA).

Extraction of Zoulang Essential Oil

Extraction of ZEO followed a three-step method. Initially, zoulang was dried in a chamber with a steady flow of dry air at 40°C. Following this, it was ground into a powder using a mill (DPA1, Moulinex, France). The essential oil was then extracted through hydrodistillation for 6 hours using a Clevenger apparatus.

Extraction of Lallemantia royleana seeds mucilage

First of all, LRS were washed thoroughly with ethanol 96% and placed in an oven (GPH-OV-50, JIM Engineering Ltd., United Kingdom) at 45 ˚C for 12 hours. Then, the seeds and water were mixed in a ratio of 1:120 and placed on a stirrer at 800rpm/min for 3h to absorb water. Then, the LRSM was separated from the seeds with a juicer (HR2820, Philips, Netherlands). To separate impurities from the mucilage, the mixture was centrifuged (VS4000D, Farzaneh Arman Co., Isfahan, Iran) at 4200 rpm for 15 minutes followed by drying in an oven (GPH-OV-50, JIM Engineering Ltd., United Kingdom) at 50 ˚C for 24h.

Film preparation

First, the dried mucilage was powdered with a mortar. Afterward, 1g of the powdered sample was added to 80 mL of distilled water and stirred on a magnetic stirrer for 2 hours to allow water absorption. It was then mixed with 0.375 mL of glycerol. Once the primary solution was prepared, ZnO nanoparticles were dissolved in 20 mL of distilled water at concentrations of 1.5%, 3%, and 4.5% based on the dried mucilage weight. The mixture was then homogenized using an ultrasonic homogenizer (FR-USC-22 LQ, Intellect, USA) at 400 W power and 40 kHz frequency for 10 minutes. This homogenized solution was subsequently added to the primary solution.

The ZEO was added in concentrations of 2%, 3.5% and 5% based on dried mucilage weight (v/w) to the solution. In the next step, 0.1 mL of tween 80 were added as emulsifier into the solution followed by ultrasonic degassing at 25 °C for 10 min. For film formation, 65 mL of each prepared solution was poured into 9 cm diameter petri dishes and placed in an oven at 30 ˚C for 48 hours. In the final step, dried films were peeled off from plates and conditioned in a desiccator with calcium nitrate (RH 55%) at 25 ˚C for 24 h.

Measuring the Thickness of the Films

The thickness of the films was measured using a digital micrometer (Mitutoyo, Japan) with a precision of 0.001 mm. Measurements were taken at 12 different locations on each

film, and the average thickness was calculated and reported.

Moisture Content

To determine the moisture content, the samples were cut into 1 cm × 1 cm pieces and placed in an oven at 120°C until a constant weight was achieved. The moisture content was then calculated using equation (1) [17].

$$M\ (\%) = \frac{Wi - We}{Wi} \times 100 \quad (1)$$

Where M is the moisture content (%), W_i is the initial weight (g) and W_e is the weight of the film at the end of drying (g).

Water Solubility

The film samples were cut into 1 cm × 1 cm pieces and dried in an oven at 95°C for 24 hours. Their weights were recorded afterward. The samples were then immersed in 50 mL of distilled water and stirred on a magnetic stirrer at 25°C for 6 hours. After stirring, the samples were filtered and dried in an oven at 110°C until a constant weight was reached. The solubility of the films was then calculated using the following equation [18].

$$\text{Solubility}\ (\%) = \frac{Wi - Wf}{Wi} \times 100 \quad (2)$$

Where W_i is the initial weight (g) and W_f is the final weight (g).

Moisture absorption

The film samples (1 cm × 1 cm) were first conditioned in a desiccator with $CaSO_4$ for 24 hours to ensure complete drying, and their weights were recorded. Subsequently, the samples were transferred to a desiccator containing a saturated calcium nitrate solution (55% relative humidity) at 25°C until they reached a constant weight. The moisture absorption was then calculated using equation 3.[19].

$$\text{Water absorption}\ (\%) = \frac{Wt - Wo}{Wo} \times 100 \quad (3)$$

Where W_t is the final weight of the samples (g) and W_o is the initial dry weight (g).

Water Vapor Permeability

First of all, films were cut in to a circle shaped figure with 2 cm diameters. Then they sealed in glass vials with 2 cm diameter and 4.5 cm length. Paraffin wax was employed around the glass to insulate the inner-environment of it. Anhydrous calcium sulfate was used to decrease the RH of vials from its initial value to 0%.

The vials were then placed in a desiccator containing saturated potassium sulfate (RH 97%). The pressure gradient created by this setup was the driving force for water vapor transfer into the vials. The vials were weighed every 6 hours for 4 days using a scale with an accuracy of 0.001g. The data was recorded and plotted, and the slope of the resulting graph (weight vs. time) was determined using linear regression. The water vapor transfer rate (WVTR) was calculated by dividing the slope (S) by the surface area of the film (A) [20].

$$WVTR = \frac{S}{A} \quad (4)$$

Water vapor permeability (WVP) was calculated by equation 5:

$$WVP = \frac{WVTR \times X}{\Delta P} \quad (5)$$

Where X is the film thickness (mm) and ΔP is the water vapor pressure difference between the inside and outside of the vial (ΔP is 3115.42 Pa).

Antioxidant activity

To measure the antioxidant activity of various treatments, 25 mg of each sample was dissolved in 5 mL of distilled water. Then, 0.1 mL of the extract was added to 3.9

mL of DPPH solution (0.04 g/L) in ethanol. The mixture was vortexed and incubated in a dark box at room temperature for 60 minutes. After incubation, the absorbance was measured using a UV-Vis spectrophotometer at 517 nm. The percentage of DPPH radical inhibition was then calculated using equation 6 [21].

$$\text{Radical scavenging activity (\%)} = 100 \times (1 - A_{sample}/A_{DPPH}) \quad (6)$$

where A_{Sample} indicates sample absorption and A_{DPPH} indicates pure DPPH solution absorption.

Fourier Transform Infrared Spectroscopy (FT-IR)

The samples were pre-conditioned at 55% relative humidity and 25°C for 24 hours. FT-IR measurements were conducted to obtain the film spectra using a THERMO NICOLET AVATAR 360 FT-IR ESP (Thermo Fisher Scientific, MA, USA). The samples were scanned across a wave number range of 600 cm^{-1} to 4000 cm^{-1} with automatic signal gain and a resolution of 4 cm^{-1}.

X-ray diffraction (XRD)

The samples were pre-conditioned at 55% relative humidity and 25°C for 24 hours. X-ray diffraction (XRD) analysis was performed using a Shimadzu XRD-6000 (Shimadzu, Japan) at 25°C, with a voltage of 40 kV and a current of 30 mA. The results were recorded over a diffraction angle (2θ) range from 0° to 60°, with a scanning rate of 3° per minute.

Scanning Electron Microscope (SEM)

The samples were conditioned for 24 hours at 25 °C and 55% relative humidity. Scanning electron microscopy (SEM) analysis of the film's surface morphology was performed using a FEI Quanta 200 SEM (Philips-FEI Co., Netherlands) with magnifications ranging from 10 to 100,000 times. To prepare the samples for imaging, they were coated with gold, and a heated tungsten cathode electron gun was employed.

Thermal Weight Analysis (TGA)

The samples were conditioned for 24 hours at 25 °C and 55% relative humidity. This analysis was conducted using a STA 504 device (BAHR, Germany), covering a temperature range from room temperature to 600 °C under nitrogen flow, with a heating rate of 10 °C/min.

Mechanical Properties

Mechanical testing was conducted using a TA.XTPlus texture analyzer (Stable Micro Systems Co., UK) in accordance with ASTM standard methods. The film samples were first cut into a dumbbell shape measuring 5.5 × 1 cm^2 and then conditioned for 48 hours in a desiccator with calcium nitrate to maintain a relative humidity of 55% at 25 °C. The initial gap between the jaws was set at 30 mm, with a movement speed of 0.83 mm/sec. Following the mechanical tests, tensile strength (TS) and elongation at break (EAB) were determined using Equations 7 and 8 [19].

$$TS = F_{max} / A \quad (7)$$

$$EAB = (L_{max} / L_0) \times 100 \quad (8)$$

Where, F_{max} is maximum load (N), A is cross section area (m^2), L_{max} is extension at the moment of rupture (m) and L_0 is initial length of the film samples (m).

Statistical Analysis

Statistical analysis of the results was performed using Microsoft Excel 2013 and SPSS Statistics 23 software (IBM Corporation, Armonk, NY, USA). Analysis of variance (ANOVA) was conducted at a significance level of 95% using Duncan's test to evaluate differences between the means. The results are presented as mean ± standard deviation.

3.Results and Discussion

Film thickness

Table 1 shows the results of thickness measurements. As it can be seen from the table, the film's thickness varied between 0.133 mm and 0.166 mm. The results indicated that the incorporation of essential oil did not significantly affect the thickness of the edible films. However, the addition of nanoparticles resulted in an increase in film thickness. This increase can be attributed to the higher dry matter content of the film [20]. An increase in thickness can significantly impact mechanical strength, water vapor permeability, light transmission, and the color of the film. [22]. Other researchers reported similar findings regarding the impact of zinc oxide nanoparticles on the thickness of pectin/alginate films [23].

TABLE 1 The thickness, moisture content, water solubility, water absorption and water vapor permeability of film samples.

sample	Thickness (mm)	Moisture content (%)	Water solubility (%)	Water absorption (%)	Water vapor permeability ($\times 10^{-7}$ g/m.h.Pa)
control	0.133 ± 0.009^{a}	28.07 ± 0.13^{a}	40.74 ± 0.73^{b}	16.66 ± 0.03^{a}	11.87 ± 0.33^{a}
A	0.165 ± 0.005^{d}	26.53 ± 0.35^{b}	37.93 ± 0.76^{a}	10.52 ± 0.18^{c}	10.40 ± 0.46^{c}
B	0.153 ± 0.007^{c}	24.61 ± 0.15^{c}	41.93 ± 1.24^{b}	9.67 ± 0.24^{c}	10.34 ± 0.24^{c}
C	0.145 ± 0.005^{b}	25.26 ± 0.18^{d}	42.85 ± 1.07^{b}	13.15 ± 0.28^{b}	11.22 ± 0.18^{b}
D	0.142 ± 0.004^{b}	24.47 ± 0.27^{c}	50 ± 0.45^{d}	8.1 ± 0.74^{d}	10.77 ± 0.34^{c}
E	0.166 ± 0.008^{d}	23.35 ± 0.54^{e}	46.66 ± 1.08^{c}	2.5 ± 0.1^{e}	7.50 ± 0.26^{d}

(A) EO= 2% N.P= 4.5%, (B) EO= 3.5% N.P= 3%, (C) EO= 2% N.P= 1.5%, (D) EO= 5% N.P= 1.5 %, (E) EO= 5% N.P= 4.5%

Moisture content

The results of the moisture content test are presented in Table 1. As the percentages of nanoparticles and essential oil increased, the moisture content of the films decreased. Due to the hydrophobic properties of zoulang essential oil, samples containing 3.5% and 5% essential oil exhibited the greatest reduction in moisture content. Similar results have been reported by other researchers. They stated that adding essential oil to the film matrix reduced the moisture content of the films [24, 25]. There was a significant difference between all groups ($P<0.05$), except for groups B and D, which did not differ significantly ($P>0.05$). In another study, it has been reported that adding nanoparticles to film can reduce the moisture content, which might be due to the ability of nanoparticles to create pores on the film surface [26].

Water solubility

Insolubility, or water resistance, is a key property of edible films that makes them suitable for food packaging. This characteristic is particularly important when the water activity of the product is high or when the edible film comes into contact with moisture during food processing [27]. As indicated in Table 1, the solubility of the control film was approximately 40%. The solubility of the films increased significantly with higher concentrations of ZEO ($p < 0.05$). This increase may be attributed to the detrimental effect of ZEO on the strength of the polysaccharide-polysaccharide bonds within the film [28]. Conversely, an increase in the concentration of nanoparticles within

the film structure leads to a reduction in solubility. This effect may be attributed to the formation of a stronger structure within the film matrix, as well as the dimensional characteristics of the nanoparticles [29].

Water absorption

The results of the water absorption test are shown in Table 1. The water absorption for the control sample was about 17%, with increasing the percentage of essential oils and nanoparticles, the water absorption decreased ($p<0.05$). There was no significant difference between groups A and B ($p> 0.05$) but the other groups had a significant difference ($p <0.05$). The samples with 5% essential oil and 4.5% nanoparticles had the lowest water absorption (2-3%). This reduction may be due to the hydrophobic nature of nanoparticles and essential oils. In addition, the formation of a lattice structure by ZEO and ZnO nanoparticles can increase water resistance [26-30]. Similar results have been reported by Jamróz et al. (2018). They observed a decrease in water absorption by adding lavender essential oil to the film composition [31]. Vaezi et al. (2018) also stated that the addition of ZnO NPs in the film matrix has reduced water absorption [32].

Water vapor permeability

Water vapor permeability is a critical functional property of packaging films, as it helps preserve food quality by minimizing moisture transfer between the food and the surrounding environment. [33], therefore, using a hydrophobic film or coating can prevent undesirable changes in the water activity of packaged food [34]. As shown in the Table 1, control samples had the highest permeability and adding ZEO and ZnO nanoparticles to the film structure, reduced its WVP significantly ($p<0.05$). Samples with the highest concentrations of ZEO and ZnO nanoparticles had the lowest permeability. Researchers have stated that the WVP depends on hydrophilic-lipophilic balance of the film [35]. So, this decrease might be due to the dominant hydrophobic properties of the ZnO nanoparticles and also ZEOs [35]. In addition, uniform distribution of small-sized ZnO nanoparticles and filling the small pores can reduce the amount of free pathways for water vapor transfer [35]. Similar results were reported by other researchers who studied the development of starch films with zinc oxide nanoparticles [15]. Moreover, the existence of lipid drops can change the vapor penetration pathways and increase their length [36]. Other researchers have reported comparable findings, investigating the effects of oregano essential oil on basil seed gum films. [37].

Antioxidant activity

The results of the DPPH radical scavenging activity are illustrated in Figure 1. A significant difference ($p < 0.05$) was observed between all film samples and the pure DPPH sample, indicating that LRSM possesses antioxidant properties as well. These results align with findings from other researchers who studied LRSM, noting that the observed antioxidant properties are attributed to the phenolic compounds present in mucilage [38].

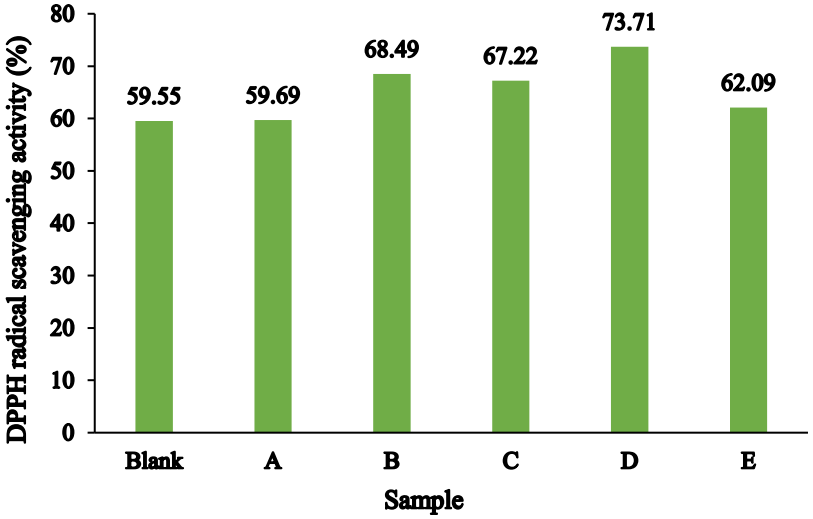

Fig 1: Antioxidant activity. (A) EO= 2% N.P= 4.5%, (B) EO= 3.5% N.P= 3%, (C) EO= 2% N.P= 1.5%, (D) EO= 5% N.P= 1.5 %, (E) EO= 5% N.P= 4.5%

There was no significant difference between sample A and the control sample ($p > 0.05$), but the other samples had a significant difference with each other ($p<0.05$). The addition of 1.5% ZnO nanoparticles to the film appears to have marginally enhanced its antioxidant properties. This slight improvement may be attributed to the transfer of electron density from the oxygen to the unpaired electron on the nitrogen atom in DPPH [39]. These findings are consistent with those of other researchers who investigated the antioxidant and antimicrobial properties of active packaging films made from carboxymethyl cellulose combined with curcumin and zinc oxide [40]. However, with increasing the percentage of nanoparticles from 1.5% to 4.5%, the antioxidant properties decreased. Other researchers have reported similar observations regarding the reduction of antioxidant properties by adding ZnO nanoparticles to the film structure [41, 42]. As demonstrated in Figure 1, the antioxidant properties of the film improve with higher concentrations of essential oil. ZEO exhibits antioxidant capabilities attributed to its phenolic content. Other researchers also confirmed the antioxidant properties of ZEO [43-45].

X-ray diffraction (XRD)

The results of the X-ray diffraction test are shown in Figure 2. The diffractogram of control film showed a peak at 2θ of 14.54°, 17.38° and 26.01° which illustrated the semi-crystalline structure of sample. As can be seen in the nanoparticle containing samples, slight peaks were observed in 2θ of 31.75°, 34.56°, 36.25°, 47.49° and 56.55°. The height of these peaks increases with increasing the percentage of ZnO nanoparticles while the position of the peaks did not change. The same results were reported in another study [46]. A similar trend was noted with the addition of ZEO to the samples, which resulted in an increase in the crystallinity of the edible films [47]. Constant position of the peaks even after adding the ZEO illustrated the compatibility of essential oil with the polymer part of the film.

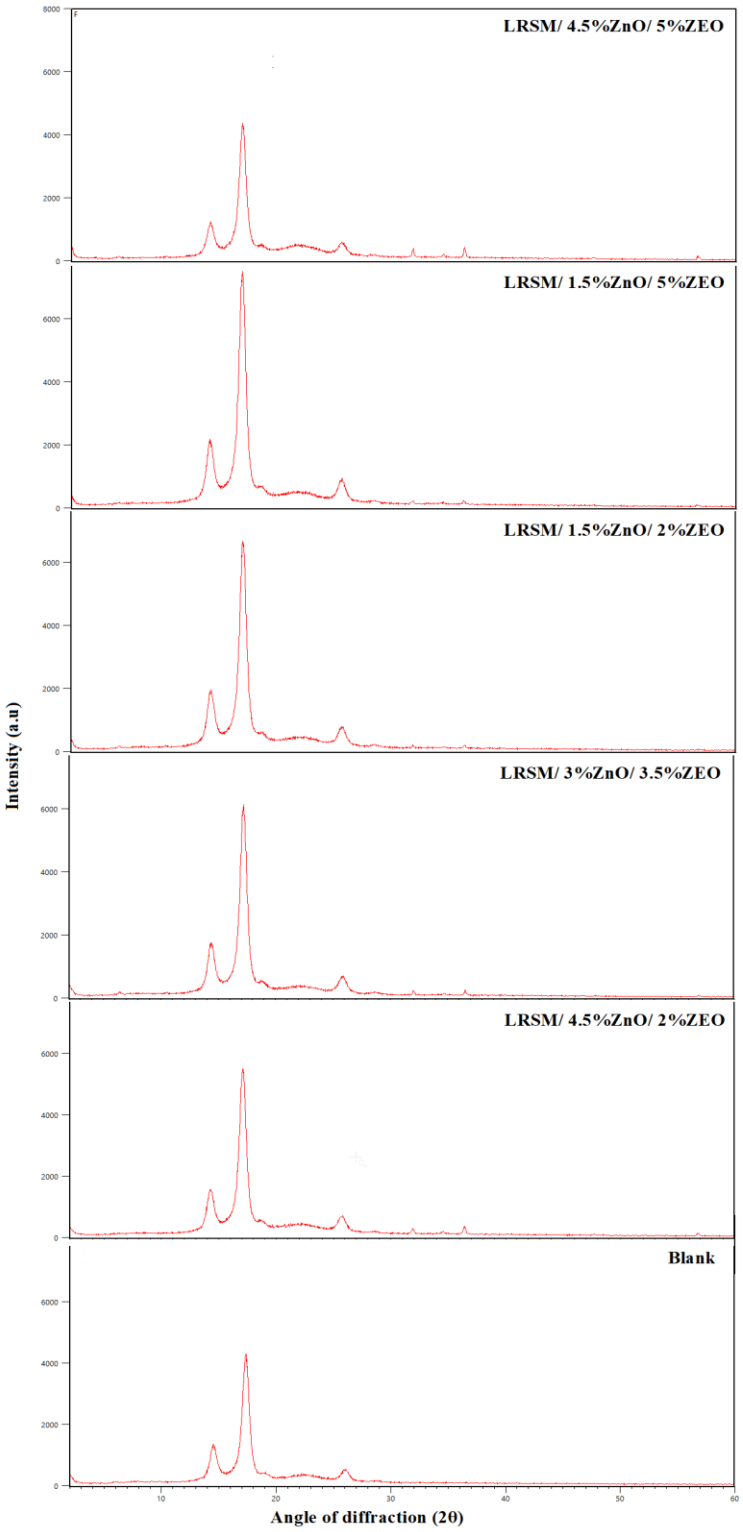

Fig 2: XRD patterns of pure LRSM and modified samples

Another study also reported an increase in crystallinity and peak intensity, with no change in peak location, when oregano essential oil was added to chitosan/gelatin films. [48]. As the concentration of ZEO increased, the peak heights also rose. However, at higher concentrations of ZnO nanoparticles, a decrease in peak height was observed compared to lower percentages of ZnO nanoparticles. This reduction may be due to the bonding interactions between the ZnO nanoparticles and the ZEO.

Scanning Electron Microscope (SEM)

The results of SEM have been demonstrated in Figure 3. Addition of ZEO into the film composition is usually followed by homogenization or adding emulsifiers to the mixture. When the film dried (as seen in the Figure 4) the ZEO can change the film structure.

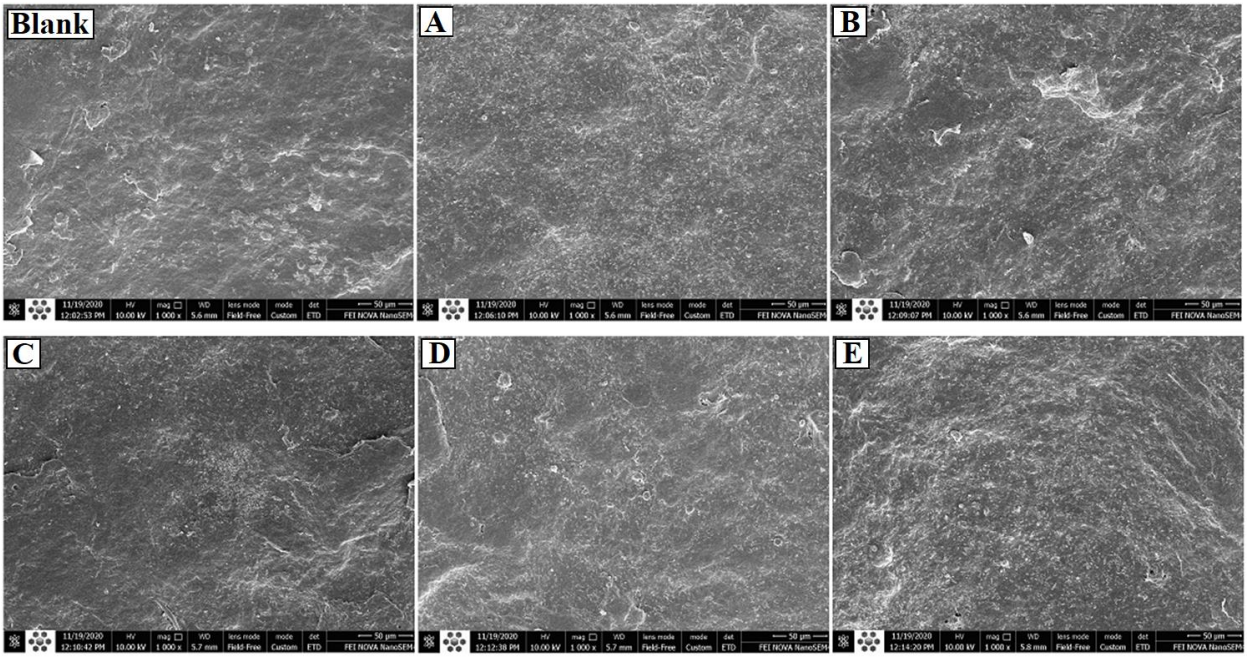

Fig 3: SEM of the surface of film samples (A) EO= 2% N.P= 4.5%, (B) EO= 3.5% N.P= 3%, (C) EO= 2% N.P= 1.5%, (D) EO= 5% N.P= 1.5 %, (E) EO= 5% N.P= 4.5%

The morphology and structural features of films affect its mechanical and gas barrier properties [49]. As shown in Figure 3, control samples have smoother and more homogenous surface compared with the other films which have ZEO and/or ZnO nanoparticles in their formulation. In addition, all samples have a wrinkled surface, which could be formed during drying process [40]. The amount of this wrinkles seems to decrease in samples with higher amounts of ZnO nanoparticles. Although Zinc oxide nanoparticles were distributed well on the film surface, increasing the percentage of these nanoparticles caused rougher and more heterogeneous film surface. Similar observations were made in another study examining starch films with zinc nanoparticles, where an increase in surface roughness was noted [15].

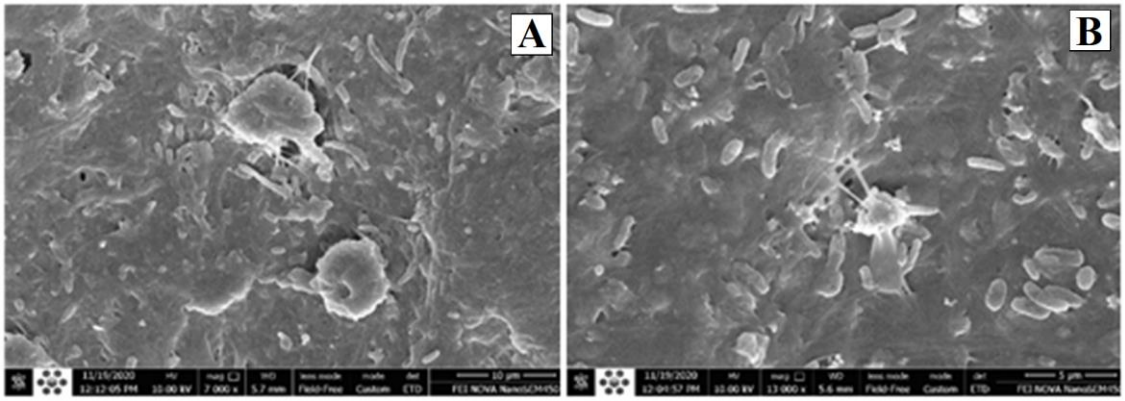

Fig 4: Surface microstructure of film sample

Adding the ZEO does a noticeable difference at the film surface. In general, it seems that the essential oil mixes well with the polymer base and is homogeneously distributed on the film surface. As the percentage of ZEO increased, the surface roughness of the film as well as their diameter increased. Also, in some places (few locations) it caused pores on the surface of the film (Figure 4A), which may be due to the lack of proper bonding between the ZEO and the film matrix due to its different nature (hydrophilic-hydrophobic).

A similar result was reported by other researchers in the development of fish gelatin film with cinnamon essential oil. They observed an increase in pores at the film surface with an increase in the percentage of essential oil, which was due to the different nature of the polymer base and the essential oil [50]. This defect may be remedied by increasing the emulsifier percentage. Due to its phenolic content, the essential oil can form new O-H bonds to some extent, which creates a bond between the essential oil and the polymer base, as a result, improves its mechanical and inhibitory properties. The essential oil also forms bonds with nanoparticles, especially at high concentrations which can increase mechanical strength as well as improve inhibitory properties (Figure 4B).

Fourier Transform Infrared Spectroscopy (FT-IR)

This method is based on the absorption of infrared light by molecular vibrations. The FT-IR spectra of the various samples are shown in Figure 5. The spectrum of film samples had specific peaks in the region of 3200-3500, 2852-2915, 1312-1573 and 800-1200 cm^{-1}. The peaks in the wavenumbers between 3200 cm^{-1} and 3500 cm^{-1} are related to O-H bonds. The addition of essential oils, attributed to their phenolic content, resulted in the emergence of strong and broad peaks in this region. It was also noted that films containing higher concentrations of essential oils exhibited broader and more intense peaks compared to those with lower concentrations [26]. Furthermore, phenols can form new O-H bonds, which may enhance the integration of ZEO with the film's polysaccharide matrix. This interaction is supported by the XRD analysis, which shows an increase in peak height without any displacement of the peaks. On the other hand, the incorporation of ZnO nanoparticles resulted in a peak shift from 3274 cm^{-1} to 3222 cm^{-1}, indicating the formation of new bonds between the nanoparticles and the essential oil. The absorption intensity at region of 2853 cm^{-1} to

2918 cm^{-1} are related to the C-H bonds, which includes the bond vibrations of C-H, $C-H_2$, $C-H_3$ and bending vibrations, symmetric, asymmetric and occasionally doubles overlapping with O-H. In addition, the peaks in regions 1312 and 1573 are related to symmetric and asymmetric C-OO carboxyl bonds, respectively.

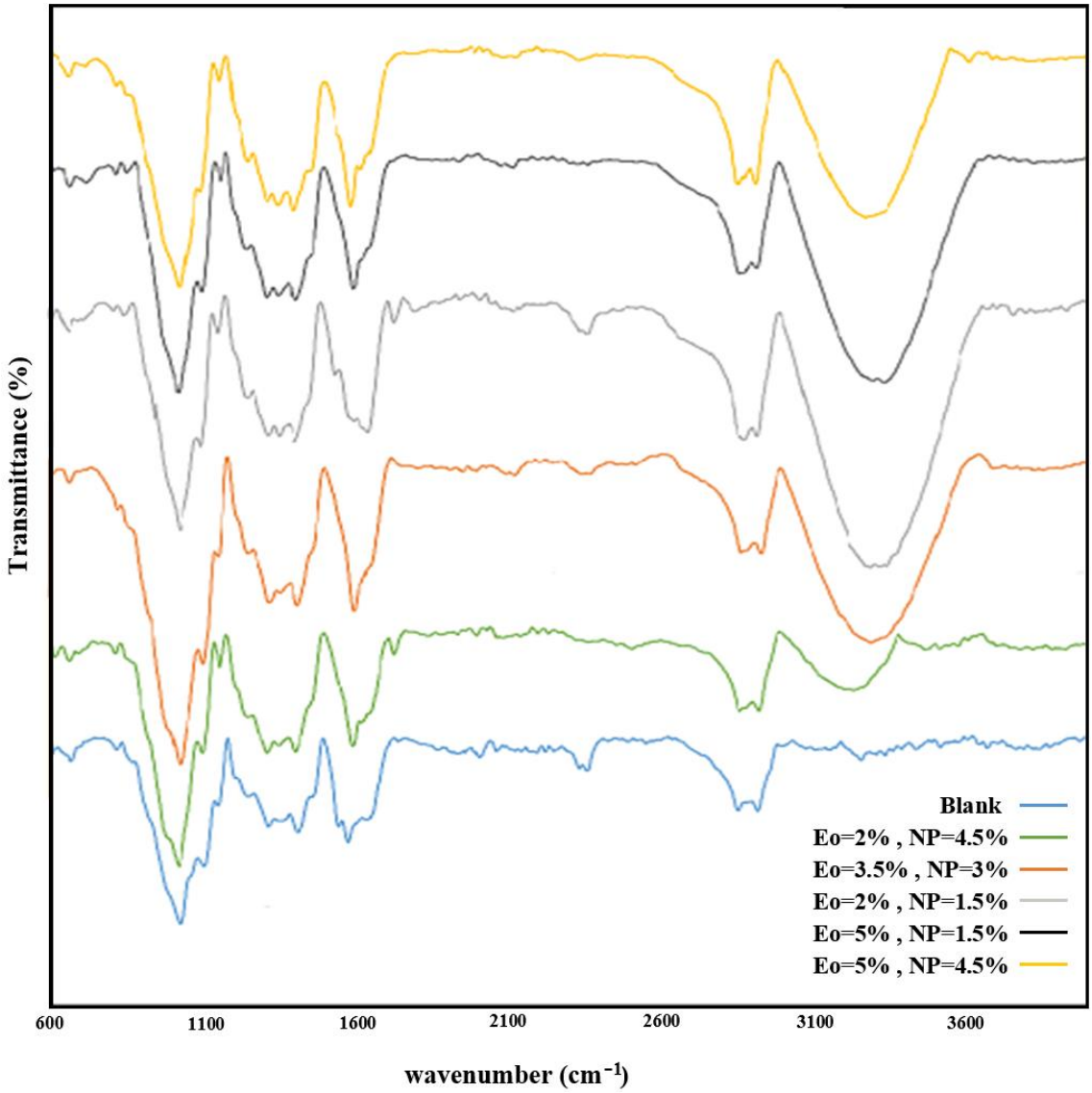

Fig 5: FT-IR spectra of film samples

The presence of these groups can indicate binding sites for ions, which can play an important role in the structure of film lattice [7]. A special peak in wavenumber 1406 cm^{-1} in all samples is due to the addition of glycerol in the structure of the films [51]. On the other hand, poor absorption in wavenumber 1250 cm^{-1} and 1300 cm^{-1} can be due to low amine percentage. Also, the strong and broad peaks that appear in the range of 800 cm^{-1} to 1200 cm^{-1}, which known as the "fingerprint" range for carbohydrates, are related to the vibrations of the C-O, C–O–C glycosidic and C–O–H stretching vibrations

of the polymer chain structure. Addition of ZnO nanoparticles to the film matrix due to the creation of new bonds between oxygen and carbon in the polymer chain, shifted these peaks from 1022 cm^{-1} and 1115 cm^{-1} to 1027 cm^{-1} and 1102 cm^{-1}, respectively. In addition, absorption at wavenumber 814 cm^{-1} and 889 cm^{-1} is probably due to the presence of β-D-manopyranose units [7].

Thermal Weight Analysis (TGA)

TGA analysis is utilized to examine the chemical and physical changes in materials in response to temperature variations. The thermograms of the film samples are presented in Figure 6. The differential thermal gravimetry (DTG) curves revealed four distinct weight loss steps across all samples. The initial weight loss, occurring between 90-110 °C, was attributed to the evaporation of water absorbed by the polysaccharide chains and glycerol, as well as the loss of low molecular weight compounds [52]. The second weight loss around 190-230 °C could be associated with water attached to ZnO NPs and decomposition of glycerol [53, 54]. The third weight loss around 300 °C was attributed to thermal decomposition of polysaccharides and also it was due to degradation of ZnO NPs [55, 56]. And the fourth stage, at about 370-410 ºC, may be due to thermal decomposition of ZEO. The aromatic structures present in the ZEO are highly stable due to the resonance of the benzene ring [52]. Additionally, this reduction may be attributed to the degradation of saccharide rings and the disintegration of the macromolecular chains in LRSM [57]. The presence of a low amount of tween 80 can also be effective in this peak [53].

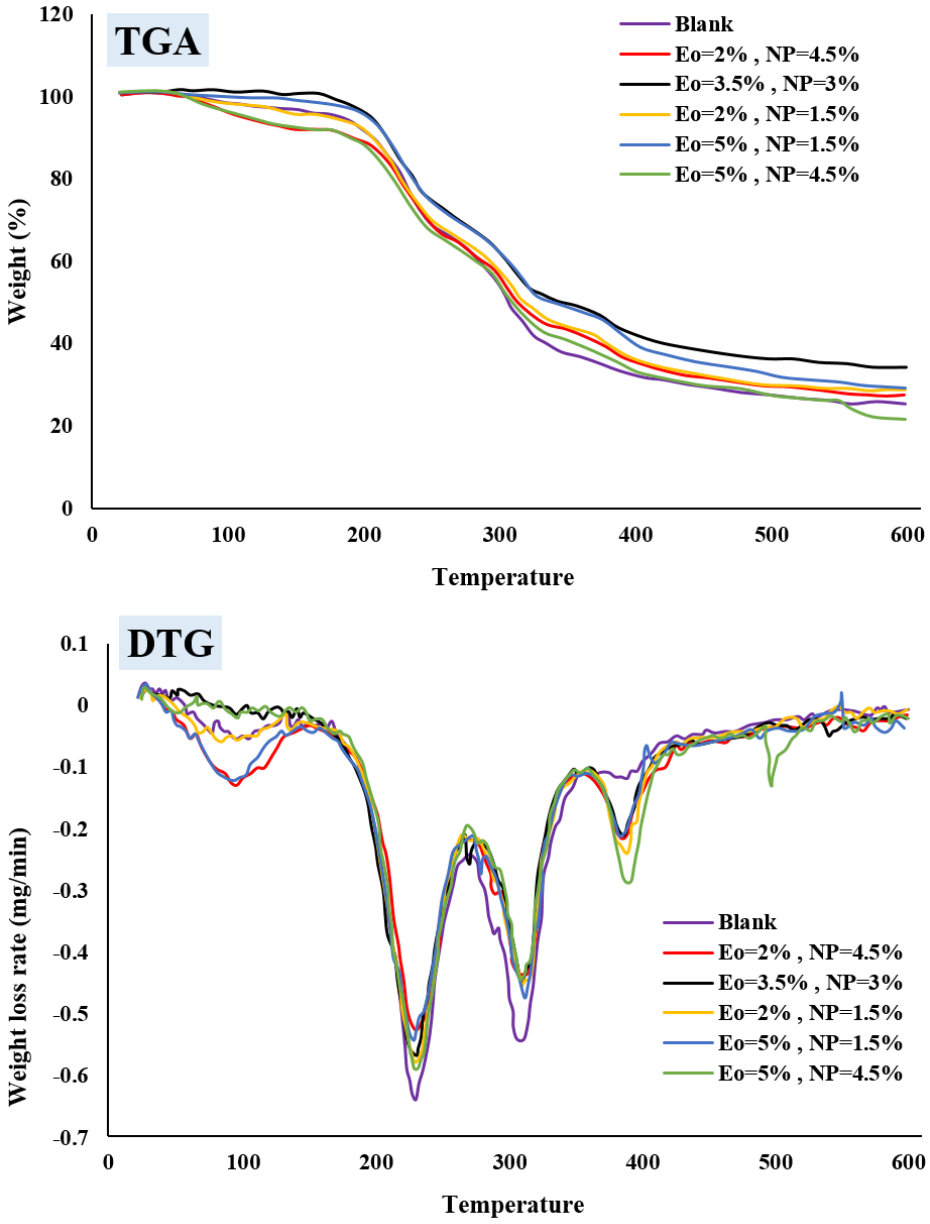

Fig 6: TGA and DTG thermograms of samples

The addition of ZnO nanoparticles decreased the thermal stability of the edible films. Although the impact of ZnO nanoparticles on thermal stability was minimal, they did contribute to a slight reduction in the films' overall stability. Consequently, samples with the highest nanoparticle content exhibited the lowest thermal stability. This decrease may be attributed to the plasticizing effects of ZnO nanoparticles and the resultant modifications to the structure of the film samples [46]. The addition of essential oil had minimal impact on the thermal properties of the films. However, it seems to have slightly enhanced these properties. This improvement may be attributed to the rearrangement of the polymer structure, particularly following the incorporation of higher concentrations of ZEO [53]. Additionally, the bioactive compounds found in essential oils, such as phenolic content, can enhance the thermal stability of edible films [58]. The results were in good agreement with the results of the XRD test, showing that with increasing the degree of crystallinity, the thermal stability was also increased, which might be due to the more energy required to break the higher crystalline structure.

Mechanical Properties

Mechanical properties are crucial characteristics of edible films. Biodegradable films need to possess adequate strength during production and transportation. Consequently, parameters such as tensile strength (TS) and elongation at break (EAB) were assessed to evaluate the films' strength. The results of the mechanical tests are presented in Table 2. Tensile strength (TS) refers to a material's capacity to withstand tensile stress until rupture. The TS value for the control sample was 17.09±0.08 MPa. Generally, the TS of the samples increased with the incorporation of ZnO nanoparticles (NPs) and ZEO. However, unexpectedly, the addition of ZnO NPs significantly decreased the TS of the film samples ($p<0.05$). The edible film containing the highest concentration of ZnO NPs exhibited the lowest TS at 17.55±0.11 MPa. This reduction in TS may be attributed to weak interfacial interactions between the ZnO nanoparticles and the film matrix. Additionally, ZnO NPs have plasticizing effects that can enhance the mobility of polymer chains, while the incorporation of nanoparticles may also diminish the electrostatic bonding among the polymer chains [46]. Unlike ZnO NPs, the addition of ZEO significantly ($p<0.05$) increased the TS of the films. The sample with 5% ZEO had the highest TS (20.63±0.05 MPa). Addition of essential oil probably increases TS due to the formation of hydroxyl bonds between the polyphenolic content and polymer chains. The formation of strong bonds between the ZEO and the carbohydrate polymer reduces the mobility of the chains, which in turn increases the TS [58]. The results of FT-IR analysis confirm the increase of hydroxyl bonds by adding ZEO.

EAB is the film’s stretch ability prior to ruptured. All samples had higher EAB than the control one. According to the results, in general, adding ZnO NPs to edible films did not have a significant effect on EAB of films ($p>0.05$), but it improved the EAB slightly. This slight increase may be due to the plasticizing properties of the nanoparticles, as well could attribute to no interruption effect of ZnO NPs in the movement of polymer chains [46].

Adding ZEO to the film matrix significantly increased EAB ($p<0.05$). As a result, the EAB increased from 4.60±0.07% for the control sample to 7.22±0.11% and 6.92±0.09% for the samples with the highest concentrations of ZEO. The incorporation of essential oil into polysaccharide-based films can enhance cohesiveness and flexibility by disrupting the inter-chain links of the polymers. Overall, adding essential oils tends to reduce the rigidity of the films, thereby improving the flexibility of the polymer

chains and increasing their stretchability [58]. Other researchers achieved similar results and reported an increase in stretchability of films by adding essential oil to the film matrix [46, 52, 59].

Conclusion

In this study, it was observed that incorporating zoulang essential oil and ZnO nanoparticles into the film structure resulted in significant changes to the physicochemical properties of the films. The addition of ZEO enhanced solubility, antioxidant properties, crystallinity, thermal properties, tensile strength, and elongation at break (EAB) in the film samples. Conversely, the moisture content, water absorption, and water vapor permeability (WVP) of the films decreased. The introduction of nanoparticles led to an increase in thickness while reducing moisture content, solubility, water absorption, WVP, antioxidant properties, crystallinity, and tensile strength. Notably, significant insights were gained regarding the new interactions within the film matrix, such as the increase in O-H bonds resulting from the addition of ZEO, which contributed to improved mechanical properties. Ultimately, zoulang essential oil shows promising potential for use in food packaging films, although the performance of ZnO nanoparticles did not meet expectations.

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Ethical approval This article does not report any studies involving human participants or animals conducted by any of the authors.

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مقاله علمی_پژوهشی

فیلم‌های خوراکی فعال مبتنی بر موسیلاژ دانه بالنگو: بررسی اثرات نانوذرات اکسید روی و اسانس گیاه زولانگ

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چکیده

در این تحقیق از موسیلاژ دانه بالنگو به عنوان پایه فیلم استفاده شد، در حالی که اسانس حاصل از برگ و ساقه گیاه زولانگ به همراه نانوذرات اکسید روی (ZnO) به عنوان افزودنی برای تولید فیلم‌های خوراکی فعال ترکیب شدند. یافته‌ها نشان داد که ترکیب اسانس حاصل از برگ و ساقه گیاه زولانگ (ZEO) قابلیت‌های آنتی اکسیدانی، نفوذپذیری، خواص حرارتی، استحکام کششی و ازدیاد طول فیلم را افزایش می‌دهد. تجزیه و تحلیل FT-IR ایجاد پیوندهای هیدروژن-اکسیژن جدید بین ZEO و زنجیره های پلی ساکارید را تایید کرد که به تقویت ساختار فیلم کمک می کند. درحالیکه افزودن نانوذرات اکسید روی باعث کاهش خواص آنتی اکسیدانی، نفوذپذیری، کریستالینیتی، مقاومت حرارتی، رطوبت و استحکام کششی لایه‌ها شد. علاوه بر این، نانوذرات ZnO به افزایش پلاستیسیته کمک کردند که باعث افزایش کشش پذیری لایه ها و ضخامت بیشتر آنها شد. تصویربرداری SEM برهمکنش بین نانوذرات ZEO و ZnOرا تأیید کرد و با یافته‌های آنالیز FT-IR همسو بود. به طور کلی، این مطالعه نشان می‌دهد که فیلم‌هایی که با استفاده از موسیلاژ دانه بالنگو در ترکیب با نانوذرات ZEO و ZnO ساخته شده‌اند، پتانسیل بالایی برای کاربرد در بسته‌بندی مواد غذایی و فیلم‌ها یا پوشش‌های فعال، به‌ویژه برای مواد آسیب‌پذیر در برابر اکسیداسیون دارند.

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Scientific Research

Physicochemical and Mechanical Properties of Edible Sodium Alginate Films

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ABSTRACT

This study aims to investigate the physicochemical and mechanical properties of edible films prepared from sodium alginate at concentrations of 0.5% and 1%, with glycerol as a plasticizer at concentrations of 10%, 30%, and 50%. Thickness, tensile strength, percentage elongation, water solubility, water vapor permeability, light permeability, transparency, and antioxidant activity were evaluated. Results showed that the thickness, elongation percentage, water solubility, and water vapor permeability of the films increased with the rise in glycerol concentration from 10% to 30%. In contrast, tensile strength, water solubility, light permeability, and transparency decreased when the sodium alginate concentration was increased to 1% at the same glycerol concentrations. Antioxidant activity increased with higher sodium alginate concentrations at the same levels of glycerol. Hence, sodium alginate can be used as a natural component in food or pharmaceutical industries

1- Introduction

The widespread plastic waste globally has hazardous toxic effects on the environment, aquatic life, and humans. Due to its non-biodegradable nature and improper disposal, significant research efforts have been devoted to finding eco-friendly alternatives that are self-degradable, edible, and health-safe. These alternatives, known as biopolymers, include proteins, carbohydrates, fats, or their combinations, derived from plant or animal sources, or as by-products of the food industry, reducing industrial waste (Rossi-Márquez et al., 2023).

Edible films have garnered global attention due to their ability to coat food items, biodegrade, and be consumed with food or removed before consumption. They maintain food quality by acting as barriers against moisture and oxygen, preventing undesired chemical, enzymatic, or microbial interactions (Farhana et al., 2022). Polysaccharides have been utilized in preparing edible films due to their affordability, availability, renewable nature, and good mechanical properties that preserve food texture, flavor, and shelf life (Cazon et al., 2017).

Sodium alginate, a biodegradable linear polyuronic acid, consists of α-L-guluronate and β-D-mannuronic acid units. Alginate has excellent gas barrier properties but poor water vapor barrier properties due to its hydrophilic nature. It is a low-toxicity carbohydrate colloid extracted primarily from brown algae, capable of forming gels, making it a vital material in applications such as packaging, tissue engineering, wound dressings, and edible films (Leyva-Jiménez et al., 2023; Li et al., 2022; Foschi and Bonoli, 2019).

The current study aims to prepare edible films using various concentrations of sodium alginate and the plasticizer glycerol and to investigate their qualitative properties.

2.Materials and Methods

Materials:

The materials employed in this study were chosen in a way that would guarantee the reliability of results in light of similar studies conducted in the future. All materials were sourced from Sigma-Aldrich (Germany) unless otherwise specified:

- Sodium alginate: Used as the primary biopolymer for film formation.
- Glycerol (pharmaceutical grade): Served as a plasticizer to enhance film flexibility and mechanical properties.
- 1,1-Diphenyl-2-picrylhydrazyl (DPPH): Used as a standard antioxidant reagent to assess the antioxidant activity of the films.
- Distilled water: Used as the solvent.

Preparation of Edible Films:

These solutions were prepared following the method of Anward et al (2013) with slight modifications This solution consist of sodium alginate 0.5% and 1.% w/v and glycerol 10%, 30% and 50% w/v in 100 ml distilled water The solutions were prepared by mixing on Gellenkamp hotplate magnetic stirrer at 80°C for about twenty minutes. Following gelation of the solution at ambient temperature, the resulting gel was poured into 9.1 cm diameter Petri dishes and allowed to dry at 25-30°C for approximately 20 – 24 hours. The films formed were subjected to relative humidity of 52% and then subsequently analyzed.

Film Thickness, Tensile Strength, and Elongation at Break:

Thickness, tensile strength, and elongation at break of the PCL/Chitosan/HA films were determined using a texture analyzer at Polymer Research Center, University of Basrah based on ASTM standard number D-882-10.

Water Solubility:

According to Jancikova et al., 2020 water solubility was carried out by weighing film squares 2x2 cm, drying them at 105°C until their constant weight was attained, immersing them in distilled water, 50 ml for 24 hours at 25°C, then drying and re-weighing. The solubility was calculated as:

$$\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \text{x } 100\%.$$

Water Vapor Permeability (WVP):

Water vapor permeability (WVP) was measured based on the ASTM E96-16 witnessing a modification as well (Mehdizadeh et al., 2020). Unglued plastic cups (3.9 cm outer diameter, 3.75 cm inner diameter, and 6.25 cm depth) were filled to a volume of 10 ml distilled water, covered with the film and rubber band and kept in desiccator containing anhydrous copper sulph. Weight loss was monitored every 6 hours until constant weight. WVP was calculated as:

$$\mathbf{WVP} = \frac{\mathbf{WVT}}{\mathbf{S} * (\mathbf{R1} - \mathbf{R2})}$$

where: G = weight loss (g), t = time (h), A = film area (m²), S = saturated vapor pressure at 25°C, R1 = relative humidity of the surrounding environment (52%), R2 = relative humidity in the desiccator (0%).

Light Permeability and Transparency:

Light permeability was measured using a spectrophotometer (Physics Department, University of Basrah) over a wavelength range of 200–900 nm, as described by Niroomand et al. (2016).

Antioxidant Activity:

Antioxidant activity was measured using DPPH following Al-Hilifi et al. (2023). One milliliter of film solution was mixed with 1 ml of 0.01% DPPH solution, incubated in the dark at 25°C for 30 minutes, and the absorbance measured at 517 nm. Antioxidant activity was calculated as:

$$\text{Antioxidant Activity (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \text{ x } 100\%$$

3.Results and Discussion

The simple films prepared from sodium alginate at concentrations of 0.5% and 1%, combined with glycerol at concentrations of 10%, 30%, and 50%, exhibited transparency, glossiness, and a smooth texture. They could be easily detached from the plate. Figure 1 illustrates the types of films prepared from sodium alginate.

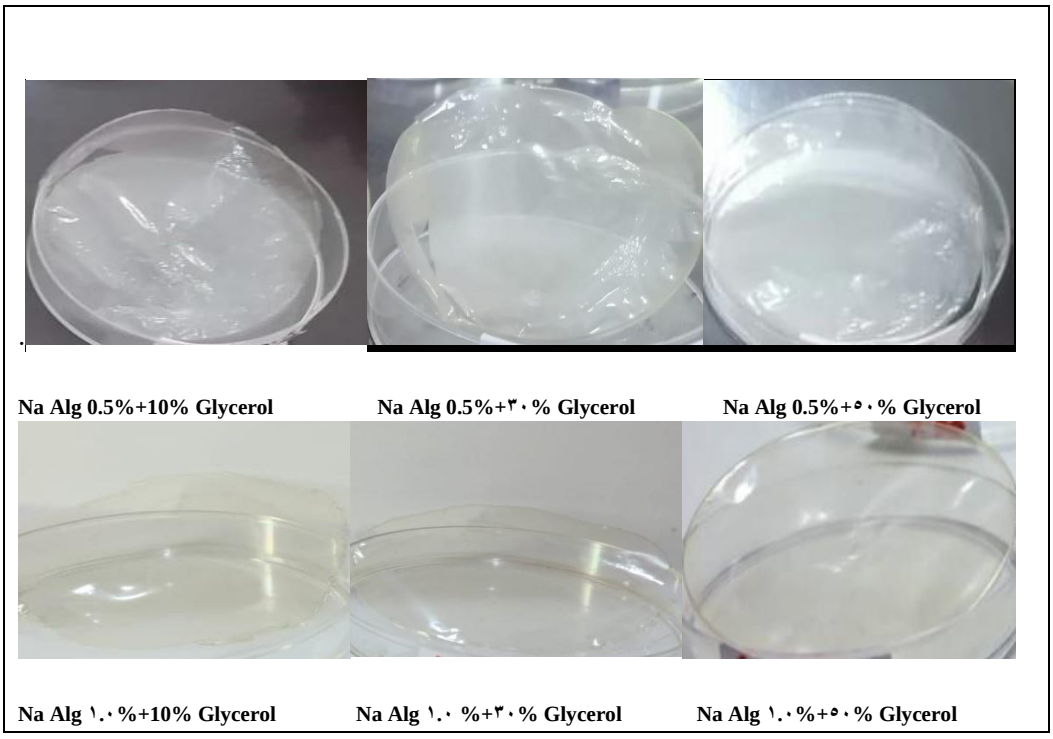

Figure 1: Simple Sodium Alginate Films

Film Thickness:

Thickness is an important factor affecting the application of films for packaging specific products.

It also influences other mechanical properties such as tensile strength, elongation, water vapor transmission rate, and solubility. Film thickness varies based on film composition; however, for practical application, it must be adjusted according to the product being packaged (Fransiska et al., 2021; Khairunnisa et al., 2018).

Figure ٢ illustrates the thickness of films prepared from sodium alginate at concentrations of 0.5% and 1%, with glycerol concentrations of 10%, 30%, and 50%. An increase in glycerol concentration led to greater film thickness at each alginate level. For films with 0.5% sodium alginate, the thickness values were 0.05, 0.06, and 0.07 mm, while for 1% sodium alginate, the thickness values were 0.06, 0.07, and 0.08 mm. This is attributed to glycerol's solubility in water, which increases solution viscosity and binds water. Higher glycerol concentrations reduced the rate of water evaporation, as some water in the film solution binds to glycerol, influencing the resulting film's thickness. Also, an increase in plasticizer content (glycerol) affects the film components and develops the thickness of the film (Khairunnisa et al., 2018).

Alginate concentration also affected film thickness; the increased sodium alginate concentration caused thicker film formation. This happens because the thickness of the film corresponds to the content of polymers in the solution. It turns out that with the increased concentration of alginate, more polymers are produced, which forms a thicker gel and film (Tavassoli-Kafrani et al., 2016). Additionally, the film thickness of the nanoparticles can also be affected by the manufacturing and drying processes (Racmayani & Husni, 2020).

The film thickness values measured in this study are acceptable based on the JIS for the maximum allowable thickness of edible films, which is 0.25 mm (Suyatno and Ariska 2015). The research data confirmed that even the thickest film used in this research corresponds to the necessary standards when being applied as the primary packaging layer within food production. In addition, with the increase in glycerol content, the thickness of the films was higher due to the interactivity of the said plasticizer with the components of the films (Maria et al., 2018). In the same manner, the thicker films observed at higher sodium alginate concentrations were due to dense polymer content, resulting in increased gel thickness (Tavassoli-Kafrani et al., 2016). It has been found that manufacturing stages affect film thickness and drying processes (Racmayani and Husni, 2020). All films formulated in this study were below the Japanese Industrial Standards (JIS) maximum thickness of 0.25 mm for edible films (Suyatno and Ariska, 2015), meeting the needed food packaging applications.

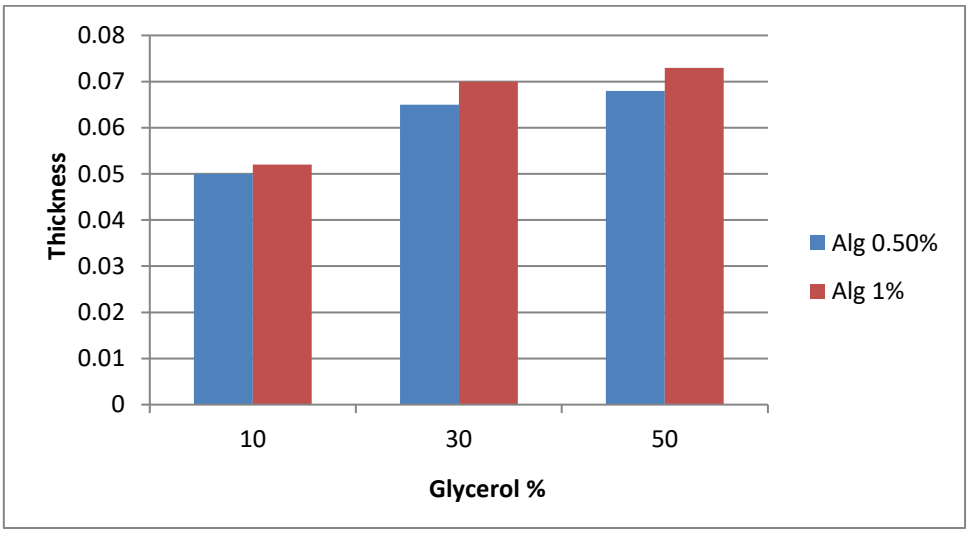

Figure 2: Thickness of Alginate Films

Tensile Strength:

Figure 3 presents the tensile strength of the films made of 0.5% and 1% of sodium alginate with the addition of glycerol at 10%, 30%, and 50%. A clear

inverse relationship between glycerol concentration and tensile strength is evident (0.5% alginate: 3.89, 3.54, 3.07 MPa; 1% alginate: 5.39, 5.27, 4.85 MPa). This decrease in strength is due to the ability of glycerol to disrupt the interactions between polymer chains within the film matrix (As et al., 2017). However, enhancing alginate concentration leads to a substantial enhancement of tensile strength in accordance with Racmayani and Husni (2020), where the alginate concentration of 2% to 6% provided 0.519MPa to 3.097MPa tensile strength correspondingly. This enhancement results from higher polymer chains and, consequentially, stronger inter-molecular force.

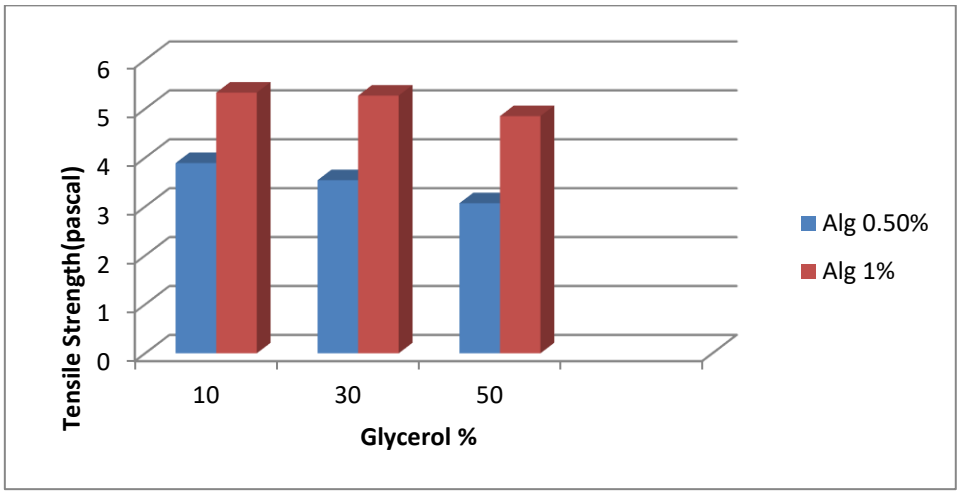

Figure 3: Tensile Strength (MPa) of Alginate Films

Elongation at Break:

High elongation values are critical for films to resist natural stress during application on food. As shown in Figure ٤, the maximum elongation was 1.7% for films prepared with 1% alginate and 50% glycerol, while the minimum elongation was 1.1% for films with 1% alginate and 10% glycerol. For films with 0.5% alginate and 10% glycerol, the elongation was 1.2%, increasing to 1.9% for 0.5% alginate with 50% glycerol. The increase in elongation with glycerol concentration is attributed to molecular interactions between the polymer and plasticizer. Glycerol molecules disrupt hydrogen bonds between polymer chains, increasing molecular flexibility and film elasticity (Sitompul and Zubaidah, 2017).

The alginate concentration also affects elongation, as higher alginate levels increase the solution's viscosity, leading to a more elastic polymer structure (Sitompul and Zubaidah, 2017).

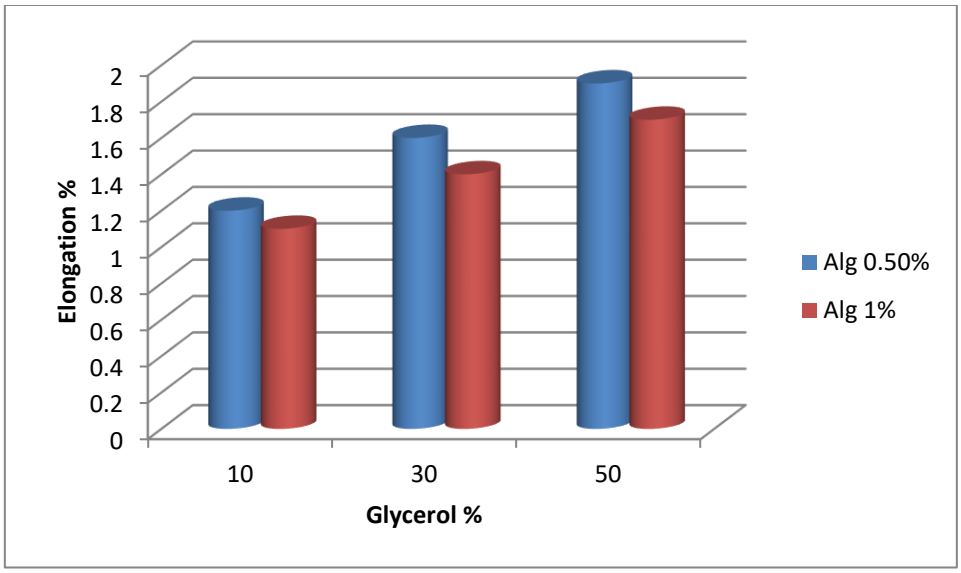

Figure 4: Elongation Percentage of Alginate Films

Water Vapor Permeability (WVP)

Figure ٥ shows that the maximum WVP was 1.82 g·mm/m²·h·kPa for films with 1% alginate and 50% glycerol, while the minimum WVP was 1.56 g·mm/m²·h·kPa for films with 0.5% alginate and 10% glycerol. WVP increased with higher glycerol concentrations (10%–50%) and alginate concentrations (0.5%–1%). This is because plasticizers disrupt hydrogen bonds within polymer chains, increasing molecular mobility and facilitating water vapor transmission (Senna et al., 2011).

Additionally, a positive correlation was observed between film formulation and permeability values. Increased polymer concentration leads to higher viscosity during mixing, causing air bubbles and larger voids in the film structure, which enhance permeability (Rachtanapun and Rattanapanone, 2011).

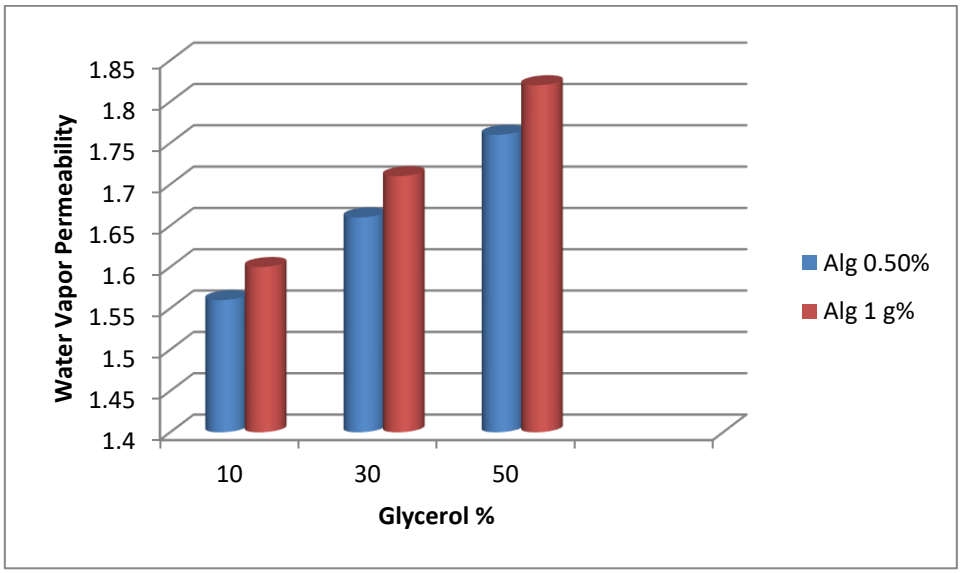

Figure 5: Water Vapor Permeability of Alginate Films

Water Solubility:

The results presented in Figure ٦ indicate the solubility of films prepared with different concentrations of sodium alginate (0.5% and 1%) and glycerol (10%, 30%, and 50%). The maximum solubility was 86.62%, while the minimum solubility was 79.49% for films with 0.5% alginate and varying glycerol levels. For films with 1% alginate, the maximum and minimum solubility values were 84.51% and 78.86%, respectively.

The solubility of the films is influenced by alginate concentration, as alginate is hydrophilic and readily absorbs water. At higher alginate concentrations, the film absorbs more water, and the increased water interaction reduces film density. Since sodium alginate is highly water-loving, it dissolves more easily in water at higher concentrations due to weaker bonds between alginate and glycerol molecules (Racmayani and Husni, 2020).

Edible films with high solubility are ideal for ready-to-eat food products, as they dissolve

easily upon ingestion. On the other hand, low solubility is a critical requirement for films used to package food products with high water activity (Fransiska et al., 2021; Cazon et al., 2017).

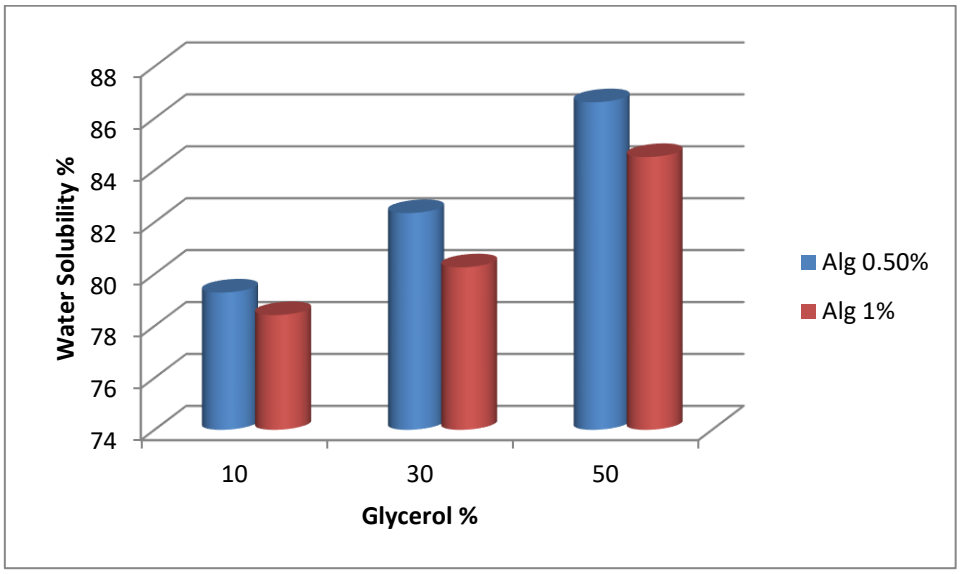

Figure 6: Water Solubility Percentage

Light Permeability and Transparency:

Figure (7) illustrates the percentage of light transmission for sodium alginate films measured at wavelengths ranging from 200 to 900 nm. The transmittance ranged between 55.98% and 82.66% for films prepared with 0.5% sodium alginate and varying glycerol concentrations (10%, 30%, and 50%). However, transmittance decreased when the sodium alginate concentration increased to 1% under the same glycerol concentrations, ranging between 48.784% and 58.566%. Transparency also declined with the increase in both sodium alginate and glycerol concentrations, reaching 3.07 at 0.5% sodium alginate and 50% glycerol and decreasing to 2.07 at 1% sodium alginate and 50% glycerol. This reduction is attributed to increased film density due to the higher solid content and enhanced cross-linking between sodium alginate and glycerol molecules, filling the spaces within the polymer matrix. Consequently, light reflection, scattering, and obstruction take place, which cause the film structure to look less bright and denser (Khairunnisa et al., 2018; Yan et al., 2022; Li et al., 2024).

Such results have similar characteristics with the study of Li et al. (2022) on the evaluation of the optical property of sodium alginate edible films reinforced by tannic acid. This they noted as important since light transmission they postulated plays an important role in the deterioration of food products through effects such as lipid oxidation and nutrient degradation which results to shorter shelf life of foods.

Likewise, the findings harmonized with those of Bhatia et al (2023) regarding the transparency of chitosan/alginate films incorporating fig extract.

Transparency is a term associated with the degree of clarity associated to a film and the capability to pass through light. Transparency can also improve the consumer acceptance of the edible films since it worked as aesthetical value in marketing edible film (Apriliyani et al., 2020).

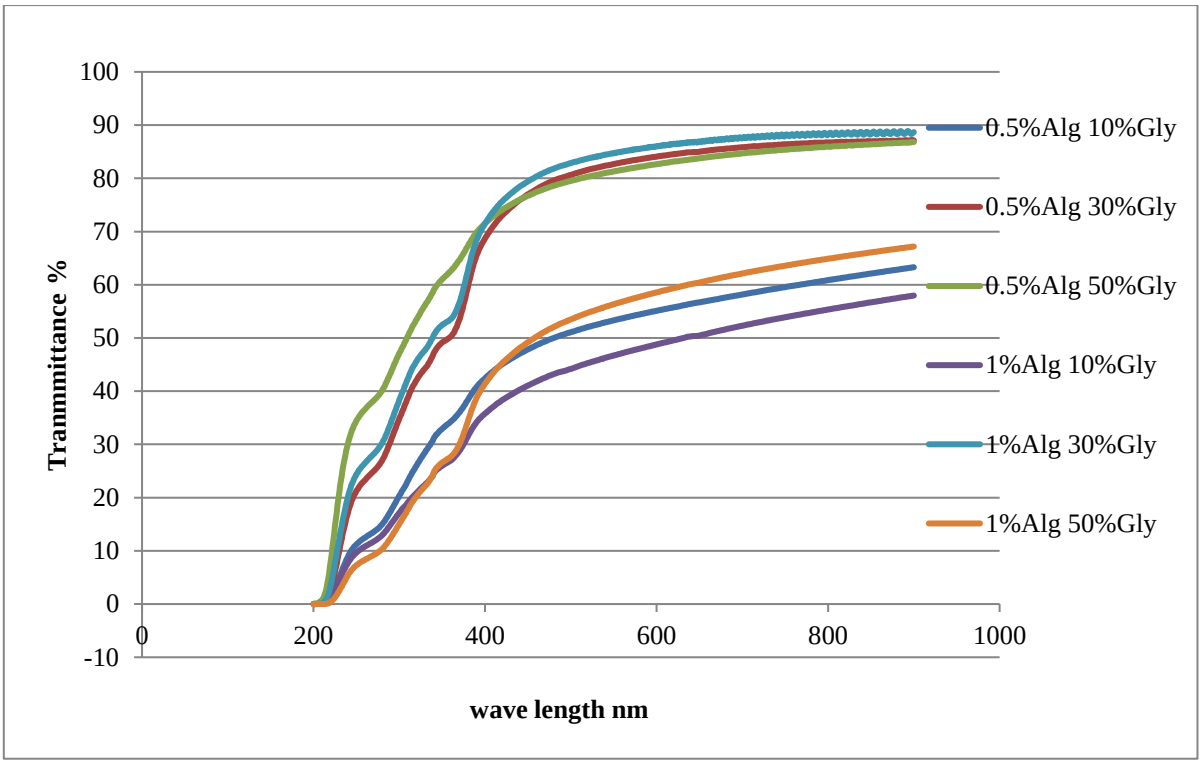

Figure 7: Light Transmission Percentage of Alginate Films

Antioxidant Activity:

The antioxidant activity of the prepared films was evaluated using a simple colorimetric method with DPPH (2,2-diphenyl-1-picrylhydrazyl). In this test, the color of the film solution changed from purple to yellow, and the intensity of the color change was measured to estimate its effectiveness in scavenging free radicals. As shown in Figure (٨), the highest antioxidant activity was 54.31% for the film prepared from 1% sodium alginate and 50% glycerol, while the lowest antioxidant activity was 48.11% for the film prepared from 1% sodium alginate and 10% glycerol. The antioxidant activity was 36.84% for the film prepared from 0.5% sodium alginate and 10% glycerol, and increased to 42.14% for the film prepared from 0.5% sodium alginate and 50% glycerol. These results are in agreement with those found by Sellimi et al. (2015), who studied the antioxidant properties of sodium alginate extract from Tunisian brown seaweed using various antioxidant tests. They observed that sodium alginate exhibited free radical scavenging activity, strong ferrous ion-reducing ability, and high protection against DNA damage. Li et al. (2022) mentioned that sodium alginate is a linear, biodegradable polysaccharide composed of a-L-guluronic acid (G) and ß-D-mannuronic acid (M) in varying ratios, which gives alginate its gelling properties. They also noted that antioxidants work by delaying or preventing food spoilage and quality deterioration caused by oxidation, such as oxidative rancidity and enzymatic browning in certain foods.

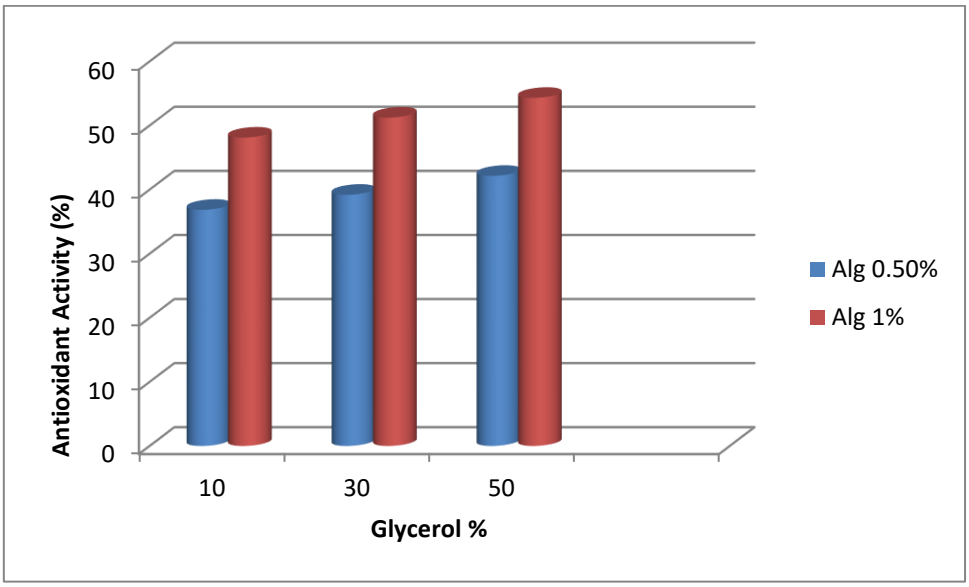

Figure 8: Antioxidant Activity (%)

4.Conclusions

This study successfully prepared edible films using sodium alginate (0.5% and 1%) and glycerol (10%, 30%, and 50%). The results demonstrate that glycerol concentration significantly impacts film properties. Increasing glycerol concentration (from 10% to 30%) led to increased thickness, elongation percentage, water solubility, and water vapor permeability. Conversely, increasing sodium alginate concentration to 1% (at the same glycerol concentrations) decreased tensile strength, water solubility, light permeability, and transparency. However, antioxidant activity increased with higher sodium alginate concentrations at all glycerol levels. All films produced met the Japanese Industrial Standards (JIS) maximum thickness for edible films, making them suitable for food packaging applications. The findings suggest that sodium alginate can be a viable natural component in food and pharmaceutical industries due to its film-forming properties and antioxidant activity. Further research could optimize the concentrations of sodium alginate and glycerol to achieve desired film properties for specific applications.

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Scientific Research

Extraction of bioactive compounds from saffron stigma into sunflower oil: A comparative study of various solid-liquid extraction methods

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ABSTRACT

Flavoring edible oils is aimed at enhancing oxidative stability and creating desirable sensory properties to expand their market. This study aimed to produce saffron-flavored sunflower oil using various solid-liquid extraction techniques including stirring-assisted maceration (SAM), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). The study aims to optimize the effective parameters in each extraction method and characterize the physicochemical properties of the flavored oils. The UAE method effectively extracted bioactive compounds of saffron stigma in a shorter time frame. However, the high energy input during the UAE process led to the deterioration of its physicochemical and sensory properties. Similarly, in the MAE method, sensitive and volatile compounds of saffron stigma were degraded. Alternatively, the SAM method, in a gentle and continuous process, managed to dissolve a high concentration of saffron compounds in the oil while preserving its physicochemical and sensory properties. The optimal concentrations of picrocrocin, safranal, and crocins were determined to be 317, 56, and 160 mg L^{-1}, respectively. The aromatized sunflower oil presents a promising novel product for diverse food applications and market expansion.

1- Introduction

Production of edible oils flavored with different herbs and spices has been increasing in recent years [1]. The global practice of flavoring oils serves several purposes, including the enhancement of oxidative stability in edible oils [2], which are prone to degradation through exposure to air, light, and heat. This oxidative process can lead to unfavorable odors, decreased nutritional quality, and potential health risks for consumers. Artificial antioxidants like TBHQ, BHT, BHA, and PG are commonly used in commercial settings to prevent this degradation, yet their safety and effectiveness are under scrutiny [3]. Therefore, there is a critical need to explore alternative methods, such as incorporating natural antioxidants from vegetables, herbs, spices, and fruits, to improve the quality and stability of edible oils [4]. In response to consumer demand for products with enhanced sensory qualities and health benefits, the practice of aromatizing edible oils with natural aromatic and flavoring substances, such as herbs, spices, vegetables, and fruits, has gained prominence [5, 6]. These substances, in various forms like powders, extracts, or essential oils, are added to oils to create products with unique sensory attributes that cater to the preferences of consumers seeking gourmet experiences. Furthermore, the incorporation of bioactive substances into oils not only enhances their nutritional value [7] but also brings about additional benefits, including antimicrobial, anticancer, and antioxidant effects, along with improvements in sensory perception and stability [5, 8].

Various methods exist for aromatization of edible oils. These methods include direct addition of essential oils and oleoresins [8, 9], maceration [10], malaxation [11], and liquid-liquid extraction (LLE) [6]. In maceration, solid parts of plants or fruits directly contact the oil [12]. Sometimes, maceration is coupled with stirring, heating [13], ultrasound [14, 15] or microwave [9] treatment to accelerate extraction.

Iran is the world's leading producer of saffron (*Crocus sativus* L.) [16], with its stigma containing bioactive compounds such as safranal, picrocrocin, and crocins, known for their aroma, bitter taste, and color respectively. Saffron has been historically used for medicinal and culinary purposes due to its unique composition [17]. Modern research has uncovered its various health benefits, including anticarcinogenic, antimutagenic, immunomodulating, neuroprotective, and antioxidant properties [18]. In Iran, saffron is widely utilized in coloring and flavoring various food products [19]. However, limited literature exists on incorporating saffron stigma into edible oils. A study by Sena-Moreno et al. blended olive oil with aqueous saffron extract, but it led to a decrease in oxidative stability due to the back-extraction of polyphenolic compounds [6]. Thus, there's a need for suitable methods to flavor edible oils with saffron stigma. Additionally, no research has explored flavoring sunflower oil (extracted from sunflower plant seeds (*Helianthus annuus*)) with saffron stigma, presenting a potential opportunity for creating a unique and valuable product.

In this study, sunflower oil was aromatized with saffron stigma for the first time.

Different methods such as stirring-assisted maceration (SAM), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) were explored and optimized to extract saffron stigma's bioactive compounds into sunflower oil. Furthermore, the physicochemical characteristics of the resulting flavored sunflower oils were evaluated.

2 Experimental section

2.1 Materials and chemicals

Saffron stigma was obtained from Ilia's company (Iran). Sunflower oil, obtained through the cold-press method, was purchased locally in Iran without any antioxidant additives. Cyclohexane and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA), while ethanol (96%) was supplied by Ameretat Co (Iran). Methanol, Folin Ciocalteu's reagent, acetic acid, potassium iodide, potassium hydroxide, starch, and phenolphthalein were obtained from Merk (Germany). Sodium carbonate was sourced from AppliChem (Germany), and isooctane was from Supelco (Germany). Deionized water was produced using the ZU101 water purification system (Zolalan Pars Co., Iran).

2.2 Aromatization of sunflower oil by saffron stigma

2.2.1 Ultrasound-assisted extraction (UAE)

Initially, saffron stigma was ground into a fine powder using a mortar. Then, 0.03 g of the powder was added to 4.5 mL of sunflower oil. The extraction process was conducted using either an ultrasonic probe (p-UAE) (UHP-400, Topsonics, Iran) or an ultrasonic bath (b-UAE) (vCLEAN 1- L2, Backer, Iran). p-UAE operated with a duty cycle of 7s on/3s off. After 10 min of operation, a 10-min resting period was implemented, followed by resumption until extraction completion. The b-UAE process involved continuous operation for 10 min followed by a 5-min resting period, with optional stirring using a magnetic stirrer during intervals.

2.2.2 Microwave-assisted extraction (MAE)

0.03 g of saffron powder was mixed with 4.5 mL of sunflower oil. The sample underwent microwave heating (M245, Butane, Iran) for 30 s at different power levels, followed by stirring on a magnetic stirrer for 5 min. This cycle of microwave heating and stirring was repeated sequentially until a total time of 60 min was reached (total 5 min microwave).

2.2.3 Stirring-assisted maceration (SAM)

0.03 g of saffron powder was mixed with 4.5 mL of sunflower oil. The mixture was then stirred using a magnetic stirrer (MR3001 K, Heidolph, Germany) at 500 rpm for one week.

2.3 Determination of saffron stigma bioactive compounds in aromatized sunflower oil

Initially, 1.5 mL of the clarified aromatized sunflower oil was transferred to a 10 mL falcon tube and diluted with 6 mL of cyclohexane, followed by shaking. LLE was conducted by adding 2 mL of 60% v/v ethanol, vigorously shaking for 3 min. The two phases were then separated by centrifugation at 4000 rpm for 10 min. The absorbance of the ethanolic phase was measured using a SP-UV500DB spectrophotometer (Spectrum, China) at

wavelengths of 257 nm, 330 nm, and 440 nm for picrocrocin, safranal, and crocins, respectively. Standard curves were constructed within the concentration range of 5-25 μg mL^{-1} for picrocrocin and 2-15 μg mL^{-1} for safranal and crocins, using 60% v/v ethanol as the solvent.

2.4 Physicochemical characterization of flavored sunflower oil

2.4.1 Acid value (AV)

20 g (m) of oil was placed in a 250 mL Erlenmeyer flask, and approximately 100 mL of a neutralized ethanol-diethyl ether mixture (1:1) was added. Then, 3 to 4 drops of phenolphthalein reagent were added and mixed thoroughly. The resulting solution was titrated with 0.1 N standard solution of potassium hydroxide (N) until a persistent pale pink color appeared. Finally, AV was calculated in milligrams of potassium hydroxide per gram of fatty acid (mgKOH g^{-1}), (equation 1) with 'V' representing the volume of titrant consumed to reach the endpoint [20].

$$AV = \frac{V \times N \times 56.1}{m} \quad (1)$$

2.4.2 Peroxide value (PV)

5 g (m) of oil was placed in a 250 mL Erlenmeyer flask. A mixture of 50 mL glacial acetic acid:isooctane (3:2) was added to the sample and mixed thoroughly until complete dissolution of the oil was achieved. Subsequently, 0.5 mL of saturated potassium iodide solution (1.75 g mL^{-1}) was added to the mixture. The Erlenmeyer flask was sealed with its lid and stirred for 1 min. After removing the lid, 100 mL of freshly boiled distilled water was added promptly to the flask. The released iodine was titrated with 0.01 N sodium thiosulfate (N) in the presence of starch reagent until the blue color of the solution disappeared, indicating the endpoint. PV was expressed in milliequivalents of active oxygen per kilogram of oil ($meqO_2$ kg^{-1}), (equation 2) with 'V' representing the volume of titrant consumed to reach the endpoint [21].

$$PV = \frac{V \times N \times 1000}{m} \quad (2)$$

2.4.3 Determination of total phenolic content (TPC)

TPC in the sunflower oil was determined using the Folin-Ciocalteu assay [22, 23]. Initially, 100 μL of oil was mixed with 2 mL of methanol and stirred for 15 min, followed by centrifugation. Subsequently, 400 μL of 10% Folin-Ciocalteu reagent was added to the methanol fraction and left in darkness for 6 min. Then, 500 μL of 7.5% sodium carbonate was introduced, and the mixture was vortexed. The resulting solution was kept in darkness for 1 h, and the absorbance was measured at 765 nm. Gallic acid (10-85 μg mL^{-1}) in methanol was reacted with 10% Folin-Ciocalteu reagent to construct the calibration curve. The results were expressed as milligrams of gallic acid equivalents per gram of oil (mg GAE g^{-1}).

2.4.4 Total carotenoid content (TCC)

Initially, 7.5 g of oil was adjusted to volume in a 25 mL volumetric flask using cyclohexane. The TCC was measured using a spectrophotometer at a wavelength of 470 nm. The concentration of carotenoid pigment was determined in milligrams per kilogram (mg Kg^{-1}) using equation 3 [24].The density of the sunflower oil was experimentally determined to be 0.915 g mL^{-1} at 25°C by measuring the weight of a known volume of oil.

$$C_{carotenoid} = \frac{Abs_{470} \times 10^6}{2000 \times 100 \times density} \quad (3)$$

2.4.5 Evaluation of the antioxidant activity

Initially, 100 µL of oil was transferred to a falcon tube, covered with foil. Then, 2 mL of a DPPH solution (100 µM) was added to the tube and vortexed thoroughly. The sample was stored in the dark at room temperature for 30 min. Finally, the absorbance of both the control sample ($A_{control}$) and the flavored sunflower oil (A_{sample}) was determined using a spectrophotometer at 517 nm, with methanol as the blank. The results were expressed as the inhibition percentage according to equation 4 [25].

$$I\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (4)$$

2.4.6 K232 and K268 extinction coefficients

0.25 g and 0.05 g of oil were individually transferred to 25 mL volumetric flasks and diluted with isooctane for the determination of K268 and K232, respectively. The absorption (E_λ) at wavelengths of 232 and 268 nm was recorded with isooctane as the blank. Specific absorption (K_λ) was calculated using equation 5, where 'C' represented the concentration of the final solution (g mL^{-1}), and 'S' denoted the cell length (cm) [26].

$$K_\lambda = \frac{E_\lambda}{100 \times C \times S} \quad (5)$$

All measurements were conducted in triplicate. Statistical analysis, including calculation of mean, standard deviation, and ANOVA analysis, was performed using GraphPad prism software. Statistically significant differences were defined as those with p-values ≤ 0.05.

3 Results and discussion

3.1 UV-Vis measurment of the saffron stigma active compounds in oil samples

One of the significant methods for quantifying saffron compounds is the ISO 321 standard method, wherein picrocrocin, safranal, and crocins are assessed at wavelengths of 257, 330, and 440 nm, respectively [27]. In this study, instead of measuring E1%, as in Sena-Moreno's work [6], calibration curves were generated using standard solutions of all three compounds. Here, calibration curves for each of the mentioned compounds in 60% ethanol were plotted in the range of 5 to 25 µg/ml for picrocrocin and 2 to 15 µg/ml for safranal and crocins, as depicted in Figure 1a. Additionally, similar to the study by Suárez et al. [28], polar compounds extracted in oil were isolated using the LLE technique with an aqueous alcoholic solution in order to prevent sunflower oil interfering compounds adsorption, and the resultant aqueous-alcoholic extract (60% ethanol) was analyzed via UV-Vis spectroscopy. The concentrations of picrocrocin, safranal, and crocins were measured at their respective maximum wavelengths, as depicted in Figure 1b, which are 257, 330, and 440 nm, respectively. As depicted in Figure 1b, the absorption spectrum pattern of the LLE extract closely resembles that of the corresponding compounds in the standard solution, indicating the success of the extraction process.

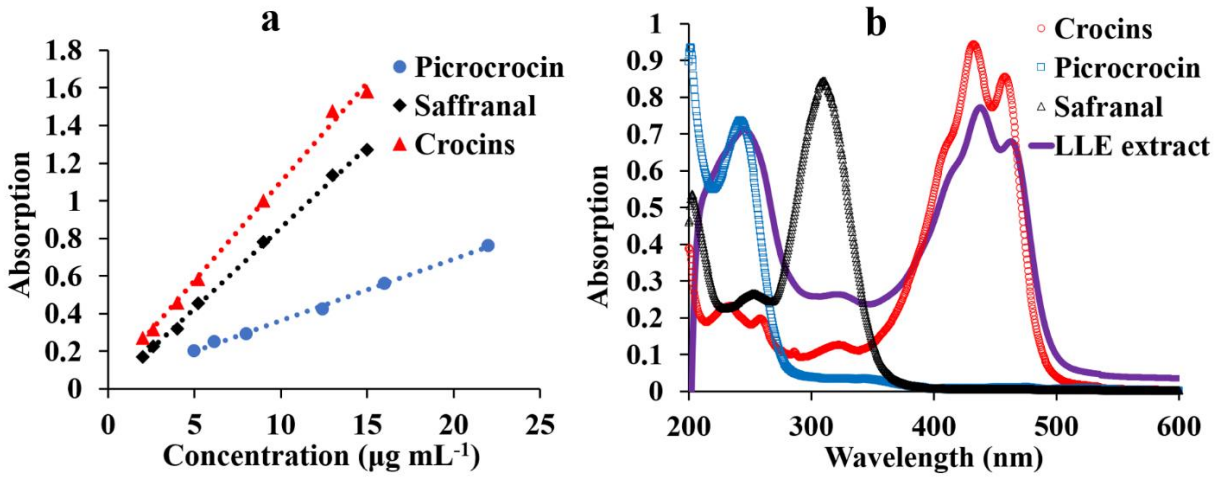

Figure 1 Calibration curves (a) and Absorption spectra (b) of picrocrocin, safranal and crocins in standard or LLE extraction solution prepared in ethanol 60%. The calibration equations for picocrocin, safranal, and crocins are as follows: $y = 0.0328x + 0.0341$ (R^2=0.9973), $y = 0.0861x - 0.0031$ (R^2= 0.9989), $y = 0.1054x + 0.0474$ (R^2=0.9965).

3.2 Ultrasound-assisted extraction (UAE)

This study focused on aromatizing sunflower oil by blending it with saffron stigma using different solid-liquid extraction techniques. UAE was chosen for its notable efficiency, as highlighted in recent research [29]. The UAE involved both a probe device and a bath, and various parameters were explored and fine-tuned to optimize the process.

3.2.1 Optimization of p-UAE process

The ultrasonic probe's power was adjusted between 50 to 200 W. Figure 2a shows that extraction levels of picrocrocin, safranal, and crocins increased as power increased. Purohit et al. found similar results with β-carotene extraction from carrot waste, noting a slower rate of increase with oil compared to other solvents [30]. Figure 2b demonstrates a linear increase in compound extraction within sunflower oil with longer ultrasonic probe time at 200 W. However, at 200 W and 45 min, extraction efficiency increased but with a noticeable burnt odor. Lara et al. also observed increased carotenoid extraction from papaya using sunflower and soybean oils with longer ultrasonic probe times from 10 to 60 min [31].

3.2.2 Optimization b-UAE process

The ultrasonic bath, operating at lower intensities, offers milder extraction conditions, reducing sample damage [32]. Subsequently, in this study, the ultrasonic bath was also used for aromatization of sunflower oil with saffron stigma, with various parameters investigated. Results in Figure 2c show that the concentration of extracted compounds is lower in b-UAE compared to p-UAE even at 50 W power, showing lower efficiency of the bath method. However, at 70°C, b-UAE's extraction improved, yielding comparable results for picrocrocin and safranal to p-UAE (200 W, 10 min), and double the amount of crocins. Crocins exhibit greater stability in the milder conditions of the ultrasonic bath, explaining the difference. Similarly, Purohit et al. demonstrated enhanced beta-carotene

extraction from carrot waste oil at higher temperatures with ultrasound [30]. In b-UAE, extraction remained relatively constant up to 1 h, increasing thereafter, with optimal extraction time considered as 30 min (Figure 2d). Goula et al. found carotenoid degradation during ultrasonic bath extraction, highlighting the importance of minimizing extraction time to less than 30 min to mitigate degradation [33].

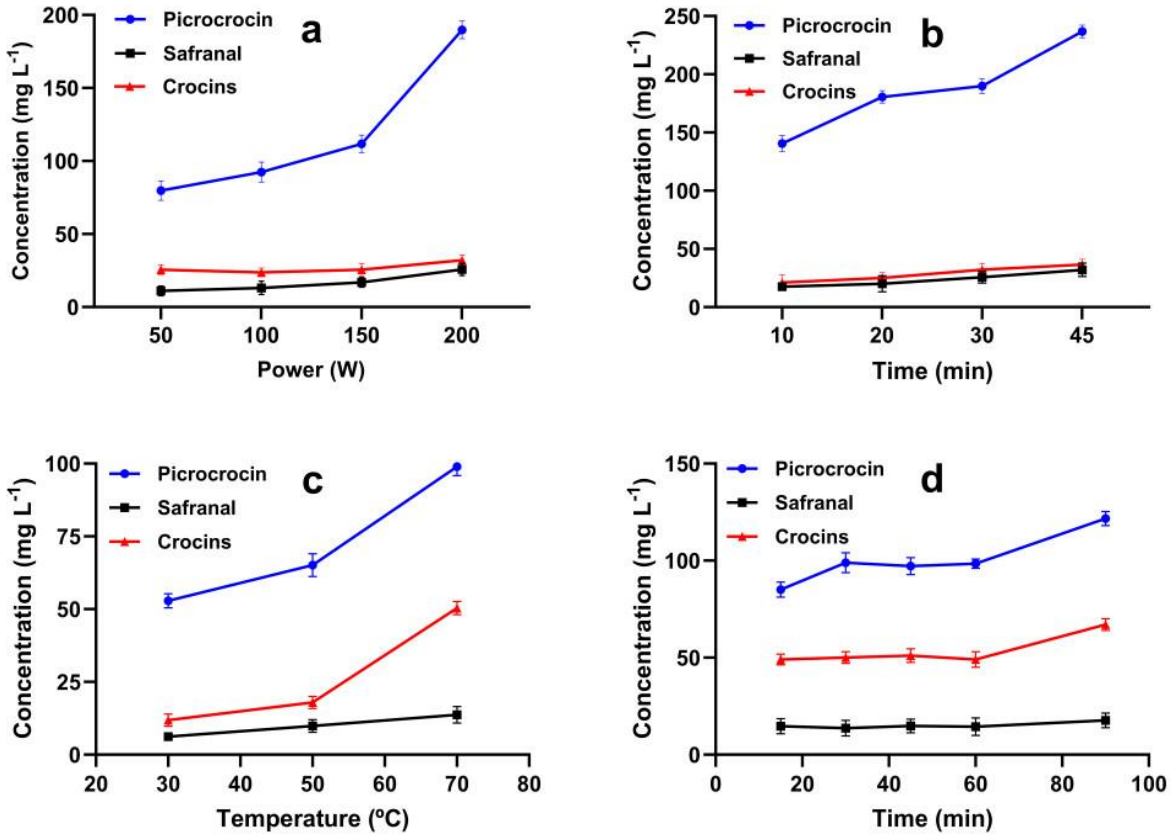

Figure 2 Optimization of the p-UAE process: (a) ultrasonic power optimization (time: 30 min), (b) time optimization (power:200 W); optimization of the b-UAE process: (c) temperature optimization (time: 30 min), (d) time optimization (temperature: 70 °C) (saffron stigma: 0.7% w/v).

3.2.3 Multi-step extraction in b-UAE

Increasing saffron stigma powder in sunflower oil up to 17.8% w/v led to a 305% increase in picrocrocin content and a 141% increase in safranal content (Figure 3). However, adding more powder beyond this point did not significantly enhance these compounds further. In contrast, higher amounts of saffron stigma powder did not improve crocin extraction efficiency in the oil. The limited extraction of crocins is attributed to their higher water solubility (containing 24 hydrogen acceptors, and 14 hydrogen donors) [34], which makes its extraction in the oil phase more difficult. On the other hand, the instability of crocins against strong ultrasonic waves can also be an important factor in the degradation of the extracted amounts, which can be observed in

comparison with the results of the SAM method.

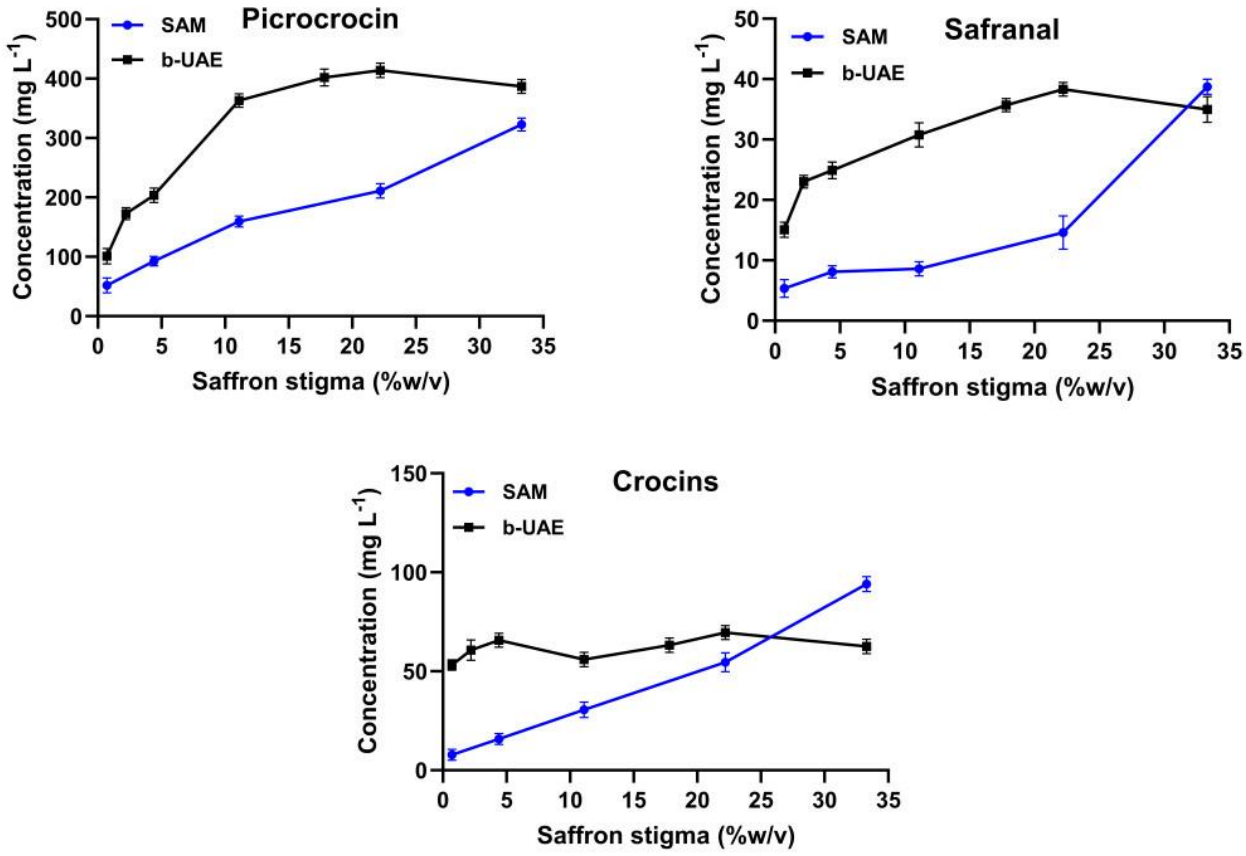

Figure 3 The impact of wight of powder on the extraction efficiency in b-UAE (70 °C for 30 min) and SAM (30 °C for 60 min).

Considering that only a small percentage of saffron stigma bioactive compounds dissolved in sunflower oil during single-step bath ultrasonic-assisted extraction (b-UAE), a multi-step extraction approach was implemented to enhance efficiency. The remaining residue from the initial extraction stage underwent further extraction under similar conditions, repeated for up to 10 cycles. Results depicted in Figure 4 show increased cumulative extracted material with each cycle. Each b-UAE cycle comprised 10 min of ultrasonication followed by 5 min off. In some samples, stirring using a magnetic stirrer occurred during the off period. Overall efficiency was higher when ultrasonication was accompanied by stirring (b-UAE+SAM) compared to ultrasonication alone (b-UAE). Stirring aided in mass transfer of extracted compounds to the solvent bulk, facilitating contact between fresh solvent and settled saffron powder in subsequent ultrasonic cycles.

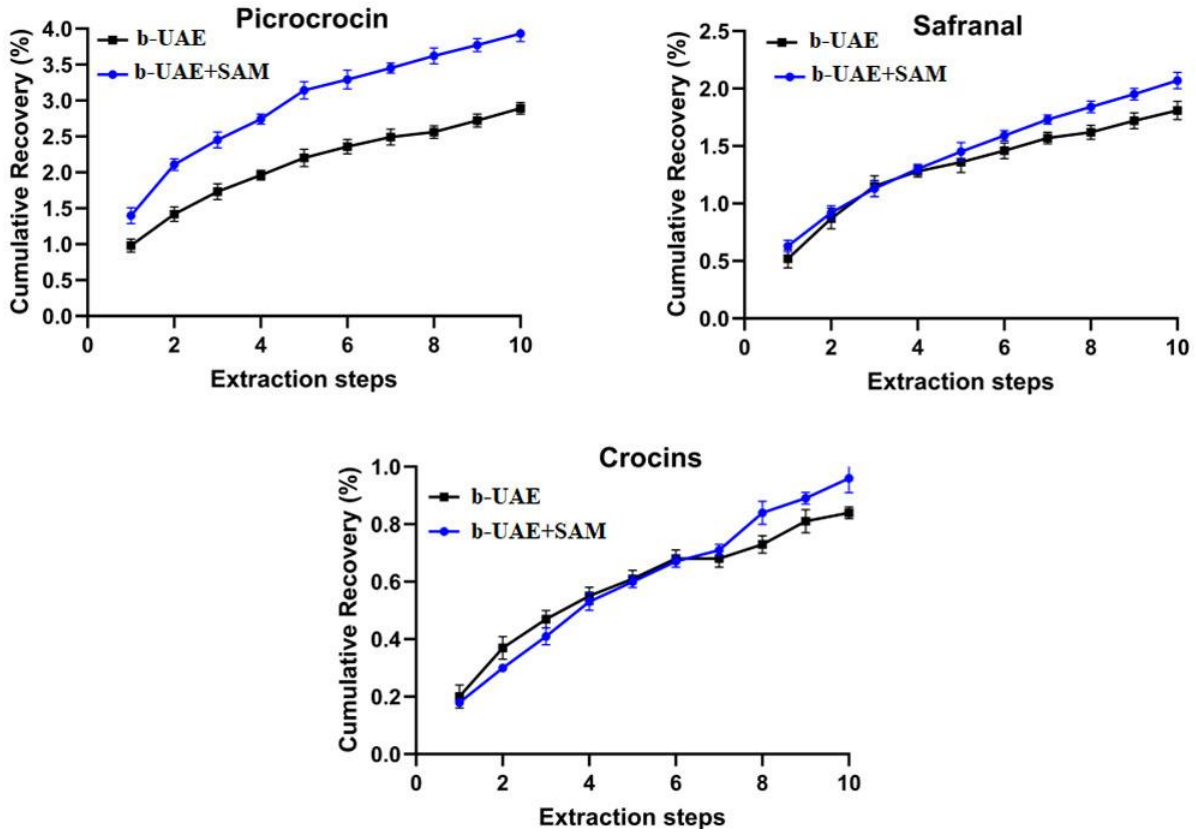

Figure 4 The impact of the number of extraction cycles on the cumulative recovery of b-UAE (70 °C and 30 min).

In b-UAE at 70°C for 30 min with 10 extraction cycles and stirring, maximum extractable amounts of picrocrocin, safranal, and crocins from stigma were 3.85, 2.0, and 0.92%, respectively. The extraction efficiency of these compounds varies according to their polarity; the more polar the compound, the lower its extraction in oil. In first cycle, 36, 31, and 19% of picrocrocin, safranal, and crocins were obtained. About 81, 73, and 64% of total recovery for picrocrocin, safranal, and crocins occurred within the first 5 cycles. Reusing residual waste from the initial extraction stage does not achieve similar efficiency as fresh powder, as most compounds remain in the waste. With each cycle, extractable amounts decrease, and after 5 uses, residue no longer yields acceptable extraction efficiency. Since sunflower oil is a non-polar solvent, its capacity to extract polar compounds is limited, and its ability to permeate and penetrate the plant surface is also reduced. As the number of extraction cycles increases, the oil's penetration into plant cells appears to diminish, leading to decreased extraction efficiency. Therefore, it cannot be expected that oil will completely extract all saffron compounds; it can only incorporate a portion of the compounds, sufficient to enhance aroma and flavor. Furthermore, the extraction efficiency of compounds in the residual plant material decreases with increasing extraction cycles. The exact reason for this decline is not clearly understood, but one possible explanation is the degradation of these compounds in the plant due to the high energy of ultrasonic waves. Nonetheless, saffron is a highly expensive plant, and this solubility limitation can increase the final cost of the oil, restricting its applications to luxury uses.

3.3 Stirring-assisted maceration (SAM)

The results indicate that UAE effectively extracts bioactive compounds from saffron stigmas into sunflower oil, albeit with a reduction in organoleptic properties. Conversely, the maceration method, being easily applicable in the industry and offering milder extraction conditions, avoids negative impacts on oil's organoleptic properties. Consequently, the study investigated sunflower oil aromatization with saffron stigmas using solvent-assisted maceration (SAM), optimizing certain influential parameters.

3.3.1 Optimization of time in SAM

Figure 5 illustrates an increasing trend in bioactive compound extraction from saffron stigmas with longer maceration times at room temperature. Optimal levels for picrocrocin, safranal, and crocins were reached after 6, 4, and 5 days of maceration, respectively, with no significant changes beyond these durations. SAM extraction for one day yielded similar results to b-UAE, while after 4 days, SAM matched p-UAE. The key difference was the absence of burnt odor in SAM-produced oil. Moreover, SAM yielded 3- to 5-fold higher crocins content compared to b-UAE or p-UAE after 4 to 5 days, indicating less crocin degradation. Safranal and picrocrocin content in SAM oil was 3- and 7.1-fold higher than b-UAE and 2.5- and 3.1-fold higher than p-UAE, respectively. Despite the longer SAM duration, resulting concentration characteristics and organoleptic properties are noteworthy. Similarly, Jović et al. found optimal extraction times for aromatic plants in extra virgin olive oil using maceration to be around 2 weeks [35].

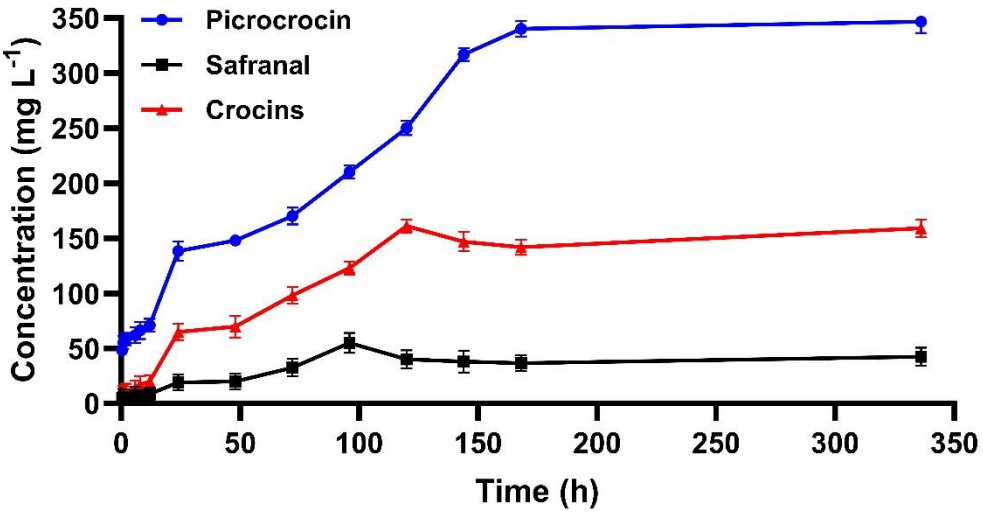

Figure 5 The impact of time on the extraction efficiency of SAM method at room temperature (saffron stigma: 0.7% w/v).

3.3.2 Optimization of power in MAE

To improve maceration extraction, microwave energy was incorporated alongside stirring. Following 5 min of stirring, the sample underwent 30 s of microwave treatment, continuing for a total of 60 min. Figure 6 shows that oil samples without microwave irradiation had higher bioactive compound content compared to microwaved samples. Microwave treatment at 30 W reduced the content of all compounds, even eliminating crocins. However, higher microwave power slightly increased extraction, with 100 W matching non-microwaved sample content. Doubling microwave time at 100 W marginally improved extraction (data not shown). This indicates microwave treatment enhances extraction but may also degrade compounds in oil, limiting its usefulness for saffron stigma bioactive compound extraction in sunflower oil.

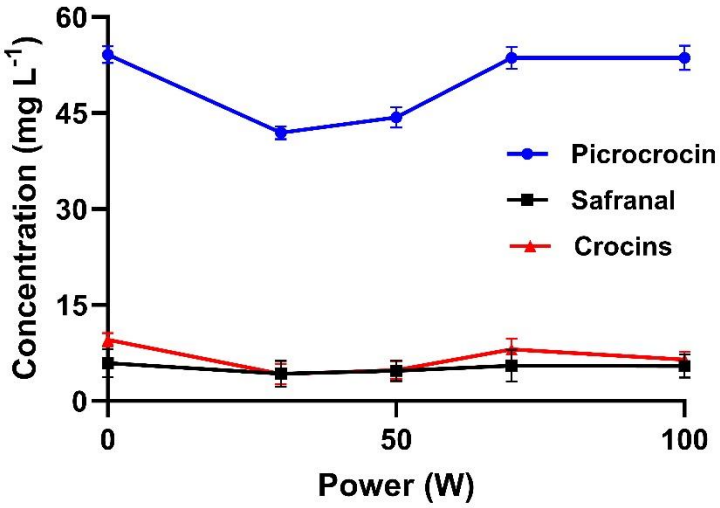

Figure 6 The effect of microwave power on the extraction efficiency of MAE. (saffron stigma: 0.7% w/v).

3.3.3 Optimization of temperature in SAM

To improve the efficiency of extracting bioactive compounds from saffron stigmas in sunflower oil using the SAM method, various temperatures were explored. Figure 7 illustrates that SAM at 50°C resulted in higher dissolution of bioactive compounds from saffron stigmas in oil during the initial 12 hours compared to 30°C. However, over longer durations, compound content decreased due to temperature susceptibility. Therefore, for extended SAM processes, a more suitable ambient temperature is recommended.

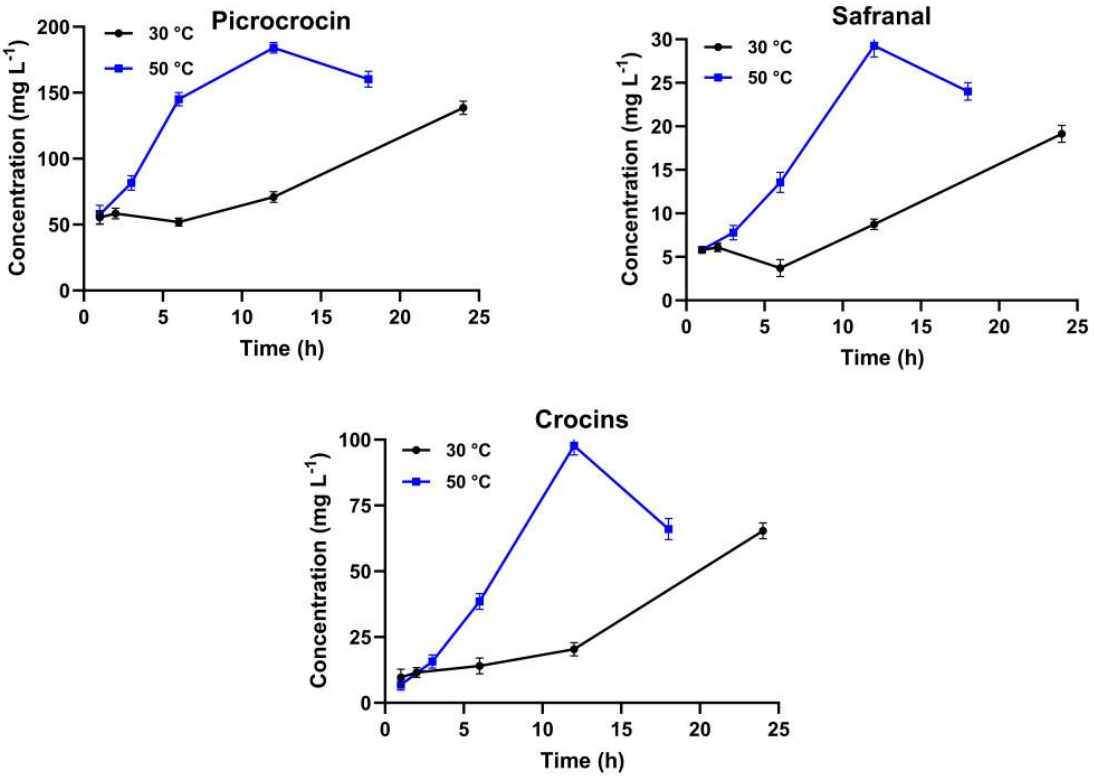

Figure 7 The effect of temperature at different time intervals in SAM method (Saffron stigmas: 0.7 % w/v).

3.3.4 Optimization of weight/volume ratio in SAM

Increasing the weight-to-volume ratio of saffron stigma powder in the SAM method results in a nearly linear increase in extracted bioactive compound content in sunflower oil (Figure 3). At lower saffron powder weights, b-UAE extraction exceeds that of SAM. However, as saffron powder weight increases, b-UAE extraction becomes limited due to compound degradation. Conversely, SAM extraction increases linearly with saffron stigma mass without degradation. Both methods are constrained by the polar nature of saffron's bioactive compounds and the non-polar nature of sunflower oil. Similarly, Noor et al. demonstrated that carotenoid extraction from tomato waste in oil increases with plant weight [36].

3.4 The optimum concentration of bioactive compounds extracted via various aromatization methods

Table 1 presents the concentration of bioactive compounds from saffron stigmas in sunflower oil extracted using b-UAE, p-UAE, and SAM (one week) methods under optimal conditions Results show that picrocrocin content in sunflower oil is higher with the SAM method compared to UAE methods. The SAM method also yields the highest levels of safranal and crocins in sunflower oil. Figure 8 illustrates the color of oils obtained from these three methods, with the SAM method resulting in the most colorful sample.

Table 1 The concentration of bioactive compounds in sunflower oil obtained under optimal conditions using various methods. (Saffron stigmas: 0.7 % w/v)

Method	Time	Temperature (˚C)	Picrocrocin (mg/L)	Safranal (mg/L)	Crocin (mg/L)
b-UAE	30 min	70	98.8 ± 1.11	14.7 ± 1.37	51.7 ± 1.23
p-UAE	45 min	RT	231 ± 2.40	31.2 ± 1.68	36.4 ± 1.55
SAM	1 week	RT	317.2 ± 0.91	55.8 ± 0.99	160.1 ± 1.36

RT= room temperature

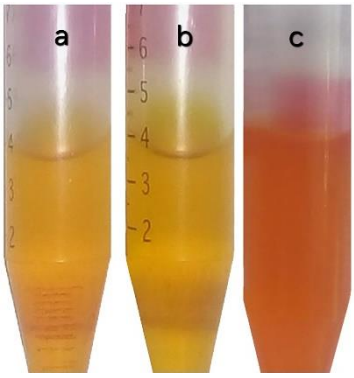

Figure 8 Color of the aromatized oils using methods: a) b-UAE, b) p-UAE c) SAM.

3.5 Physicochemical properties of saffron stigma-aromatized sunflower oil

For the optimized oils, various physicochemical parameters were measured, including TPC, TCC, antioxidant capacity using the DPPH method, AV, PV, K232 and K268. The results in Table 2 show that TPC in aromatized sunflower oils is 1.4 to 1.8 times higher than the control, while TCC is 4 to 10 times higher. TPC and TCC from p-UAE are higher than b-UAE, likely due to the ultrasonic probe's higher extraction power. TPC from b-UAE with SAM is approximately equal. However, TCC using SAM is higher than UAE methods, suggesting slower, more stable extraction compared to UAE methods, which may degrade sensitive compounds like carotenoids. Hosseini et al. demonstrated the detrimental effect of ultrasonic probes on beta-carotene in sunflower oil, where increasing power over 5 min reduced beta-carotene levels [37].

The aromatized sunflower oils displayed greater DPPH free radical scavenging activity compared to the control, with oils from b-UAE and SAM methods exhibiting the highest antioxidant activity. This increase is likely attributed to higher phenolic and carotenoid contents, enhancing antioxidant capacity. Similarly, Bhimjiyani et al. found that flaxseed oil enriched with sea buckthorn had superior antioxidant capacity compared to the original oil [15]. Hamad et al. also demonstrated that adding spices like turmeric to palm oil using the maceration method improved antioxidant capacity [5].

According to Gondkar et al., the acceptable AV range for sunflower oil is between 0.7 and 2.0 [38]. The AV of the control oil in this study fell within this range, consistent with typical properties of sunflower oil (1.17 ± 0.26). A slight increase in AV was observed during the aromatization process with saffron stigma, yielding values between 1.4-1.8. PV of the control oil was 9.7 ± 0.8, aligning with previous reports for sunflower oil [39]. Aromatizing the oil with saffron stigma resulted in a reduction in PV, indicating increased oil stability, likely due to decreased formation of primary oxidation compounds. As Table 2 shows, sunflower oil aromatized using the p-UAE method had higher TPC levels compared to oil obtained through b-UAE, resulting in a lower PV. Similarly, Moustakime et al. demonstrated that aromatizing olive oil with *Pimpinella anisum* using the maceration method increased AV and decreased PV [14]. Soares et al. showed that aromatizing extra virgin olive oil with rosemary and basil using b-UAE increased the PV [40], while Sousa et al. found that flavoring olive oil with garlic increased the AV and decreased the PV, with no significant effect observed for hot chili pepper aromatization [41]. Reports by Hosseini and Halim suggest that the ultrasonic process can increase PV of oil due to its destructive effect [37] [42]. However, saffron-aromatized sunflower oil in this study exhibited lower PV and higher stability compared to the control, attributed to its increased content of TPC and TCC introduced during the UAE process.

Table 2 Physicochemical properties of saffron stigma-aromatized sunflower oil using different methods.

Sample	TPC (mgGAE g oil^{-1})	TCC (mg Kg^{-1})	DPPH (%inhibition)	AV (mgKOH/g)	PV (meqO_2/kg)	K232	K268
Control**	7.81 ± 1.11	0.22 ± 0.04	33.43 ± 0.65	1.17 ± 0.26	9.67 ± 0.85	11.6 ± 0.35	1.92 ± 0.25
b-UAE	10.69 ± 1.77	0.91 ± 0.05	53.09 ± 2.76	1.68 ± 0.30	5.05 ± 1.48	14.74 ± 0.40	1.75 ± 0.07
p-UAE	13.69 ± 2.01	1.71 ± 0.96	38.42 ± 1.28	1.45 ± 0.78	4.95 ± 1.91	16.13 ± 0.90	3.13 ± 0.01
*SAM	11.64 ± 0.76	2.24 ± 0.74	49.60 ± 1.63	1.79 ± 0.64	5.30 ± 1.98	11.09 ± 0.81	1.85 ± 0.07

*Stirred for 1 week, **control= unflavored sunflower oil

The extinction coefficients K232 and K268 are crucial indicators of oil stability and purity, with K232 reflecting primary oxidative degradation products and K268 indicating secondary products. Analysis of these coefficients in Table 2 reveals that both short-term and long-term stability indices of saffron stigma-aromatized sunflower oils obtained by the UAE method surpass the control, suggesting deteriorative conditions accompanied by a burnt odor. Due to the more rigorous conditions of p-UAE compared to b-UAE, the K232 and K268 indices for sunflower oil obtained through p-UAE exceed those obtained through b-UAE. Previous studies by Hosseini and Halim demonstrated that ultrasonic processes can elevate the K232 coefficient, reflecting ultrasound's destructive effect on sunflower oil [37, 42]. Conversely, these indices slightly decrease for saffron-aromatized sunflower oil obtained by the SAM method, attributed to the mild extraction conditions that preserve the oil's integrity during extraction, allowing saffron bioactive compounds to dissolve gently. Moustakime et al. also observed a slight reduction in K232 for aromatized oil compared to the control, with higher values noted for UAE compared to maceration [14]. Additionally, they reported an increase in K268 for aromatized oils, with a more pronounced increase observed for UAE compared to maceration. Similarly, Sousa et al. showed that K232 decreased slightly for aromatized oil with garlic and oregano compared to the control, while K268 remained relatively unchanged [41].

4 Conclusions

Various methods including ultrasonic waves, maceration, microwave, heat, and stirring were employed to enhance the dissolution efficiency of saffron stigma's active compounds in sunflower oil. While the ultrasonic method yielded good extraction efficiency in a shorter time, it also resulted in oil destruction, leading to undesirable properties in the oil. Substituting the ultrasonic probe with a bath reduced the destruction intensity but still couldn't prevent damage. Similarly, the microwave method only caused compounds degradation. On the other hand, the simpler and more cost-effective maceration method was also employed, which can be easily applied in the industry. Despite its slow process, this method achieved good extraction results comparable to the ultrasonic extraction method. It provided the highest concentrations of safranal, crocins, and picrocrocin without causing compound degradation. The resulting saffron-aromatized sunflower oil had superior physicochemical properties, including increased TPC, TCC, and antioxidant capacity, indicating enhanced oxidative stability. Moreover, the PV and extinction coefficients K232 and K268 decreased significantly, indicating less formation of degradation products. Therefore, the saffron-aromatized sunflower oil produced by the SAM method was deemed the most suitable for various applications, offering high concentration of active compounds, desirable color and aroma, and considerable oxidative stability. Moreover, the final product exhibited a desirable aroma and flavor, and no color changes were observed during the storage period.

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Conflict of Interest

The authors have declared no conflict of interest.

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استخراج ترکیبات زیست فعال کلاله زعفران در روغن آفتابگردان: یک مطالعه مقایسه‌ای روش‌های مختلف استخراج جامد-مایع

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چکیده

معطر کردن روغن‌های خوراکی با هدف افزایش پایداری اکسیداتیو و ایجاد ویژگی‌های حسی مطلوب برای گسترش بازار آنها انجام می‌شود. هدف این مطالعه تولید روغن آفتابگردان معطر با طعم زعفران با استفاده از روش‌های مختلف استخراج جامد-مایع از جمله استخراج خیساندن به کمک همزدن (SAM)، استخراج به کمک فراصوت (UAE)، و استخراج به کمک مایکروویو (MAE) بود. این مطالعه با هدف بهینه‌سازی پارامترهای مؤثر در هر روش استخراج و شناسایی ویژگی‌های فیزیکوشیمیایی روغن‌های معطر حاصل انجام شده است. روش UAE به طور موثر ترکیبات زیست فعال کلاله زعفران را در زمان کوتاه‌تری استخراج کرد. با این حال، اعمال انرژی بالایی در طول فرآیند UAE منجر به تضعیف ویژگی‌های فیزیکوشیمیایی و حسی آن شد. به طور مشابه، در روش MAE، ترکیبات حساس و فرار زعفران تجزیه شده و از بین رفتند. به عنوان جایگزین، روش SAM با یک فرآیند ملایم و مداوم، موفق به انحلال غلظت بالای ترکیبات زعفران در روغن شد در حالی که ویژگی‌های فیزیکوشیمیایی و حسی آن را نیز حفظ کرد. غلظت بهینه‌ی پیکروکروسین، سافرانال، و کروسین به ترتیب ۳۱۷، ۵۶، و ۱۶۰ $mg\ L^{-1}$ در روغن آفتابگردان بدست آمد. روغن آفتابگردان معطر شده حاصل، یک محصول نوآورانه و پر امید برای کاربردهای متنوع غذایی و گسترش بازار می‌باشد.

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Scientific Research

Evaluation of probiotic, antifungal, and antioxidant properties of the predominant yeast isolated from acorn sourdough

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ABSTRACT

There is always the possibility of encountering probiotic yeasts with functional capabilities in natural habitats that have been less studied. In the present study, the predominant yeast from acorn sourdough was isolated and identified using PCR. The probiotic properties of the isolate, as well as its antifungal and antioxidant activities were also investigated. Sequencing results of PCR products led to the identification of *Pichia kudriavzevii* as the predominant yeast isolate. The survival rate of the isolate in simulated gastrointestinal conditions was 91.93%. The auto-aggregation ability of the isolate was equal to 84.65%, and its hydrophobicity against hexane and xylene was 35.15% and 21.70%, respectively. The antibacterial activity of *P. kudriavzevii* studied in this research against *Listeria monocytogenes* was 85.58%, which was significantly (P<0.05) higher than other studied foodborne bacteria. However, the co-aggregation ability of the yeast isolate against tested pathogens showed no significant difference. Furthermore, the isolate showed no hemolytic activity, and it was resistant to all tested antibiotics, but showed relative sensitivity to the antimycotic agents including itraconazole, ketoconazole, and natamycin, while being resistant to potassium sorbate. The antifungal activity of the isolate against *A. flavus* was also confirmed, with antioxidant activity measured at 78.67%. Accordingly, *P. kudriavzevii* yeast isolate can be introduced as a suitable candidate for use as a probiotic and/or protective culture in fermentation industries.

1- Introduction

Probiotics are live microorganisms that positively affect human health when consumed in an adequate population. Some of the benefits of probiotics include improved digestion, maintaining gut microbiome balance, modulation of the immune system, reducing symptoms of gastrointestinal diseases, and even lowering the risk of various infections [1,2]. Probiotic microorganisms include lactic acid bacteria and yeasts. Unlike probiotic bacteria, the probiotic properties of yeasts have been less studied. These eukaryotic microorganisms offer potential benefits such as antimicrobial activity, resistance to acid and bile salts, adhesion to the mucosal surfaces of the digestive tract, antioxidant properties, and the ability to reduce fungal biohazards. One key advantage of yeasts over probiotic bacteria is their resistance to antibiotics and the absence of gene transfer for resistance, which allows them to remain active during antibiotic treatment and help restore gut microbial flora without the risk of transferring antibiotic resistance genes, as sometimes observed with probiotic bacteria [3,4].

To date, research has been conducted on isolating probiotic yeasts from non-dairy substrates, particularly fermented cereals and pseudo-cereals. For example, Shahryari et al. [5] reported that the isolated yeast from buckwheat sourdough demonstrated good survival in simulated gastrointestinal (SGI) conditions, and in addition to suitable adhesion and hydrophobicity, significantly inhibited foodborne pathogens. Shruthi et al. [6] also isolated 73 yeasts from traditional fermented foods in India and screened them based on antimicrobial activity, selecting 10 yeasts for further probiotic evaluation. According to their report, all 10 isolates showed resistance to SGI conditions, with a survival rate of over 50%. Additionally, the isolated yeasts had adhesion abilities exceeding 40% and exhibited strong antioxidant properties. Similarly, Greppi et al. [7] isolated probiotic yeast from traditional African fermented foods and identified *Pichia kudriavzevii* M28, reporting that the yeast had a survival rate of 40.1% in gastric juice and 19.5% in pancreatic juice, with an auto-aggregation rate of 38.9%.

Alkalbani et al. [8] also isolated 12 yeasts, including *Saccharomyces cerevisiae* OK441070 and *P. kudriavzevii* OK441060, from fermented dairy and non-dairy products, and after conducting probiotic tests, reported that the survival rate of the tested strains ranged from 69% to 89%. Moreover, all the isolates exhibited proper antimicrobial properties, hydrophobicity, and auto-aggregation ability. In another study, Lara-Hidalgo et al. [9] evaluated the probiotic properties of *P. kudriavzevii* IPNFG1, *Wickerhamomyces anomalus* IPNFG3, and *Hanseniaspora opuntiae* IPNFG2 isolated from fermented foods and reported that their adhesion to mucin and survival rates in SGI conditions were comparable to commercial strains, with auto-aggregation rates exceeding 90%. The study also confirmed the co-aggregation ability with pathogens, antioxidant capacity, and antimicrobial activity of the yeasts.

Based on the literature review, no reports of probiotic yeast isolation from acorn sourdough have been documented so far. Accordingly, this study aims to evaluate the probiotic, antifungal, and antioxidant properties of the predominant yeast isolated from acorn sourdough.

2-Materials and Methods

Preparation of raw materials

The foodborne microorganisms used in this study (*Escherichia coli* PTCC 1399,

Staphylococcus aureus PTCC 1112, *Listeria monocytogenes* PTCC 1298, *Salmonella enterica* PTCC 1709, and *Aspergillus flavus* PTCC 5018) were purchased from the Persian type culture collection (PTCC) of the Iranian research organization for science and technology. The microbial culture media and chemicals were purchased from commercial brands with analytical grade like Merck (Germany) and Chromagar (France). For acorn flour preparation, acorns were procured from a local market, and after removing the outer and inner shells, the kernels were milled and sieved into flour. The properties of the acorn flour were determined based on standardized methods [10]. The acorn flour used in the present study contained 5.7% fat, 6.4% protein, 6.8% reducing sugars, and 8.3% moisture.

Spontaneous fermentation of acorn

A dough yield of 160 (equivalent to 100 g of flour and 60 mL of sterile distilled water) was prepared, and the mixture was fermented for 24 h at 25 °C [11]. To determine pH and total titratable acidity (TTA), 10 g of the sourdough sample was mixed with 90 mL of distilled water and titrated using 0.1 N sodium hydroxide (NaOH) to reach a pH of 8.5. TTA was expressed based on the volume of NaOH used [12], and the pH was measured with a pH meter.

Isolation and identification of the predominant yeast isolate

For isolation, serially ten-fold dilutions of the spontaneously fermented acorn were prepared in ringer solution, and then it was surface-plated on yeast glucose chloramphenicol (YGC) agar medium. After incubation for 24-72 h at 25 °C, single colonies were obtained using the streak plate method [13]. For molecular identification, the predominant yeast's DNA was extracted using a commercial kit (Geneall, South Korea). The DNA was amplified using PCR with internal transcribed spacer (*ITS*) primers *ITS1*: 5′-TCCGTAGGTGAACCTGCGG-3′ and *ITS4*: 5′-TCCTCCGCTTATTGATATGC-3′ [14], and then the PCR products were separated using electrophoresis in 1.5% agarose gel (for 40 min at V= 90 at Tris boric acid EDTA or TBE buffer) and subsequently they were sequenced (Pishgam, Iran). The obtained data was compared with the data available in the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLASTn) algorithm.

Survival of the predominant yeast in simulated gastrointestinal conditions

The yeast isolate was adjusted to a population of 10^8 colony forming units (CFU)/mL and exposed to SGI conditions (pH 2 with 0.1% pepsin for 2 h, followed by pH 8 with 0.3% bile salt and 0.1% pancreatin for 3 h). Survival was determined by surface plating of serially ten-fold diluted samples on YGC agar compared to untreated control sample [15].

Hemolytic activity of the yeast isolate

The yeast isolate was cultured on blood agar containing 5% sheep blood to observe any hemolysis signs or color changes [16].

Auto-aggregation ability of the yeast isolate

The yeast population was adjusted in phosphate-buffered saline (PBS) to 10^8 CFU/mL, and the suspension was incubated at 25 °C for 24 h. The absorbance of the yeast suspension at 600 nm was measured using a spectrophotometer (PGI, UK) and auto-aggregation was calculated using the following equation:

$$\text{Auto-aggregation\%} = [1- (A_f/A_0)] \times 100$$

Where, A_f and A_0 are the absorbance values at the end and beginning of the incubation period, respectively [17].

Hydrophobicity of the predominant yeast

The yeast population was adjusted in PBS (10^8 CFU/mL), and 3 mL of the yeast suspension was mixed with 1 mL of xylene or hexane. After vortexing for 30 seconds and incubating at 25 °C for 4 h, the absorbance of the yeast suspension was measured at 600 nm. Hydrophobicity was also calculated using the following formula:

Hydrophobicity% = $[(A_a - A)/A_a] \times 100$

Where, A_a is the absorbance at the beginning and A at the end of the incubation period [18].

Antibacterial activity of the isolate

Fresh 24-hour cultures of foodborne pathogens (*E. coli*, *S. aureus*, *L. monocytogenes*, and *S. enterica*) were prepared and their populations were adjusted. Then, equal populations of the yeast isolate and each bacterium (10^8 CFU/mL) were mixed and incubated for 24 h in brain heart infusion (BHI) broth. Serially ten-fold dilutions of the suspension were surface-plated on specific chromogenic media. After 24 h of incubation at 37 °C, colonies were counted and compared to the control [19].

Co-aggregation ability of the isolate

Equal volumes of yeast and selected foodborne bacterium suspensions (10^8 CFU/mL) were mixed and incubated for 4 h. Absorbance at 600 nm was recorded, and co-aggregation was calculated using the following formula:

Co-aggregation% = $[(A_p+A_y)/2 - (A_{mix})/(A_p+A_y)/2] \times 100$

Where, A_p is the absorbance of the bacterial suspension, A_y is the absorbance of the yeast suspension, and A_{mix} is the absorbance of the yeast-bacteria mixture [9].

Antibiotic and antimycotic susceptibility of the isolate

YGC agar plates with surface cultured yeast were overlaid with antibiotic and antimycotic disks. The inhibition zones diameters were measured and ≤ 14 mm, 15-19 mm and ≥ 20 mm diameters indicating resistance, intermediate sensitivity and sensitivity, respectively [20]

Antifungal activity of the yeast isolate

The overlay method was used against *A. flavus*. After 48 h incubation of yeast cultured on YGC agar, *A. flavus* spores (10^4 spores/mL) were mixed with potato dextrose agar (PDA) and poured onto the yeast cultures. Plates were then incubated at 25 °C until the control plate was fully covered by fungal growth. The inhibition percentage was determined using Image J software [21].

Antioxidant activity

The 24-hour yeast culture was centrifuged (3000 g, 10 min), and the yeast cells were re-suspended in PBS. Then, 800 µL of this suspension was mixed with 1 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) and kept in the dark for 30 min. After centrifugation (3000 g, 10 min), the absorbance of the supernatant was measured at 517 nm, and the DPPH scavenging activity was calculated using the following formula according to Gil-Rodriguez et al. [17].

DPPH scavenging activity% = $[1-(A_{sample}/A_{blank})] \times 100$

Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA). All tests were performed in triplicate, and data analysis was conducted using SPSS (version 20). Microsoft Office Excel 2019 was used for drawing the charts. Mean comparisons were performed using the least significant difference (LSD) test at $P<0.05$ confidence level.

3-Results and Discussion

Identification of the predominant yeast isolate

The predominant yeast isolate was identified through sequencing of the PCR products compared with NCBI database records, revealing *P. kudriavzevii* PA01 with 96% similarity. Specific amplification of the target sequence was verified in the agarose gel electrophoresis as shown in Fig. 1 In some studies, this yeast has been isolated from other sourdoughs. For example, in a study on buckwheat sourdough, *P. kudriavzevii* was isolated, and its probiotic characteristics were verified [5]. In the same vein, *P. kudriavzevii* MK044080.1 was isolated as a potential probiotic yeast from Ethiopian injera sourdough by Muche et al. [22]. The ability of yeasts to maintain in sourdough's acidic environment and their adaptation to specific conditions of stressful sourdough ecosystem are key factors in their dominance during sourdough fermentation [2].

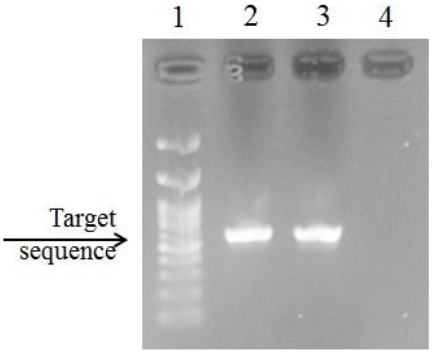

Fig. 1 Gel electrophoresis of the PCR products obtained from amplification of the target sequence (the *ITS* region) of predominant yeast isolated from acorn sourdough (lane 2) compared to the positive and negative control samples (lane 3 and 4, respectively) and 100 bp DNA ladder (lane 1).

Survival and adhesion capabilities of the yeast isolate

The survival rate of *P. kudriavzevii* isolate under SGI conditions was equal to 91.93 ± 0.91%, demonstrating its low tolerance to the environments of the gastrointestinal tract. Additionally, the yeast showed a strong auto-aggregation capability (84.65 ± 1.59%), which is an essential characteristic for its survival and probiotic functionality. Similarly, the survivability of the *P. kudriavzevii* isolate under SGI conditions was equal to 79.26% in the study of Shahryari et al. [5]. These data are in agreement with those recently reported by Rahimi et al. [23] for probiotic yeast isolated from sourdough. Accordingly, probiotic yeasts have the ability to form biofilms, helping them to protect cells from harsh environmental conditions. By utilizing surface cellular compounds, these yeasts enhance their auto-aggregation and survival capabilities, making them more resistant to stomach acidic conditions and bile salts of the intestine.

Antibacterial and co-aggregation abilities of the isolate

P. kudriavzevii isolate showed a significantly (P<0.05) higher inhibitory effect on *L. monocytogenes* compared to the other tested foodborne bacteria studied (Fig. 2) Moreover, there was no significant difference among co-aggregation ability of the isolate with *E. coli*, *S. aureus*, and *S. enterica*.

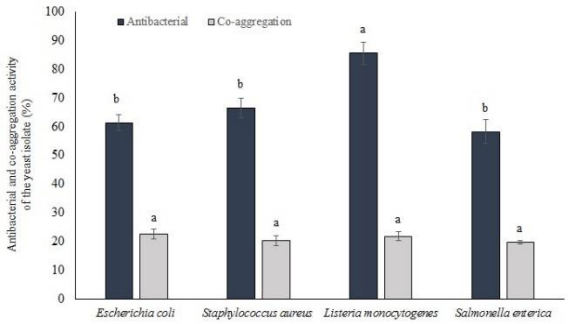

Fig. **2** Inhibitory activity (%) of the *P. kudriavzevii* isolate against *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. enterica* in comparison with the co-aggregation ability of the yeast isolate with the tested foodborne bacteria. Different letters indicate significant differences at $P<0.05$ in terms of each capability.

The antimicrobial activity against foodborne pathogens is considered one of the most important probiotic features. In the present study, the inhibitory effect of the isolate on Gram-positive bacteria was higher than those of Gram-negative bacteria studied. Several studies have confirmed the antimicrobial properties of probiotic yeasts against foodborne pathogens. For example, Kim et al. [24] verified the antibacterial activity of the probiotic yeast *S. cerevisiae* KU200270 against *L. monocytogenes*, while Chen et al. [25] reported that *S. cerevisiae* (isolated from koumiss) exhibited significant antimicrobial activity against *E. coli*, attributing this effect to organic acids such as citric acid and propionic acid. Younis et al. [26] also demonstrated the antimicrobial effects of probiotic yeasts against *S. aureus* and *E. coli*. Similarly, Al-Sahlany et al. [27] identified an antimicrobial peptide in *S. cerevisiae* ATCC 36858 that was resistant to heat (50-90 °C for 30 min) and that was effective against *E. coli* and *Klebsiella aerogenes*. Accordingly, action modes of antibacterial activity in probiotic yeasts include competition for nutrients and epithelial cell binding sites, as well as production of antimicrobial metabolites like organic acids, hydrophobic peptides, and ethanol [3]. In addition, the correlation between co-aggregation ability and antibacterial activity of probiotic yeast has been verified in the study of Rahimi et al. [23], which was in agreement with our findings. The co-aggregation ability as an important mechanism in antibacterial activities of probiotic yeasts is associated with competition between yeast and bacteria for adhesion to epithelial surfaces, and reducing the availability of nutrients for harmful bacteria [2]. In a similar fashion, Menezes et al. [28] reported that probiotic yeasts form a yeast-bacteria complex, leading to the destruction of pathogens. Generally, type I flagella plays a crucial role in the adhesion of pathogenic bacteria to the intestinal epithelium, often using mannose as a receptor. Since yeast cell-walls are rich in mannose, probiotic yeasts can act as alternative receptors, preventing pathogenic bacteria from attaching to gastrointestinal cell receptors [29].

Hemolysis and antibiotic susceptibility of the isolate

According to the results, the *P. kudriavzevii* isolate exhibited no hemolytic activity. Similar studies on the hemolytic activity of probiotic yeasts, such as those by Fadda et al. [18] and Suvarna et al. [30] reported comparable results. The lack of hemolytic activity in yeasts may be due to the absence of hemolytic enterotoxin-producing genes,

resulting in no production of blood-destroying toxins [31]. Additionally, the yeast isolate demonstrated resistance to the antibiotics tested. Antibiotic resistance in probiotic yeasts has been reported in numerous studies, including research by Banik et al. [32], Fadda et al. [18], and Perricone et al. [33]. The lack of horizontal gene transfer of this resistance to other yeasts and bacteria makes yeasts, suitable candidates for probiotic applications. In the present study, the predominant yeast isolate was semi-sensitive to natamycin, ketoconazole, and itraconazole, and resistant towards potassium sorbate and calcium propionate (Table 1).

Table 1. Susceptibility of the yeast isolated from acorn sourdough towards antimycotic compounds. Different letters show significant differences at $P<0.05$.

Antimycotic agent (µg of effective component)	Sensitivity	The diameter of inhibition zone (mm)
Itraconazole (10)	semi-sensitive	16.32 ± 1.54^{a}
Ketoconazole (20)	semi-sensitive	17.23 ± 1.59^{a}
Natamycin (30)	semi-sensitive	18.71 ± 0.54^{a}
Potassium sorbate (60)	resistance	0.00^{b}
Calcium propionate (60)	resistance	0.00^{b}

Similar findings regarding the relative sensitivity of probiotic yeasts to antimycotic agents such as ketoconazole and itraconazole have been reported in various studies [32]. Some of yeasts mechanisms for this phenomenon include altering cell membrane permeability, inhibiting mitochondrial protein synthesis, mutation, and reducing ATP hydrolysis activities. Resistance to antifungal compounds may also result from changes in the target site of the antimycotic agents, inhibition of cellular RNA and DNA synthesis, and activation of the proton pump system in the cell membrane [34].

Antifungal and antioxidant activity of the isolate

The inhibitory effect of the predominant yeast isolate on *A. flavus* after 4 days of incubation compared to the control sample is shown in Fig. 3. As can be seen, the yeast isolate inhibited the growth of the target fungus (42.98 ± 0.84%) and prevented its sporulation as color-less growth zone.

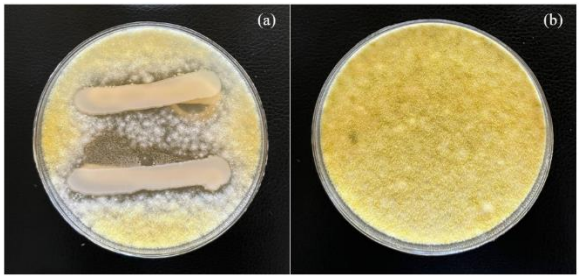

Fig. 3 Antifungal activity of the *P. kudriavzevii* isolate against *A. flavus* (a) compared to the control sample containing the target mold (b) in overlay bioassay.

A similar study found that some yeasts such as *S. cerevisiae* strains exhibited antifungal activities ranging from 2.7% to 100% against *A. flavus*, *Penicillium citrinun*, *Penicillium griseufulvun*, *Aspergillus niger*, and *Aspergillus fumigatus* [21]. Additionally, Alasmar et al. [35] approved the antifungal

activity of the yeast *Kluyveromyces marxianus* QKM-4 against 17 fungal species from the genera *Aspergillus*, *Penicillium*, and *Fusarium*, attributing this effect to volatile organic compounds produced by the yeast. Other antifungal mechanisms of probiotic yeasts include the production of metabolites such as carbon dioxide, ethanol, protein compounds, or low molecular weight peptides [21]. The DPPH radical scavenging ability of the yeast isolate in the present study was also equal to 78.67% ± 1.88%. In a study by Romero-Luna et al. [36], this ability was reported to be 63.0%. Probiotic yeasts are capable of producing antioxidant compounds like glutathione, superoxide dismutase, and catalase, which help protect against oxidative damage. Certain compounds produced by yeasts, such as hydrogen peroxide not only possess antioxidant properties but also can damage the cell membrane of fungi, preventing their growth [37].

4-Conclusion

Exploring the properties of probiotic yeasts isolated from substrates that have been less studied opens the possibility of discovering unique characteristics. Based on available data, there has been no prior report on the isolation of potential probiotic yeast from acorn sourdough and the evaluation of its properties. This study demonstrated that *P. kudriavzevii*, the predominant yeast isolated from acorn sourdough, possesses suitable probiotic, antifungal, and antioxidant capabilities. Moreover, there was a direct correlation between co-aggregation and antibacterial activities of the isolate, as well as between its antifungal and antioxidant capabilities. Interestingly, these correlations show potential applications of the isolate for a wide range of purposes after in depth characterization. Given these findings, *P. kudriavzevii* can be proposed as a suitable candidate for usage as a potential probiotic and/or protective functional culture in food and fermentation industries.

Funding

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Data availability

Data will be available based on the request.

Conflict of interest

All authors declare that there is no conflict of interest.

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مقاله علمی_پژوهشی

ارزیابی ویژگی‌های پروبیوتیکی، ضد قارچی و آنتی‌اکسیدانی مخمر جدا شده از خمیرترش بلوط

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چکیده

احتمال مواجهه با مخمرهای پروبیوتیک که از قابلیت‌های عملکردی مناسبی برخوردار باشند در بسترهای طبیعی که کمتر مورد مطالعه قرار گرفته‌اند وجود دارد. در این پژوهش، مخمر غالب از خمیرترش بلوط، جداسازی و با استفاده از PCR شناسایی شد. سپس ویژگی‌های پروبیوتیکی شامل زنده‌مانی در شرایط شبیه‌سازی شده دستگاه گوارش، قابلیت خود اتصالی و دگر اتصالی، آبگریزی، اثر ضد باکتریایی، مقاومت آنتی‌بیوتیکی، آنتی‌مایکوتیکی و قابلیت همولیز خون و همچنین قابلیت آنتی‌اکسیدانی و اثر ضدقارچی این جدایه مخمری بر روی *Aspergillus flavus* مورد مطالعه قرارگرفت. توالی‌یابی محصولات PCR منجر به شناسایی مخمر *Pichia kudriavzevii* به عنوان جدایه مخمری غالب خمیرترش بلوط شد. میزان زنده‌مانی جدایه مذکور در شرایط شبیه‌سازی شده دستگاه گوارش ۹۱/۹۳ درصد بود. قابلیت خود اتصالی این جدایه برابر با ۸۴/۶۵ درصد و میزان آبگریزی آن در برابر هگزان و زایلن به ترتیب ۳۵/۱۵ و ۲۱/۷۰ درصد بود. همچنین اثر ضد باکتریایی مخمر *P. kudriavzevii* مورد مطالعه در این پژوهش در برابر *Listeria monocytogenes* معادل ۸۵/۵۸ درصد و به شکل معنی‌داری ($P<0/05$) از سایر عوامل بیماری‌زای مورد آزمون، بیشتر بود. کمترین اثر ضد باکتریایی نیز در برابر *Salmonella enterica* مشاهده شد. میزان قابلیت دگر اتصالی جدایه مخمری غالب در برابر عوامل بیماری‌زای مورد آزمون، تفاوت معنی‌داری نداشت. علاوه بر این، جدایه مذکور فاقد فعالیت همولیتیکی بود و نسبت به تمامی آنتی‌بیوتیک‌های مورد بررسی، مقاوم بود اما در مقابل ترکیبات آنتی‌مایکوتیک ایتراکونازول، کتوکونازول و ناتامایسین، حساسیت نسبی نشان داد و همچنین نسبت به سوربات پتاسیم مقاوم بود. اثر ضد قارچی این جدایه در برابر *A. flavus* تایید شد و میزان قابلیت آنتی‌اکسیدانی آن ۷۸/۶۷ درصد بود. بر این اساس، جدایه *P. kudriavzevii* از قابلیت مناسبی برای استفاده به عنوان کشت پروبیوتیک جهت تولید محصولات غذایی تخمیری برخوردار است.

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Scientific Research

Utilization of Red Lentil Flour Substrate for the Production of Nattokinase Enzyme by *Bacillus subtilis* Natto

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ABSTRACT

In this research, the optimal production conditions of nattokinase using *Bacillus subtilis* Natto on red lentil flour substrate were studied. To optimize the fermentation process, the effects of three variables of fermentation time (24, 48 and 72 h), molasses concentration (3, 5 and 7%) and water content (50, 75 and 100 ml) on fibrinolytic and protease activities were investigated. The central composite design (CCD) was employed, and the results were modelled and analyzed using response surface methodology (RSM). Coefficients of determination, R2, of fitted regression models for fibrinolytic (quadratic model) and protease (linear model) activities were 97.15 and 90.12%, respectively, and their lack-of-fit was not significant at 95%. Hence, the models for all the responses were highly adequate. Fibrinolytic activity increased significantly ($p\leq0.05$) with increasing water content and fermentation time, while increasing the amount of molasses decreased fibrinolytic activity ($p\leq0.05$). With increasing the amount of water, protease activity also increased, but this increase was not significant, while increasing the amount of molasses and fermentation time led to a significant decrease and increase in protease activity ($p\leq0.05$), respectively. Concerning optimization, the optimal fermentation conditions were determined as 92.38 ml of water, 3.66% molasses, and 70.90 h of fermentation. Under these conditions, the activities of fibrinolytic enzymes and proteases were predicted to be 2476.03 and 1.68 U/g, respectively. For validation of the model, the optimal sample was produced, and the experimental responses were compared with the responses predicted by the model. The experimental values obtained were quite close to those predicted by the model, indicating the validity of the model. The results of this study showed that red lentil could be used as a substrate for fermentation by *B. subtilis* to produce the enzyme nattokinase.

1- Introduction

Fermentation is a process in which microorganisms are used to produce enzymes, food products, and nutraceuticals. Shelf life, safety, functionality, and sensory and nutritional properties of food are improved during fermentation. In addition, fermentation has beneficial effects on health by reducing the risk of various diseases such as type 2 diabetes and cardiovascular diseases [1].

Nattokinase (EC 3.4.21.62), also known as subtilisin NAT, is one of the most important extracellular enzymes produced by *B. subtilis* Natto. This enzyme, which belongs to the subtilisin family, is a serine protease consisting of 275 amino acids. The direct fibrinolytic activity of nattokinase is the main reason for interest in this enzyme [2]. Fibrinolytic enzymes have a significant effect on preventing the accumulation of fibrin platelets and preventing thrombosis. Not only does nattokinase break down blood clots, but it also breaks down accumulated fibrin, which is associated with heart disease [3].

B. subtilis is one of the gram-positive bacteria active in the production and secretion of various enzymes, and hence it is used in food-pharmaceutical biotechnology on an industrial scale. In fermented foods, this bacterium hydrolyzes the substrate and produces enzymes such as nattokinase, phytase, amylase, protease and lipase [4, 5].

Lentil, with the scientific name *Lens culinaris* Medik, is an edible pulse that is an important source of dietary protein in developing countries. Lentil is a highly nutritious legume with a sufficient amount of carbohydrates and a good amount of protein, minerals, vitamins and dietary fibres. The appropriate amounts of carbohydrate and protein make lentil a suitable substrate for the growth of *B. subtilis* and the production of nattokinase [6].

B. subtilis needs carbon and nitrogen sources to grow and produce nattokinase. Lentil is a good source of carbon and nitrogen, and as far as we know, there has been no study on the use of lentil flour substrate for the production of nattokinase by *B. subtilis*. In the present study, red lentil flour was used as the base fermentation substrate for nattokinase production by *B. subtilis*. To optimize the fermentation process, the effects of three variables of fermentation time, molasses concentration and water content on the fibrinolytic and protease activities of nattokinase were investigated using the response surface methodology (RSM).

2- Materials and methods

2. 1. Preparation of bacterial strain

B. subtilis subsp. Natto (ATCC23857.strain 168, DSM402, LMG 19457) was purchased from the Iranian Biological Resource Center (IBRC), Tehran, Iran, as IBRC-M:115.

2. 2. Reactivation of bacterial strain

At first, *B. subtilis* was cultured on nutrient agar (Oxoid, Altrincham, UK) medium by streaking technique, and then the cultured medium was incubated at 37°C for 24 hours. Next, one loop of cells was transferred from the plate to the nutrient broth medium (Oxoid, Altrincham, UK) and again placed in a 37°C incubator for 24 hours. Finally, bacteria grown in a nutrient broth medium were used to inoculate the substrate [7].

2. 3. McFarland solution quality control

The standard absorbance of 0.5 McFarland at 625 nm should be between 0.08 and 0.1. The density of McFarland solutions was checked by a spectrophotometer (CT-8200 Double beam, ChromTech) and the correctness of the standard was confirmed [8].

2. 4. Fermentation process

Red lentil was purchased from a local store and then ground using a home grinder. For discontinuous fermentation, 10 grams of lentil flour was poured into a 250 ml Erlenmeyer flask and mixed with some distilled water. After shaking the flask to mix

the contents, its lid was closed and autoclaved. After autoclaving and cooling the flask, under sterile conditions, 2 ml of cultured bacteria from the previous day were added to it using the McFarland method. After that, the flask was placed in a 37°C incubator shaker for 48 hours. Finally, the produced nattokinase was extracted and its fibrinolytic and protease activities were measured.

2. 5. Extraction of nattokinase

To extract nattokinase, normal saline was added to lentil flour inoculated with *B. subtilis*, which was incubated at 37°C for 24, 48 and 72 hours. Then the obtained mixture was shaken for 1 hour at 37°C using a shaker at 150 rpm. After that, the supernatant was removed and poured into 2 ml microtubes and centrifuged at 14000 rpm for 15 min at 4°C. Finally, the supernatant, the liquid containing nattokinase, was used to measure fibrinolytic and protease activities.

2. 6. Determination of fibrinolytic activity

The fibrinolytic activity of nattokinase was performed according to the method described by Gowthami and Madhuri [9]. Human plasma was used to extract fibrin for the fibrinolytic test. The reaction mixture containing 2.5 ml of fibrin solution, 2.5 ml of 0.1 M Tris buffer (pH = 7.8) and 1 ml of purified enzyme solution was incubated for 15 min at 37°C before adding 5 ml of 0.1 M trichloroacetic acid (TCA). The mixture was then kept at room temperature for 20 min. After centrifugation for 10 min at 12000 rpm, the supernatant was collected and its absorbance was determined at 275 nm.

2. 7. Determination of protease activity

Briefly, 1 ml of enzyme solution was added to 5 ml of casein (1% w/v in 50 mM potassium phosphate buffer, pH 7.5) and incubated at 37 °C for 10 min. After 10 min, 5 ml of 0.4 M TCA was added and incubated again for 30 min. Then, the obtained mixture was filtered with a 0.45-micron syringe filter. After filtration, 5 ml of 500 mM sodium carbonate was added to 2 ml of the filtrate, and then 1 ml of phenol reagent (Folin–Ciocalteu phenol solution: D–H2O=1:2) was added and incubated at 37°C for an additional 10 min. Finally, the absorbance of the filtered sample was measured at 660 nm [10].

2. 8. Experimental design and statistical analysis

The statistical Design Expert software version 11.0.3.0 (Stat-Ease Inc., Minneapolis, MN, USA) was used to design the experimental plan and data analysis. Twenty experiments (including 6 center points, 6 axial points and 8 factorial points) were carried out according to a quadratic central composite design (CCD) with three numerical variables (water content (X_1), molasses concentration (X_2) and fermentation time (X_3)) at three levels. The variables had 3 levels, coded as – 1, 0 and +1. The levels of independent variables are shown in Table 1. Twenty runs of experiments at different combinations of variables along with the actual and predicted responses (fibrinolytic activity (Y_1) and protease activity (Y_2)) were given in Table 2. Statistical analysis was performed using analysis of variance (ANOVA) test to estimate the statistical parameters and determine the significance of the model.

Table 1- Independent variables and their coded and uncoded levels according to central composite design

Independent Variables	Symbol	Coded levels		
		-1	0	+1
Water content (ml)	X_1	50	75	100
Molasses concentration (%)	X_2	3	5	7
Fermentation time (h)	X_3	24	48	72

Table 2- Experimental design matrix and responses (actual and predicted) by central composite design CCD)

Run	Variable			Response			
				Y_1 (U/g)		Y_2 (U/g)	
	X_1 (ml)	X_2 (%)	X_3 (h)	Actual	Predicted	Actual	Predicted
1	75	5	48	1876.47	1990.69	1.04	1.20
2	100	7	24	2228.83	2249.91	0.79	0.7632
3	32.96	5	48	570.43	656.93	1.22	1.11
4	75	1.64	48	2165.20	2366.70	1.50	1.41
5	75	5	48	1998.07	1990.69	1.07	1.20
6	75	8.36	48	2179.66	2165.96	0.99	0.9978
7	50	7	72	1106.65	1121.05	1.44	1.40
8	75	5	88.36	2344.21	2251.98	1.24	1.71
9	75	5	48	1894.79	1990.69	1.11	1.20
10	75	5	48	2120.65	1990.69	1.21	1.20
11	75	5	48	1937.87	1990.69	1.33	1.20
12	75	5	7.64	1696.14	1774.69	0.75	0.5756
13	50	3	24	1776.72	1713.57	0.43	0.8147
14	50	7	24	1271.10	1193.65	0.55	0.6521
15	100	3	24	1925.24	1920.51	0.83	1.01
16	100	3	72	2473.63	2560.75	1.78	1.76
17	100	7	72	2398.82	2471.64	1.52	1.51
18	50	3	72	2070.88	2059.47	1.61	1.64
19	70	5	48	2113.98	1990.69	1.19	1.20
20	117.05	5	48	2066.47	1966.34	1.42	1.30

3- Results and discussion

3. 1. Effects of independent variables on fibrinolytic activity

The results of the ANOVA showed that the quadratic model was the best model for predicting fibrinolytic activity, and the corresponding F value (34.10) also indicated the significance of the model ($p \leq 0.05$) (Table 3). According to the table, the effects of independent variables (X_1, X_2 and X_3) on fibrinolytic activity were significant ($p \leq 0.05$). The interaction effects of variables (except X_1X_3) on fibrinolytic activity were also significant ($p \leq 0.05$) in the mentioned model. The square of variables of water and molasses contents also had significant effects on the model ($p \leq 0.05$). The square of variables of water and molasses contents also had significant effects on the model ($p \leq 0.05$). It should be noted that the effects of X_1X_3 and x_3^2 on the presented model were not significant ($p > 0.05$).

After removing insignificant terms, the quadratic model obtained for fibrinolytic activity (Y1) in terms of coded factors was given as follows:

$$Y_1 = +1990.69 + 389.38\,X_1 - 152.26\,X_2 + 141.91\,X_3 + 212.33\,X_1X_2 - 104.63\,X_2X_3 - 240.11\,X_1^2 + 152.73\,X_2^2$$

(Eq. 1)

The positive and negative signs in front of each term indicate synergistic and antagonistic effects, respectively. Based on this, the water content and its square showed the most positive and negative impacts on fibrinolytic activity, respectively. The value of R2 (0.9715) for the above equation indicates a good correlation between the measured and predicted values by the model.

Table 3- Analysis of variance (ANOVA) for response surface quadratic model for fibrinolytic activity (Y_1)

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	F-value	*P* value (Probe > *F*)	
Model	3.861 E+06	9	4.290 E+05	34.10	<0.0001	significant
X_1	2.071 E+06	1	2.071 E+06	164.58	<0.0001	
X_2	2.073 E+05	1	2.073 E+05	16.48	0.0028	

X_3	2.750 E+05	1	2.750 E+05	21.86	0.0012	
X_1X_2	3.607 E+05	1	3.607 E+05	28.86	0.0005	
X_1X_3	43316.55	1	43316.55	3.44	0.0965	
X_2X_3	87573.22	1	87573.22	6.96	0.0270	
X_1^2	7.905 E+05	1	7.905 E+05	62.83	<0.0001	
X_2^2	1.957 E+05	1	1.957 E+05	15.55	0.0034	
X_3^2	878.78	1	878.78	0.0698	0.7975	
Residual	1.132 E+05	9	12581.55	-	-	
Lack of Fit	56057.39	4	14014.35	1.23	0.4054	not significant
Pure Error	57176.57	5	11435.31	-	-	
Cor Total	3.974 E+06	18	-	-	-	

Fig. 1 shows the effects of each independent variable on fibrinolytic activity separately. According to the figure, increasing the water content led to an increase in fibrinolytic activity. Increasing the amount of water up to about 90 ml was associated with a steep slope of the graph, and with an increase of more than 90 ml, the enzyme activity remained almost constant. Also, increasing the concentration of molasses decreased fibrinolytic activity. The decrease in enzyme activity up to 6% molasses was associated with a steep slope of the graph, and after 6% no significant change in fibrinolytic activity was observed. Increasing the fermentation time also resulted in an increase in fibrinolytic activity, so the highest fibrinolytic activity was observed at the end of the process (72 hours of fermentation).

Microorganisms grow and reproduce the most in their optimal moisture content. The moisture level plays an important role in the biosynthesis and secretion of many types of enzymes [11]. The best moisture content for the production of enzymes in bacteria is the condition in which the bacterial growth rate is optimal and the presence of water molecules does not prevent the proper release of oxygen and the transfer of nutrients into the bacterial cells [12]. Ding, Yao [13], investigating the fermentation conditions of tea residue using mixed strains, reported that the growth rate of bacteria increased by increasing the amount of water to 55% and then decreased with a gentle slope. They stated that the lower moisture content reduces nutrient diffusion and enzyme stability and causes matrix swelling, which harms the growth of fermenting microorganisms inside the environment. High moisture content also weakens the effect of air circulation, which is likely to cause bacterial contamination.

In the present study, the highest fibrinolytic activity was achieved at the concentration of 3% molasses. In a study, Zeng, Li [14] used a medium containing soybean residue and cane molasses to investigate the fibrinolytic activity and stated that the use of 2% cane molasses increased the production of enzymes. Li, Zhang [15] also reported that the use of more than 3% molasses led to the inhibition of the growth of *B. subtilis*, and the reason for this was an increase in the viscosity of the medium and a decrease in the diffusion of nutrients.

Wang, Chen [16] used shrimp skin substrate as a carbon source to investigate the production and activity of the nattokinase enzyme and stated that 48-hour fermentation had the highest nattokinase activity and after that, the enzyme activity gradually decreased. Wang, Torng [17] also optimized the fermentation conditions of *B. subtilis*, intending to achieve the highest activity of nattokinase, and stated that the optimal fermentation time was 37.07 hours. Pan, Chen [7] reported that if soybean meal and cassava starch are used as substrates, nattokinase activity reaches its maximum after 72 hours of fermentation, which is consistent with the results of this study.

Guo, Jiang [18] optimized fibrinolytic activity by culturing *B. subtilis* natto on Ginkgo seeds substrate and obtained the highest fibrinolytic activity in conditions of relative humidity of 80%, the initial water content of 73%, fermentation temperature of 38°C, inoculum volume of 18% and fermentation time of 38 hours. They also found that long-term fermentation causes a lack of nutrients needed for bacterial growth, as well as the accumulation of toxic inhibitors, and finally suppresses the fermentation process.

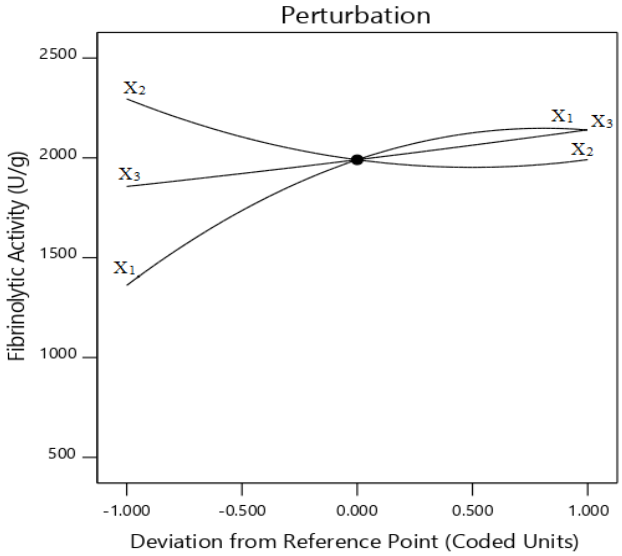
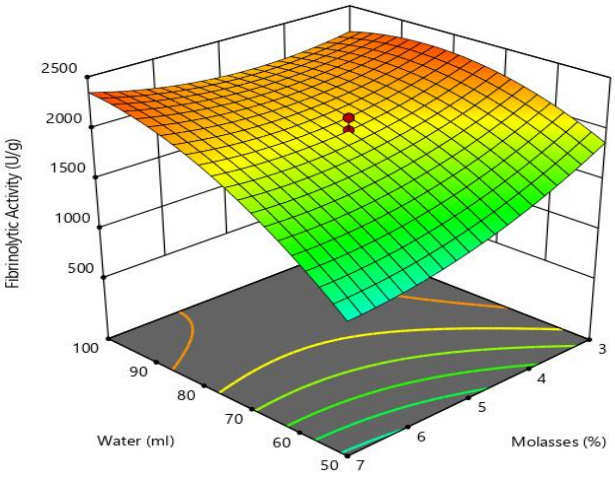


Fig. 1. The individual effects of independent variables of water content (X_1), molasses concentration (X_2) and fermentation time (X_3) on fibrinolytic activity in the center point.

Fig. 2 shows the combined effects of water content and molasses concentration on the fibrinolytic activity at constant fermentation time (48 hours). As it is known, the fibrinolytic activity increased in all molasses concentrations with increasing water content. In the amount of water below about 75%, increasing the concentration of molasses led to a decrease in fibrinolytic activity, although in higher amounts of water, with the increase in the concentration of molasses, the enzyme activity first decreased and then increased. Based on this, the role of water content in increasing fibrinolytic activity was greater than molasses concentration, and high fibrinolytic activity can be achieved in lower molasses concentrations by increasing water content.

a



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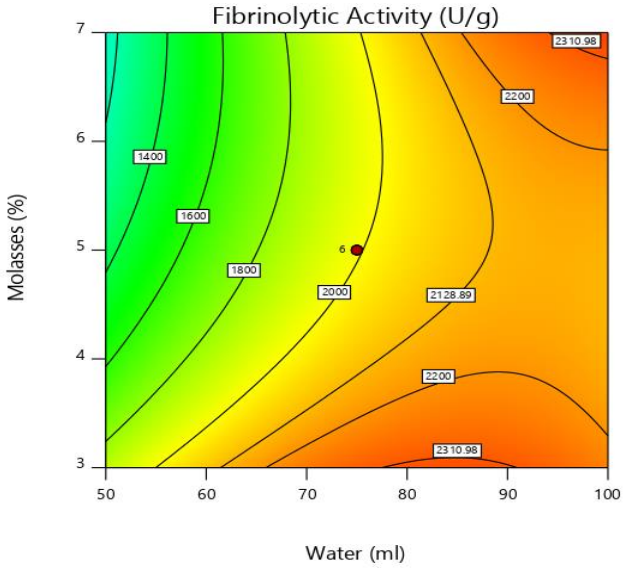
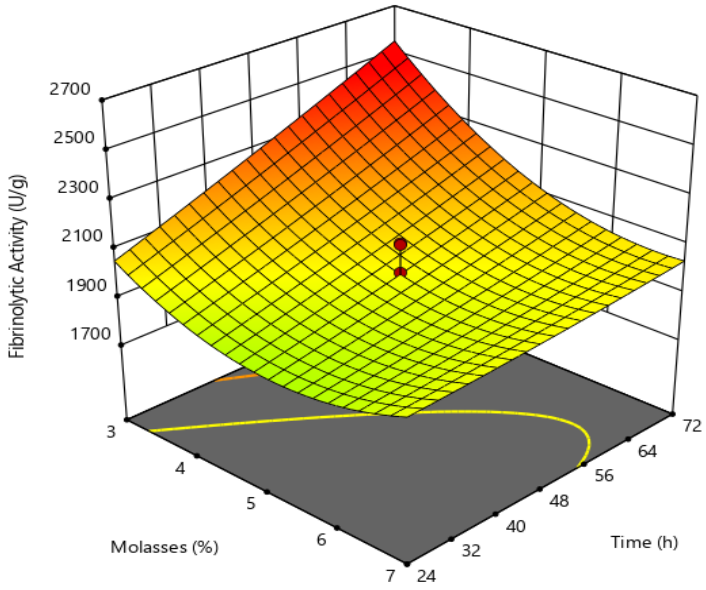


Fig. 2. The combined effects of water content and molasses concentration on fibrinolytic activity at constant fermentation time (48 hours); a: 3D; b: counter.

The combined effect of molasses concentration and fermentation time on the fibrinolytic activity at constant water content (75 ml) was shown in Fig. 3. According to the figure, with increasing fermentation time, fibrinolytic activity increased in all concentrations of molasses, especially in concentrations below 4%, so the highest enzyme activity was related to the concentration of 3% molasses and fermentation time of 72 hours. In lower amounts of molasses, the increase in fibrinolytic activity during fermentation was higher compared to higher concentrations of molasses. At all fermentation times, especially at lower times, increasing the amount of molasses first caused a decrease in enzyme activity, although, at high concentrations of molasses, fibrinolytic activity increased again to some extent. Anggraeni and Poernomo [19] evaluated the effect of different concentrations of molasses on the activity of fibrinolytic enzymes produced by *B. subtilis*. In this study, the highest fibrinolytic activity was observed at 0.5% concentration of molasses and at 30 hours of fermentation. According to the results, increasing the concentration of molasses up to 0.5% and increasing the fermentation time led to an increase in enzyme activity, and fibrinolytic activity decreased at molasses concentrations higher than 0.5%.

a



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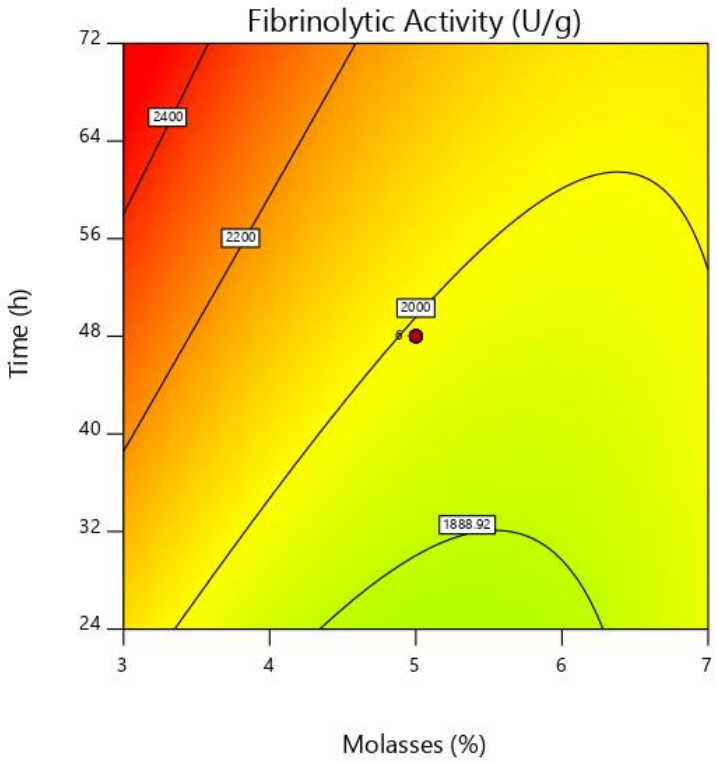


Fig. 3. The combined effects of molasses concentration and fermentation time on fibrinolytic activity at constant water content (75 ml); a: 3D; b: counter.

3. 2. Effects of independent variables on protease activity

In Table 4, the results of the linear model analysis of variance to determine the effect of independent variables and their interactions on protease activity are shown. The F value of the model was 42.58, which means that the model is significant ($p \leq 0.05$). According to

the table, molasses concentration and fermentation time had significant effects on the model ($p \leq 0.05$), while the influence of water content was not significant. The non-significance of the lack-of-fit factor confirmed the correctness of the model for predicting protease activity.

The linear model of protease enzyme activity (Y2) in terms of the coded factors and after removing non-significant terms is as follows:

$$Y_2 = +1.20 - 0.1227\ X_2 + 0.3737\ X_3$$

(Eq. 2)

Among the variables, fermentation time showed the greatest effect on the increase of protease activity.

Table 4- Analysis of variance (ANOVA) for response surface linear model for protease activity (Y_2)

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	F-value	*P* value (Probe > *F*)	
Model	1.66	3	0.5532	42.58	<0.0001	significant
X_1	0.0383	1	0.0383	2.95	0.1080	
X_2	0.1865	1	0.1865	14.36	0.0020	
X_3	1.35	1	1.35	103.61	<0.0001	
Residual	0.1819	14	0.0130	-	-	
Lack of Fit	0.1246	9	0.0138	1.21	0.4393	not significant
Pure Error	0.0573	5	0.0115	-	-	
Cor Total	1.84	17	-	-	-	

Individual effects of independent variables on protease activity are shown in Fig. 4. As can be seen, the water content had no significant effect on the protease activity, while the high slope of the graph of the other two variables indicated their significant effect on the enzyme activity, and among them, the influence of the fermentation time was higher. Increasing fermentation time led to a significant increase in protease activity, while the relationship between protease activity and molasses concentration was the opposite and increasing the amount of molasses significantly decreased enzyme activity.

B. subtilis reached the stationary phase of the growth curve after 72 hours, and the maximum accumulation of enzymes was also observed in this phase. Also, the decrease in enzyme production after 72 hours may be due to the release of a high level of intracellular proteases at the same time as endospore formation [12].

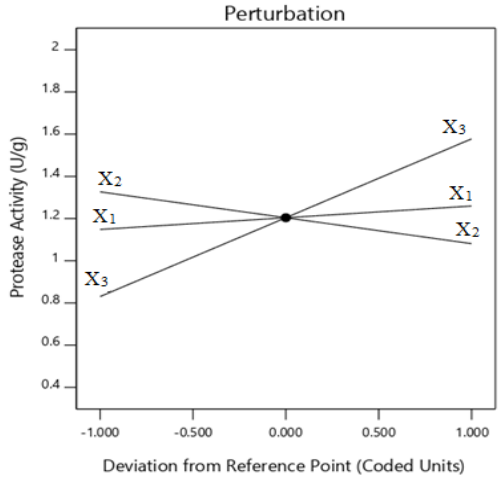
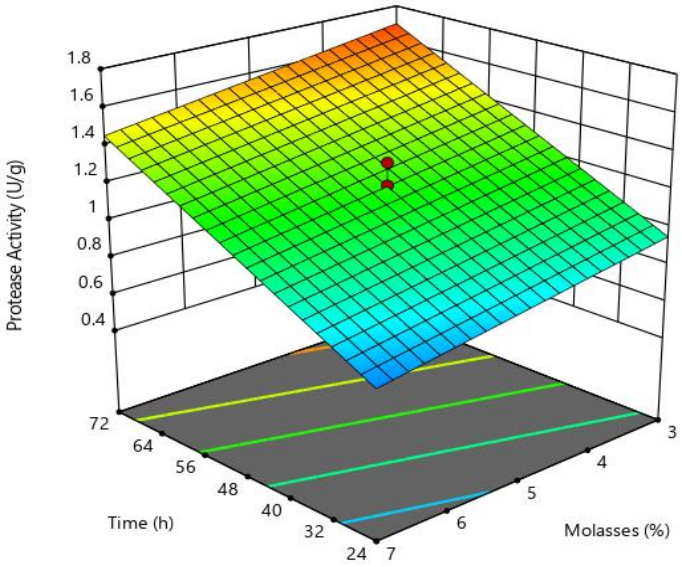


Fig. 4. The individual effects of independent variables of water content (X_1), molasses concentration (X_2) and fermentation time (X_3) on protease activity in the center point.

In Fig. 5, the effects of molasses concentration and fermentation time on protease activity in a constant amount of water (75 ml) are shown. According to the figure, with the increase in fermentation time, the protease activity increased significantly in all amounts of molasses, especially in its low concentrations, so the highest enzyme activity was related to the concentration of 3% molasses and fermentation time of 72 hours. Enzyme activity decreased with increasing amounts of molasses in all fermentation times, so the lowest enzyme activity was observed in a 7% concentration of molasses during 24 hours of fermentation. In a similar study, Sharan and Darmwal [20] investigated the activity of alkaline protease produced by *Bacillus pantotheneticus* on molasses substrate and showed that the use of molasses during 24 hours of fermentation increased the activity of the protease enzyme and further increase in fermentation time led to decrease enzyme activity.

a



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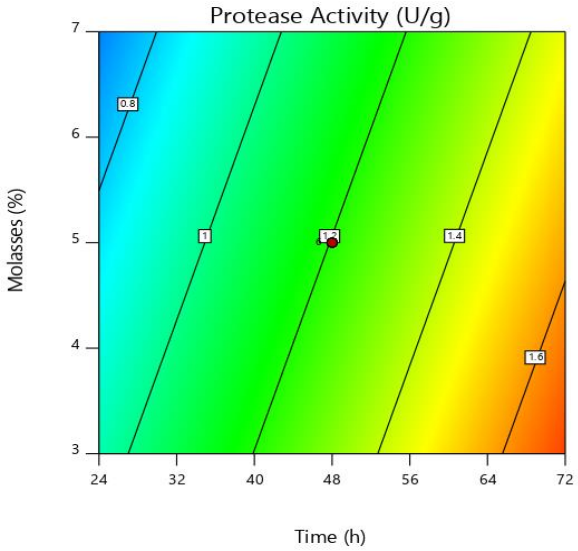

Fig. 5. The combined effects of molasses concentration and fermentation time on protease activity at constant water content (75 ml); a: 3D; b: counter.

3. 3. Process optimization

The optimization parameters are presented in Table 5. The optimal fermentation conditions of lentil flour were obtained using the numerical optimization feature of the software. For optimization, the values of the independent variables were set in the "In range" feature. Due to the relationship between nattokinase production and fibrinolytic activity and the high importance of this enzyme activity, the fibrinolytic activity was considered to be the maximum with an importance factor of 5. The "In range" feature was also considered for protease activity.

The optimal treatment was selected based on the highest degree of desirability in order to achieve the desired responses. Based on this, the optimal fermentation conditions predicted by the model were: 92.38 ml of water, 3.66% molasses and 70.90 hours of fermentation, in which fibrinolytic and protease activities were predicted as 2476.03 and 1.68 U/g, respectively. To validate the accuracy of the model, fermentation was carried out under optimal conditions, and the responses obtained were compared with those predicted by the model (Table 6). As it is clear, the experimental values were quite close to the values predicted by the model, indicating the validity of the model.

Table 5- Parameters for numerical optimization

Name	Goal	Lower Limit	Upper Limit	Importance factor
Water content	is in range	50	100	3
Molasses concentration	is in range	3	7	3
Fermentation time	is in range	24	72	3
Fibrinolytic activity	maximize	570.43	2473.63	5
Protease activity	is in range	0.55	1.78	3

Table 6- Model validation

Water content, X_1 (ml)	Molasses concentration, X_2 (%)	Fermentation time, X_3 (h)	Fibrinolytic activity, Y_1 (U/g)		Protease activity, Y_2 (U/g)	
			Experimental	Predicted	Experimental	Predicted
92.38	3.66	70.90	2462.45	2476.03	1.69	1.68

In their research, Moharam, El-Bendary [21] studied the optimization of fibrinolytic enzyme production by *B. subtilis* and stated that the incubation period of 96 hours was the most optimal time to achieve maximum enzyme activity. Nguyen and Nguyen [5] optimized the content of protease produced from soybean fermentation by *B. subtilis* and obtained the highest amount of protease during 48 hours of fermentation at 32°C. Sahoo, Mahanty [22] also used cheese whey substrate to produce nattokinase enzyme by *B. subtilis* and declared the optimal fermentation time to be 51 hours. Thu, Khue [23] investigated the fibrinolytic activity of *B. subtilis* serine protease on shrimp skin substrate and found that the highest fibrinolytic activity was related to the 16th hour of fermentation.

Bajaj, Singh [24] studied different carbon sources to optimize the production of fibrinolytic protease enzyme using *B. subtilis*. The results showed that the enzyme activity was the highest in the presence of molasses. Al Mamun, Mian [25] reported that the addition of 0.92% molasses along with soybean flour increased protease activity. Gaddad [26] optimized the fermentation conditions to increase the alkaline protease activity. The study showed that the addition of 0.75% molasses resulted in a significant increase in protease activity.

4- Conclusions

This research investigated the optimal conditions for the production of nattokinase

using *B. subtilis* on red lentil flour substrate in a fermentation method. The results of ANOVA showed that the best models for predicting fibrinolytic and protease activities were quadratic and linear models, respectively. In both models, with the increase in water content, molasses concentration and fermentation time, fibrinolytic and protease activities increased, decreased and increased, respectively. Also, the optimal fermentation conditions were 92.38 ml of water, 3.66% molasses and 70.90 hours, in which the fibrinolytic and protease activities were predicted as 2476.03 and 1.68 U/g, respectively, which confirmed the accuracy of the model due to its closeness to the experimental data. Overall, the findings of this study indicated that red lentil flour was a suitable substrate for the production of the nattokinase enzyme through fermentation by *B. subtilis*. Further investigations are recommended to optimize the conditions of fermentation in order to increase the yield of enzyme produced using red lentil flour substrate.

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استفاده از سوبسترای آرد عدس قرمز برای تولید آنزیم ناتوکیناز توسط *باسیلوس سابتیلیس* ناتو

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چکیده

در این پژوهش شرایط تولید بهینه ناتوکیناز با استفاده از باکتری *باسیلوس سابتیلیس* بر روی سوبسترای عدس دال مورد بررسی قرار گرفت. برای بهینه‌سازی فرایند تخمیر، اثر سه متغیر زمان تخمیر (۲٤، ٤۸ و ۷۲ ساعت)، غلظت ملاس (۳، ۵ و ۷ درصد) و مقدار آب (۵۰، ۷۵ و ۱۰۰ میلی‌لیتر) بر میزان فعالیت فیبرینولیتیکی و پروتئازی مورد بررسی قرار گرفت. نتایج در قالب طرح مرکب مرکزی (CCD) بررسی و به روش سطح پاسخ (RSM) مدل‌سازی و تجزیه و تحلیل شد. ضریب تبیین مدل‌های رگرسیونی برازش شده برای فعالیت‌های فیبرینولیتیک (مدل درجه دوم) و پروتئازی (مدل خطی) به‌ترتیب ۹۷/۱۵ و ۹۰/۱۲ درصد بوده و فاکتور عدم برازش آنها در سطح اطمینان ۹۵ درصد معنی‌دار نبود، از این رو صحت مدل‌ها برای برازش اطلاعات تایید گردید. با افزایش میزان آب، ملاس و مدت زمان تخمیر، فعالیت فیبرینولیتیک به‌ترتیب افزایش، کاهش و افزایش یافت ($p\leq0.05$). با افزایش مقدار آب، فعالیت پروتئازی نیز افزایش یافت، اما این افزایش معنی‌دار نبود، در حالی‌که افزایش میزان ملاس و مدت زمان تخمیر به‌ترتیب منجر به کاهش و افزایش معنی‌دار فعالیت پروتئازی گردیدند ($p\leq0.05$). در ارتباط با بهینه‌سازی، شرایط بهینه تخمیر عبارت بود از: ۹۲/۳۸ میلی‌لیتر آب، ۳/٦٦ درصد ملاس و ۷۰/۹۰ ساعت تخمیر که در چنین شرایطی میزان فعالیت آنزیم‌های فیبرینولیتیک و پروتئاز به‌ترتیب ۲٤۷٦/۰۳ و ۱/٦۸ واحد بر گرم پیش‌بینی گردید. برای اعتبارسنجی مدل، تخمیر تحت شرایط بهینه انجام شد و پاسخ‌های به‌دست آمده با پاسخ‌های پیش‌بینی شده توسط مدل مقایسه گردید. نزدیکی داده‌های آزمایشگاهی به مقادیر پیش‌بینی شده توسط مدل صحت مدل را تایید نمود. نتایج این پژوهش نشان داد که از عدس دال می‌توان به‌عنوان سوبسترای تخمیر توسط باکتری *باسیلوس سابتیلیس* جهت تولید آنزیم ناتوکیناز استفاده نمود.

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The role of Selenium in mitigating salt-induced effects on growth, Chlorophyll content, and antioxidant enzyme activity in garlic production

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ABSTRACT

This study aimed to evaluate the effect of Se application under salinity conditions on the yield and physiological characteristics of garlic. The experiment was conducted using a completely randomized design with three replications. Treatments included four levels of Se (0, 4, 8, 16 mg L^{-1}) and four salinity levels (0, 30, 60, and 90 mM sodium chloride) in a factorial arrangement. Results indicated that low concentrations of Se positively influenced vegetative characteristics. Increasing Se concentration to 8 mgL^{-1} enhanced growth across all studied vegetative traits. Relative water content of the leaves decreased with increasing NaCl concentration compared to the control. Se-treated plants showed increased levels of chlorophyll a, b, total chlorophyll and carotenoids compared to the control. Additionally, superoxide dismutase enzyme activity significantly increased with 8 mgL^{-1} Se treatment at 30 mM sodium chloride compared to the control. Interaction analysis revealed the highest and lowest catalase activity at 8 and 4 mgL^{-1} Se and 90 mM sodium chloride, respectively. Future research should explore the long-term effects of Se application on garlic under varying environmental conditions. Investigating the molecular mechanisms behind Se protective role against salinity stress could provide deeper insights. Additionally, examining the impact of Se on other economically important crops under salinity stress would be valuable.

1- Introduction

Soil salinity is a major factor limiting agricultural productivity globally, impacting approximately 7% of the world's land [1]. Enhancing plant tolerance to salinity is therefore essential [2]. Many horticultural crops are particularly sensitive to salinity and can only withstand low levels of salt stress [3]. Plants in a saline environment face two main factors: excessive salts present in the soil, which reduce the soil's osmotic potential and cause a decrease in water uptake and shortage of water in plants [4]. This leads to disruption in cell division, enlargement of cells, and affects all metabolic reactions in plants [5]. The other factor is the excessive amounts of sodium and chloride ions, which reduce the uptake of essential ions such as potassium, calcium, ammonium, and nitrate, as well as decreasing enzyme activity and damaging the membrane structure [6]. These effects result in a decrease in plant metabolic activities, including photosynthesis, and reduce plant growth in saline environments [7]. Due to ionic toxicity and osmotic stress, secondary stresses such as oxidative damage may occur in plants [8]. Salinity stress leads to the formation of Reactive Oxygen Species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH), and singlet oxygen, which cause oxidative damage to lipids, proteins, and nucleic acids [9].

A multitude of strategies have been identified to enhance the resilience of plants to high salt conditions [10]. These strategies include employing water-soluble substances, utilizing nitric oxide, incorporating polyamines, applying brassinosteroids, adding silicon, using melatonin, and introducing Se. While the effect of Se in mitigating environmental stress is well-documented in human and animal studies, its impact on plant stress is less explored [7]. Se is integral to the antioxidant defenses and hormonal regulation in humans and animals, and it similarly contributes to the antioxidant activities in plants [8].

Research indicates that low levels of Se can shield plants from a range of biological challenges, including temperature extremes, water scarcity, saline conditions and toxic metal exposure [11]. The application of Se has been shown to counteract the detrimental effects of salt stress, which typically include stunted plant growth, diminished photosynthetic activity and reduced chlorophyll levels [12]. Investigations into various crops like barley, pumpkin, wheat, cabbage, and rapeseed have revealed that Se, at minimal concentrations, can be advantageous [13]. Recent studies have highlighted the multifaceted role of Se in enhancing plant resilience. For instance, Hasanuzzaman et al. (2020) reported that Se supplementation can improve the antioxidant defense system in plants, thereby mitigating oxidative stress caused by environmental challenges [14]. Additionally, Schiavon et al. (2017) found that Se can enhance the synthesis of sulfur and nitrogen compounds, which are crucial for plant responses to abiotic stress [8]. Moreover, Se has been shown to stimulate root growth and increase water absorption capacity, which is particularly beneficial under drought conditions. According to Sharma et al. [15], Se can enhance the activity of aquaporins, proteins that facilitate water transport across cell membranes, thereby improving plant water status under stress. Furthermore, The role of Se in modulating the uptake and translocation of essential nutrients has been documented, contributing to overall plant health and productivity [16]. Garlic, scientifically known as (*Allium sativum* L.*)* and belonging to the *Alliaceae* family, is a prominent member of the genus *Allium* [17]. It is the second most consumed plant from this genus, after onion, and is highly valued for its rich mineral content [18]. Among plant species, garlic is particularly sensitive to salinity [19]. A two-year study reported that the salinity threshold for garlic is 3.9 dSm^{-1}, and at 7.4 dSm^{-1}, the yield decreases by 50% [20]. All yield components, including bulb weight and diameter, shoot biomass per unit area, and the percentage of dry matter, which is a major component of bulb quality, decrease with increasing salinity [16].

The primary goal of this study was to assess the impact of Se application on garlic's yield and physiological traits under salinity stress. The objectives included determining the optimal Se concentration for enhancing vegetative growth

and evaluating the interaction between Se and salinity levels. The study aimed to identify how Se affects chlorophyll content, carotenoids, and enzyme activities such as superoxide dismutase and catalase under different salinity conditions. By analyzing these factors, the research sought to provide insights into Se potential as a mitigative agent against salinity stress in garlic. The findings could inform future agricultural practices and guide further research on the role of Se in improving crop resilience under adverse environmental conditions.

2-Materials and Methods

This research was conducted in a greenhouse at the University of Tabriz, Iran, starting on September 30, 2019, and concluding on May 25, 2020. Data analysis and report finalization were completed by May 20, 2023. It is important to acknowledge that the dataset was gathered by the article's first author, Rozita Khademi Astaneh, at the university's greenhouse. This ensures proper credit for the data collection effort. The dataset comprises solely observational data of the plants, with results extracted in the university's lab. The consent procedure was approved by the University of Tabriz's ethics committee, ensuring compliance with all ethical guidelines and requirements for research involving human participant

Within the greenhouse, temperatures were maintained at 32°C during the day and 18°C at night. Natural variations in light and day length were observed, with relative humidity fluctuating between 23% and 65%. The research utilized garlic cloves sourced in February from the Maryanaj area in Hamedan Province, known for producing the 'Hamedan white' garlic variant. The experimental setup followed a factorial arrangement within a completely randomized design, consisting of three replications. After a chilling period, individual garlic cloves, still attached to their basal plates, were submerged in Se solutions at varying concentrations (0, 4, 8, 16 mg L^{-1}) for 26 hours. Following this immersion, the cloves were transferred to plastic containers filled with perlite, with three cloves planted per container. Throughout the study, the plants were manually watered daily with 800 mL of a modified Hoagland nutrient solution. Salinity stress was gradually introduced to the plants, which had reached the six-leaf stage, over a span of 10 days using nutrient solutions at four different salinity concentrations (0, 30, 60, 90 mM). This stress application continued for a total of 40 days.

Morphological parameters measured included the leaf count at the end of vegetative growth, the emergence of the final leaf, stem length and girth, the number of bulbs, and the dimensions of the bulbs post-harvest. The weight of the bulbs and roots was determined using a scale accurate to 0.1 gr. For dry weight assessment, the samples were oven-dried at 70°C, with roots dried for 48 hours and bulbs for 72 hours.

Determination of relative water content

Relative leaf water content was measured according to the method proposed by Ritchie et al. (1990) [21]. Leaf samples were taken using scissors from the mature leaves of all experimental treatments and their fresh weight was measured using an accurate balance. The samples were then placed in distilled water and stored at 4°C for 24 hours. After this period, the fresh weight of the leaves was measured again and the leaves were placed in an oven at 70°C for another 24 hours to determine their dry weight. The RWC is calculated by:

$$RWC = \frac{FW - DW}{TW - DW} \times 100 \qquad (1)$$

Where, FW is the weight of fresh sample, TW is the weight of turgid sample and DW is the weight of dry sample (all units is gr).

Chlorophyll and carotenoids determination

To assess the levels of chlorophyll in leaves (specifically chlorophyll a, chlorophyll b and total chlorophyll content), the procedure outlined by Arnon (1949) was employed [22]. This involved pulverizing 1 gr of fresh leaf tissue with liquid nitrogen into a fine powder, then mixing it with 20 mL of 80% acetone. The mixture was passed through Whatman No.2 filter paper to obtain a clear solution. Additional washings of the mortar, pestle and residual plant matter were conducted with 10 mL of 80% acetone and the combined filtrate was made up to a final volume

of 20 mL. The quantification of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids was based on measuring the absorbance of light at 645 nm and 663 nm for chlorophylls and at 470 nm for carotenoids, using a Spekol 1500 Analytik Jena AG spectrophotometer, comparing the readings against a control of 80% acetone.

Superoxide dismutase activity

The enzymatic function of superoxide dismutase was evaluated by its capacity to inhibit the photoreduction of Nitroblue Tetrazolium (NBT). The assay mixture for measuring this enzyme's activity included 50 mM potassium phosphate buffer at pH 7.0, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 12 mM methionine, 75 µM nitroblue tetrazolium, 2 µM riboflavin, 50 mM sodium carbonate and the enzyme sample. This mixture was exposed to a 40-watt light source, positioned 30 centimeters away to initiate the reaction. Enzyme activity was quantified as the amount of enzyme required to achieve a 50% reduction in NBT photoreduction at an absorbance of 560 nanometers, relative to controls lacking the enzyme. Activity levels were expressed in enzyme units per milligram of protein content.

Catalase activity

Catalase enzyme activity was assessed using the technique established by Aebi [23]. The assay involved a reaction solution comprising 50 mM potassium phosphate buffer (pH 7.0), 10 mM hydrogen peroxide and distilled water. This mixture, combined with the enzyme sample, was introduced into a 2-milliliter quartz cuvette. Catalase activity was quantified by the decomposition rate of hydrogen peroxide, expressed in micromoles per minute per milligram of protein, measured at an absorbance of 240 nanometers using a spectrophotometer.

Statistical analysis was performed using SPSS software, where the average results of the different treatments were evaluated through Duncan's multiple range test, with significance determined at both 1% and 5% levels. Also, the results were visually represented through charts created with Excel software.

3-Results

As indicated in **Table 1**, variance analysis revealed that Se application had a notable impact on stem thickness, bulb count per plot and both the fresh and dry mass of bulbs and roots, with statistical significance at the 1% level. Conversely, the influence on bulb size was not statistically significant. Salinity levels significantly affected all measured growth parameters, except for bulb size, with a 5% significance threshold. Also, the combined influence of salinity and Se was significant across all growth parameters at the 1% level.

Table 1. Variance analysis of Se and salinity effects on vegetative characteristics of garlic plants (number of leaves, stem diameter, bulb diameter and height, number of cloves per bulb, fresh and dry weight of bulb and root)"

		Mean of squares								
							bulb		root	
Sources of variation	df	Number of leave	Stem diameter (mm)	Garlic bulb diameter (mm)	Garlic stem diameter (mm)	number of cloves per bulb	DW(gr)	FW(gr)	DW(gr)	FW(gr)
Se	3	18.5*	8.969**	5.062^{ns}	8.969**	38.809**	43.141**	1109.416**	4.061**	179.796**
Salinity	3	75.167**	8.377**	8.606*	8.377**	30.483**	26.352**	1773.046**	12.170**	643.040**
Se× Salinity	9	48.267**	6.736**	25.138**	6.736**	27.038**	29.178**	1061.755**	6.486**	536.670**
Error	32	164.667	7.555	120.593	7.555	22.653	140.795	823.677	7.818	669.062

**, * and ns: Significantly difference at the 1 and 5 % of probability levels and non-significantly difference, respectively.

According to the results of **Table 2**, the treatment with 16 mgL^{-1} Se had the highest number of leaves. The treatment with 8 mgL^{-1} Se had the highest bulb diameter and number of bulbs per plot. The treatment with 4 mgL^{-1} Se showed the highest fresh and dry weight of roots. The treatment with 16 mgL^{-1} Se had the highest dry weight and length of bulbs at sodium chloride concentrations of 90 mM and 60 mM, respectively and the highest fresh weight of bulbs in non-saline conditions.

Table 2- Comprehensive parameters of garlic plants grown under various Se and salinity (NaCl) treatments

Selected biometric parameters										
Treatments		Number of leave	Stem diameter (mm)	Garlic bulb diameter (mm)	Garlic bulb height (mm)	Number of cloves per bulb	Bulb		Root	
Se (Se) ($mg\ L^{-1}$)	NaCl (mM)						FW(gr)	DW(gr)	FW(gr)	DW(gr)
0	0	43.66^{cd}	7.65^{f}	45.06^{bcd}	36.5^{ab}	15.06^{ab}	61.8^{def}	21.4^{de}	8.94^{ghi}	6.1^{cdefg}
	30	46.66^{cd}	8.35^{e}	45.96^{abc}	33.47^{bcd}	9.1^{f}	53.9^{fg}	20.3^{de}	9.67^{efg}	5.2^{gh}
	60	41^{d}	7.18^{f}	45.78^{abcd}	33.84^{bcd}	10.5^{ef}	83.5^{bc}	22.9^{bcde}	10^{def}	7.7^{b}
	90	42^{d}	8.63^{de}	43.56^{bcde}	36.68^{ab}	12.06^{cd}	64.8^{de}	14.7^{f}	7.7^{j}	6.2^{cdef}
4	0	43^{d}	8.69^{de}	44.99^{bcd}	36.09^{ab}	9.4^{f}	70.1^{d}	24.1^{abcd}	10.53^{de}	8.02^{b}
	30	44^{cb}	9.49^{e}	46.76^{ab}	36.71^{ab}	12.2^{c}	100.9^{a}	25.7^{abc}	11.7^{c}	9.91^{a}
	60	50.33^{ab}	7.3^{f}	40.67^{e}	32.23^{d}	9.2^{f}	66.9^{d}	22.2^{cde}	9.88^{def}	6.6^{c}
	90	45^{cd}	5.63^{g}	42.06^{de}	26.91^{e}	10.63^{def}	56.4^{efg}	20.5^{de}	9.51^{fgh}	5.5^{efgh}
8	0	49^{b}	11.88^{a}	42.2^{de}	36.05^{ab}	16.2^{a}	78.6^{c}	26.5^{ab}	14.42^{a}	8.07^{b}
	30	47.33^{bc}	9.61^{c}	48.97^{a}	35.93^{abc}	16.53^{a}	70.4^{d}	26.4^{ab}	11.97^{c}	6.6^{c}
	60	42^{d}	7.43^{f}	42.78^{cde}	33.11^{ab}	14.3^{b}	62.2^{def}	19.4^{e}	8.41^{ij}	5.6^{defg}
	90	37^{e}	9.26^{cd}	42.56^{cde}	32.16^{d}	6.96^{g}	50.8^{g}	21.8^{cde}	10.7^{d}	4.6^{h}
16	0	43.33^{cd}	10.55^{b}	47.23^{ab}	34.9^{bcd}	14.6^{b}	89.95^{b}	22.7^{bcde}	12.9^{7b}	9.24^{a}
	30	53.3^{a}	7.8^{f}	37.36^{e}	33.94^{bcd}	16.3^{a}	63.9^{de}	23.8^{abcd}	$8.71^{h}i$	6.43^{cde}
	60	42.66^{d}	9.36^{c}	46.91^{ab}	39.25^{a}	15^{ab}	67.1^{d}	22.5^{bcde}	10.59^{de}	6.56^{cd}
	90	41^{d}	9.29^{cd}	43.91^{bcde}	32.3^{cd}	11.6^{cde}	61.6^{def}	26.9^{a}	7.5^{k}	5.3^{fgh}
Salinity treatment (S)		❋*	❋*	*	❋❋	**	❋❋	**	❋❋	❋❋
Se treatment (SNP)		❋	❋❋	NS	❋	❋❋	❋❋	❋❋	❋❋	❋❋

S×Se	**	**	**	**	**	**	**	**	**

The averages of the traits, which have the same letters in each column, have no significant difference at the 5% probability level.

The application of Se resulted in an increase in stem diameter, with the highest stem diameter observed in the treatment with 8 mgL^{-1} Se under non-saline conditions. Stem height was affected by salinity treatment and increased with the concentration of sodium chloride in non-Se conditions compared to the control. The highest stem height was recorded at a sodium chloride concentration of 60 mM and with the treatment of 8 mgL^{-1} Se.

Relative Water Content (RWC)

The simple and interactive effects of salinity and Se were significant at the 1% probability level on the RWC of leaves (**Table 3**). As shown in **Fig 1**, with increasing salt concentration, the RWC of leaves decreased compared to the control plant. In non-saline conditions, the application of 16 mgL^{-1}Se significantly increased the RWC of garlic plant leaves compared to the control.

The simple and interactive effects of salinity and Se were significant at the 1% probability level on the RWC of leaves (**Table 3**). As shown in **Fig 1**, increasing salt concentration led to a decrease in the RWC of leaves compared to the control plants. Under non-saline conditions, the application of 16 mgL^{-1} Se significantly increased the RWC of garlic plant leaves compared to the control.

Table 3. Variance analysis of Se and salinity effects on RWC, Chlorophyll content, carotenoids, and activities of SOD and CAT enzymes in garlic plants

		Mean of squares						
Source of variation	df	RWC	Chl a	Chl b	Total Chl	Carotenoid	SOD	CAT
Se	3	92.50**	0.025**	0.097**	0.228**	0.020**	226.228**	91.308**
Salinity	3	212.944**	0.30**	0.150**	0.403**	0.018**	53.917**	60.972**
Se×Salinity	9	174.689**	0.016**	0.105**	0.183**	0.015**	114.213**	73.226**
Error	32	45.333	0.024	0.018	0.076	0.001	116.309	0.000218

**, * and ns: Significantly difference at the 1 and 5 % of probability levels and non-significantly difference, respectively.

The lowest RWC of leaves was first observed in the treatment with 4 mgL^{-1} Se without salinity stress, followed by the highest sodium chloride concentration (90 mM) without Se treatment. The highest RWC was observed in the treatment with 8 mgL^{-1} Se and 30 mM sodium chloride concentration, which did not differ significantly from the 60 mM sodium chloride concentration (**Fig 1**).

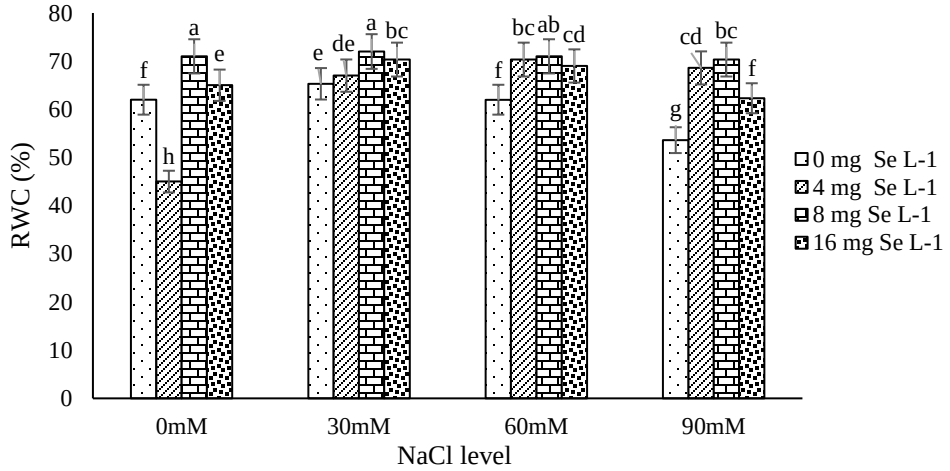

Fig.1. Impact of Se and sodium chloride on the RWC of garlic leaves

Chlorophyll content

Chlorophyll a

The study demonstrated that both the individual and combined impacts of salinity stress and Se supplementation had a statistically significant influence on chlorophyll a levels with a less than 1% probability of random occurrence. An inverse relationship was observed between chlorophyll a content and increasing levels of sodium chloride in samples not treated with Se. Conversely, in samples without salinity stress, chlorophyll a initially increased with Se supplementation but decreased with higher Se doses. The highest chlorophyll a concentration was found in samples treated with 4 mgL^{-1} Se and 30 mM sodium chloride, while the lowest concentration was observed in samples treated with 16 mgL^{-1} Se at a sodium chloride level of 60 mM (**Fig 2**).

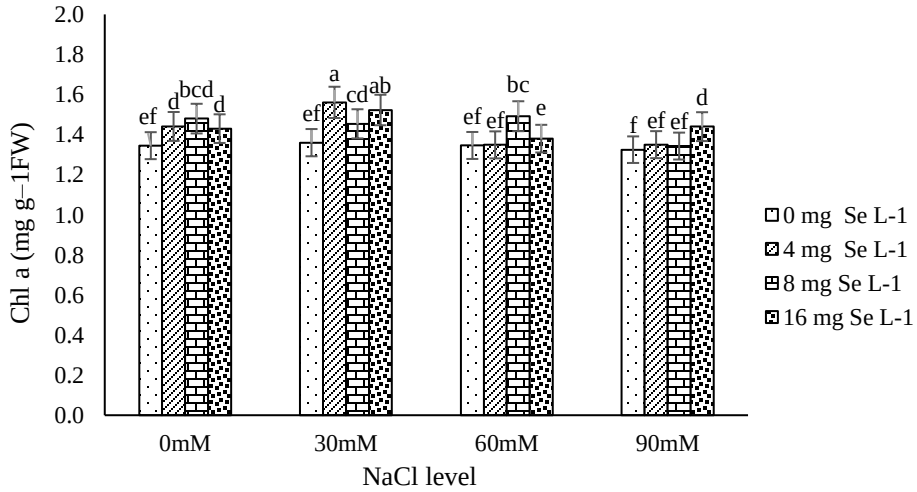

Fig.2. Impact of Se and sodium chloride on chlorophyll a content in garlic leaves

Chlorophyll b

The experimental data, analyzed through variance, indicated that both the individual and combined effects of salinity and Se had a statistically significant impact on chlorophyll b levels at the 1% probability level, as shown in **Table 3**. An upward trend in chlorophyll b content was observed with increasing Se levels in

environments free of salinity. Conversely, in the absence of Se, an increase in sodium chloride led to a reduction in chlorophyll b levels. The highest chlorophyll b content was found in the treatment with 4 mgL^{-1} Se at a 30 mM sodium chloride concentration, while the lowest content was observed in the treatments with 0 and 4 mgL^{-1} Se at a 90 mM sodium chloride concentration (**Fig 3**).

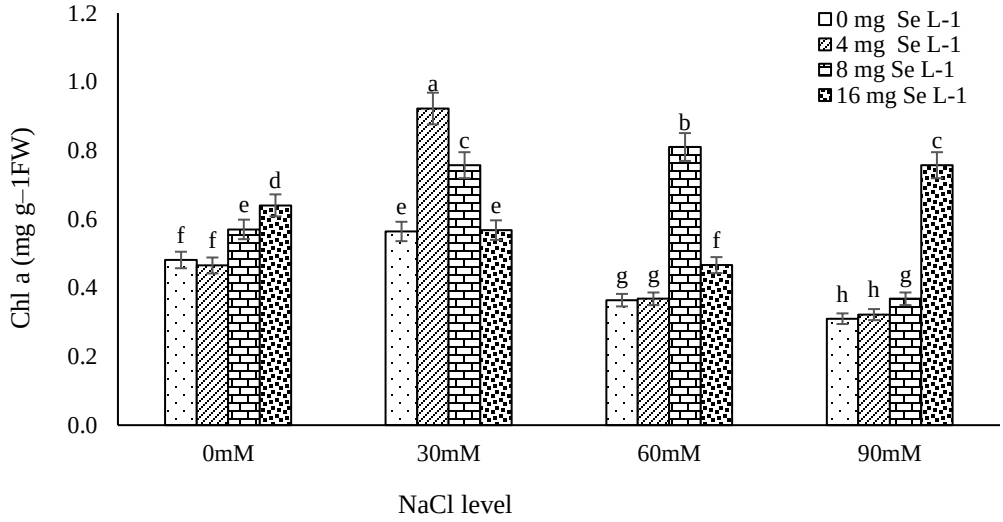

Fig.3. Impact of Se and sodium chloride on chlorophyll b content in garlic leaves

Total Chlorophyll

The simple effects of salinity stress and Se treatment, as well as their interactive effects, were statistically significant at the 1% probability level on total chlorophyll content (**Table 3**). Se treatment in non-saline conditions led to a significant increase in total chlorophyll content. In the absence of Se treatment, total chlorophyll content decreased with increasing salinity levels. The interactive effects showed that the highest total chlorophyll content was recorded in the treatment with 4 mgL^{-1} Se and 30 mM sodium chloride concentration. The lowest total chlorophyll content was observed in the treatments with 0, 4, and 8 mgL^{-1} Se at a 90 mM sodium chloride concentration (**Fig 4**).

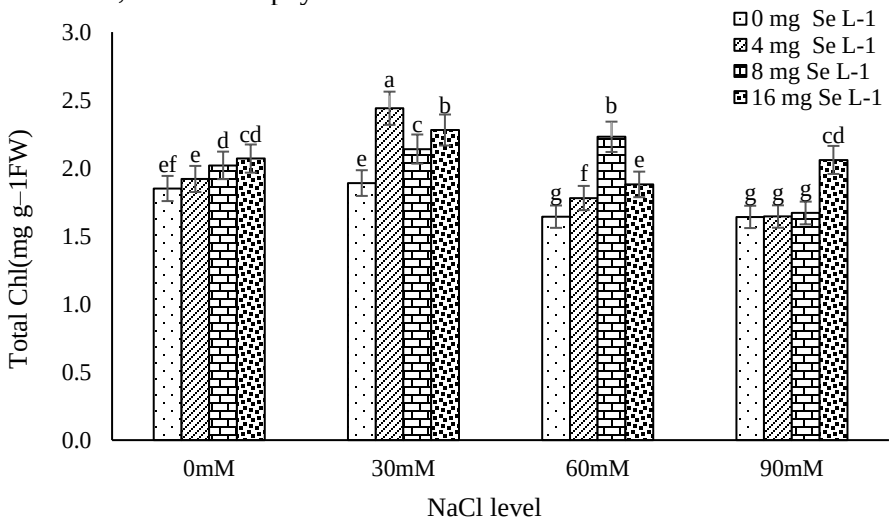

Fig.4. Impact of Se and sodium chloride on total chlorophyll content in garlic leaves

Carotenoid

The simple effects of salinity stress, Se treatment and their interactive effects were statistically significant at the 1% probability level on leaf carotenoids (**Table 3**). Increasing sodium chloride concentration led to a decrease in carotenoid content. The application of Se in non-saline conditions significantly increased carotenoid content (**Fig 5**). The interactive effects showed a significant increase in carotenoid content with the application of Se (4, 8, and 16 mgL^{-1}) at different salinity levels. The highest carotenoid content was recorded at 30, 60, and 90 mM sodium chloride concentrations with the application of 4, 8, and 16 mgL^{-1} Se, respectively (**Fig 5**).

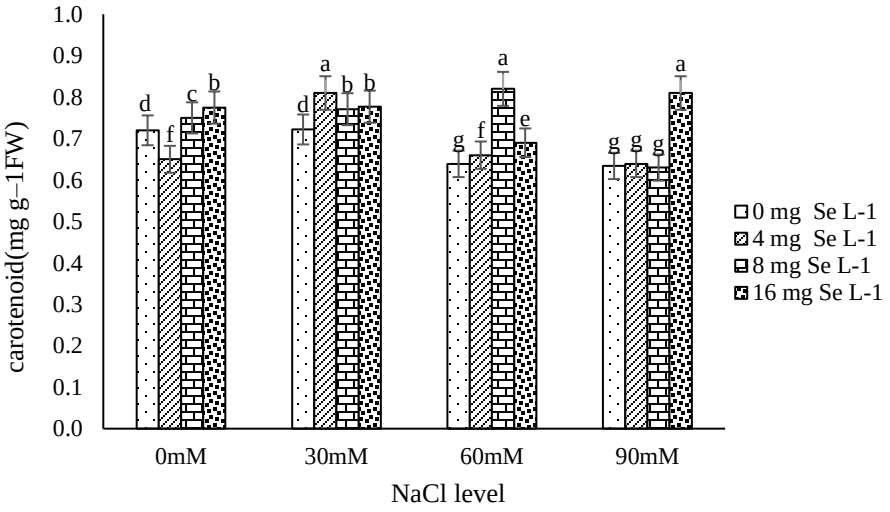

Fig.5. Carotenoid content in garlic under salinity stress with various Se treatment levels"

Superoxide dismutase activity

The interactive effects of salinity and Se were statistically significant at the 1% probability level on Superoxide Dismutase (SOD) activity (**Table 3**). Based on the results of the simple effects, SOD activity increased with rising sodium chloride concentrations and Se levels. The highest SOD activity was observed in the treatment without Se at a 60 mM sodium chloride concentration. Considering the interactive effects of salinity and Se, the application of 8 mgL^{-1} Se at 30 mM sodium chloride concentration significantly increased SOD activity compared to the control (**Fig 6**).

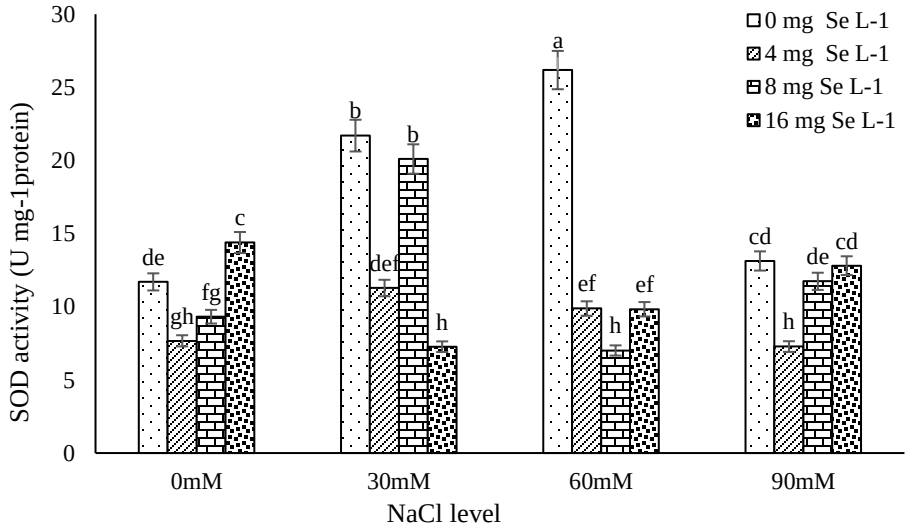

Fig.6. Superoxide dismutase enzyme activity in garlic under salinity stress with various Se treatment levels

Catalase activity

The interactive effects of salinity and Se were statistically significant at the 1% probability level on Catalase (CAT) activity (**Table 3**). CAT activity increased with rising Se concentrations. Salinity stress also significantly increased CAT activity, with the highest activity observed at a 60 mM sodium chloride concentration without Se treatment (**Fig 7**). According to the results of the interactive effects, the highest CAT activity was recorded in the treatment with 8 mgL^{-1} Se and 90 mM sodium chloride, while the lowest activity was observed in the treatment with 4 mgL^{-1} Se and 90 mM sodium chloride.

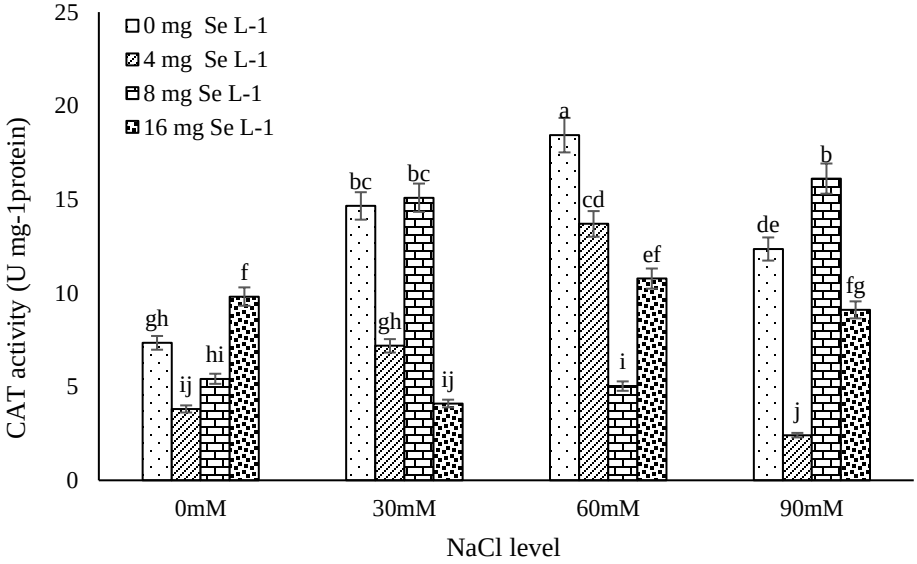

Fig.7. Catalase enzyme activity in garlic under salinity stress with various Se treatment levels

4-Discussion

The results of this study demonstrated that Se application at low concentrations, positively influenced growth characteristics. Specifically, increasing Se concentration up to 8 mgL^{-1} enhanced all growth traits studied, but higher concentrations led to a decline. These findings

align with recent studies on cucumber and watermelon under saline stress [4-6]. The higher growth characteristics observed at the 8 mgL^{-1} treatment may be due to increased leaf area, which in turn provides more assimilates for plant growth.

Se application up to a certain level also increased the dry weight of the stem, consistent with findings in other crops [8]. This increase in Se content enhances plant performance by protecting chlorophyll [24]. Plants possess antioxidant enzymes such as SOD and CAT to combat ROS. This increase in Se content enhances plant performance by protecting chlorophyll [25]. Se increases the activity of these two enzymes at low concentrations, which could potentially increase leaf production in plants through increased antioxidant activity. Additionally, selenium's growth-promoting effects may result from increased starch accumulation in chloroplasts and cell protection [26]. Se application in the form of selenate and selenite has been shown to be beneficial for plants under saline stress [6, 27]. The significant differences in leaf water content between Se-treated plants indicate varying effects on water uptake or loss through stomata and the ability to regulate osmotic pressure to maintain tissue turgor [14]. Se stimulates root growth and increases water absorption capacity, leading to higher leaf water content [28]. Additionally, Schiavon et al. (2017) noted that Se can enhance plant growth and stress tolerance by inducing the synthesis of sulfur and nitrogen compounds, which are vital for plant responses to abiotic stress [8]. Khan et al. (2023) further emphasized that Se, even in trace amounts, can significantly improve plant growth and development by acting as an antioxidant and stimulator [25].

The observed decline in chlorophyll levels under saline conditions is likely due to a shift in nitrogen metabolism, favoring proline production over chlorophyll. However, Se introduction under these conditions increases chlorophyll levels, suggesting Se alleviates damage to the photosynthetic apparatus and cell membranes [29]. Studies have shown that Se-treated plants exhibit higher levels of chlorophyll a, b, and total chlorophyll compared to untreated plants [30]. Excessive Se, however, can reduce chlorophyll, carotenoid, and xanthophyll levels, indicating a delicate balance in the effects of Se on plant physiology [4].

Carotenoids play a crucial role as biological antioxidants, protecting plant tissues. Se application increases chlorophyll and carotenoid contents, consistent with recent findings [5]. This increase may be due to the inhibition of ROS by antioxidant enzymes [14]. Se can improve the strength of cellular organelles and the membrane system in leaf cells under salinity stress. One of the most significant biochemical changes in plants under stress is the production of ROS and hydroxyl radicals in chloroplasts and mitochondria. ROS are produced during vital processes such as photorespiration, photosynthesis, and respiration, disrupting normal plant metabolism and potentially leading to cell death. Plants have various protective mechanisms to eliminate or reduce ROS, including antioxidant enzyme systems. Higher levels of antioxidants in plants correlate with greater resistance to oxidative damage [31]. One of the most significant biochemical changes that occur in plants under stress is the production of ROS and hydroxyl radicals in chloroplasts and mitochondria [32]. ROS are produced during vital processes such as photorespiration, photosynthesis and respiration and can disrupt normal plant metabolism, ultimately leading to cell death through water decomposition and electron transfer disorders. Plants have various protective mechanisms to eliminate or reduce ROS, which are effective at different stress levels [31]. Antioxidant enzyme systems are one of these protective mechanisms. Plants with higher levels of antioxidants exhibit greater resistance to oxidative damage. The activities of SOD and CAT enzymes under Se and salinity treatments are shown in **Figs 6 and 7**.

Antioxidant enzymes protect membranes, proteins, and macromolecules against oxidative damage caused by reactive oxygen species and contribute to the resistance and stability of plants under environmental stresses such as salinity [32]. Salinity stress, similar to other environmental stresses, leads to the accumulation of ROS in cells. Plants have an efficient defense

system consisting of antioxidant enzymes to combat the oxidative stress caused. Antioxidant enzymes protect membranes, proteins and macromolecules against oxidative damage caused by ROS and contribute to the resistance and stability of plants under environmental stresses such as salinity [33]. Therefore, the antioxidant capacity of plants is directly related to their tolerance to stress [8]. Sairam et al. [34] reported an increase in the activity of the SOD enzyme in plants under salinity stress compared to the control. The findings of Ashraf and Ali (2008) suggest that the presence of saline conditions enhances the action of the antioxidant enzyme SOD in mustard plant foliage. This heightened enzyme activity could be attributed to an accelerated synthesis of DNA and proteins within the plant tissues [31]. Additionally, when Se is administered to plants subjected to saline environments, there is a notable rise in the activity of the SOD enzyme [15].

5-Conclusion

The application of Se significantly enhanced the weight of fresh shoots, dry root weight, number of shoots per plant, and shoot diameter and length compared to control plants. Se at low concentrations positively influenced growth characteristics, with all traits improving up to a concentration of 8 mgL^{-1}, beyond which they declined. Increased salinity levels reduced the relative water content of leaves, but Se treatment at 8 mgL^{-1}improved water content at 30 and 90 mM sodium chloride levels. Se regulated plant water status by stimulating root growth and enhancing water absorption capacity, thereby increasing leaf water content. It also protected chloroplast enzymes, leading to higher chlorophyll and photosynthetic pigment levels. Under salinity stress, the activity of superoxide dismutase and catalase enzymes increased, with Se treatment further boosting antioxidant enzyme activity. This increase may be attributed to enhanced DNA and protein synthesis in tissues. Future research should investigate the long-term effects of Se application on garlic under diverse environmental conditions. Exploring the molecular mechanisms behind Se's protective role against salinity stress could provide deeper insights. Additionally, examining the impact of Se on other economically important crops under salinity stress would be valuable. Field trials to validate these findings under real-world agricultural conditions are recommended.

6-Acknowledgement

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مقاله علمی_پژوهشی

نقش سلنیوم در کاهش اثرات ناشی از نمک بر رشد، محتوای کلروفیل و فعالیت آنزیم آنتی‌اکسیدانی گیاه سیر

رزیتا خادمی آستانه*، صاحبعلی بلندنظر، فریبرز زارع نهندی

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چکیده

هدف این مطالعه ارزیابی اثر کاربرد Se در شرایط شوری بر عملکرد و ویژگی‌های فیزیولوژیکی سیر بود. این آزمایش در قالب طرح کاملا تصادفی با سه تکرار انجام شد. تیمارها شامل چهار سطح ۰ ، ٤ ، ۸ ، ١٦ میلی‌گرم سلنیوم و چهار سطح نمک (۰ ، ۳۰ ، ٦۰ و ۹۰ میلی متر کلرید سدیم) بود. نتایج نشان داد که غلظت‌های پایین Se به طور مثبت بر ویژگی‌های گیاهی سیر تأثیر می‌گذارد. افزایش غلظت Se به ۸ میلی‌گرم بر لیتر، رشد را در تمام صفات رویشی مورد مطالعه افزایش داد. محتوای آب نسبی برگ‌ها با افزایش غلظت NaCl در مقایسه با تیمار شاهد، کاهش یافت. گیاهان تحت تیمار Se در مقایسه با شاهد، سطح کلروفیل a ، b و کاروتنوئیدها را افزایش دادند. علاوه بر این، فعالیت آنزیم سوپراکسید دیسموتاز با مصرف ۸ میلی‌گرم بر لیتر Se و ۳۰ میلی موس در مقایسه با کنترل به طور قابل توجهی افزایش یافت. نتایج این مطالعه نشان داد که بالاترین و پایین‌ترین فعالیت کاتالاز در ۸ و ٤میلی‌گرم بر لیتر Se و ۹۰ میلی موس کلرید سدیم است. تحقیقات آینده باید اثرات بلند مدت استفاده از Se را بر سیر در شرایط محیطی متفاوت بررسی کند. بررسی مکانیسم‌های مولکولی نقش محافظتی Se در برابر استرس شوری می‌تواند بینش عمیق‌تری را فراهم کند. همچنین، بررسی تاثیر Se بر سایر محصولات مهم اقتصادی تحت استرس شوری قابل پیشنهاد است.

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The Economic and Agricultural Significance of Phoenix dactylifera: Challenges and Opportunities for Sustainable Development in Morocco's Date Palm Sector

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ABSTRACT

The present study discusses the date palm (Phoenix dactylifera) in Morocco concerning its economic and agricultural importance, genetic diversity, and ecological adaptability; its importance within the economies of oases is underlined. The paper focuses on the eighteen main varieties, which highlight that the date palm economic sector in Morocco has a high risk because of various causes, such as water shortage, climate change, and market fragmentation. Through morphological, cultural, and economic analyses, the major growth constraints affecting the sector were identified. It further assesses the effectiveness of government initiatives to enhance productivity and sustainability. It reveals that improved water management, market access, and varietal recognition will raise the status of Morocco in the date global market. The paper concludes with strategic recommendations on how to balance traditional agriculture with modernization to make it sustainable in the long term.

1- Introduction

The date palm, scientifically referred to as Phoenix dactylifera, is a plant species important for both ecological and economic reasons. It plays an integral role in arid and semi-arid agricultural ecosystems across North Africa up into the Middle East. This perennial fruit tree is renowned for its hardiness toward harsh climate conditions, its socioeconomic importance, and its ability to produce dates a staple food in many cultures. Of all its species, only Phoenix dactylifera is cultivated for its fruit and has played a very important role in maintaining a population in situ to maintain agriculture.
The date palm is the central plant in the oasis system of agriculture in Morocco, but particularly in the region of Drâa-Tafilalet. Though traditionally famous for date-palm cultivation, several challenges confront this sector, including climate change, water shortage, and competition from imported varieties at relatively cheap prices. Such are to be identified and overcome in order to improve productivity, quality, and enhance economic value for the different varieties of Moroccan palm dates. The main goals of the investigation will be:

- The morphological and genetic variabilities of Phoenix dactylifera cultivars within Morocco will be assessed for adaptability to different environmental conditions.
- The economic contribution of date palm cultivation in Morocco will be analyzed based on the local economy, especially in the oasis zones.
- The critical challenges concerning climate constraints, water management issues, and market competition will be highlighted for the Moroccan date palm sector, with strategies to address them being suggested.

Assess the contribution of modern agricultural practices and government initiatives to the improvement in yield and quality characteristics in date palm production in Morocco.
It intends to present an overview of the date palm sector in Morocco regarding its sustainable development at both the local and international market levels, coupled with the identification of opportunities to improve the socio-economic impact of date palm farming.
Through this comprehensive study, we aim to provide insight into the sustainable development of the Moroccan date palm sector and give practical recommendations on how to optimize its contribution to the agricultural economy.

2-METHODOLOGY

The methodology applied is focusing on both primary and secondary data. The sources include peer-reviewed journal articles, research handbooks, conference proceedings, and reports from international organizations such as the FAO. Additionally, government reports and strategic documents on agricultural development in Morocco form a crucial part of the resource base. The references span a wide range of years, with many recent studies published between 2020 and 2024, offering updated insights into modern agricultural practices and challenges. Older foundational research from the 1990s and early 2000s is also incorporated, especially in sections focusing on the botanical and morphological aspects of the date palm.
The review covers a diverse array of topics. These include agronomy, where various cultivation techniques, soil and water management strategies, and varietal

differences are discussed. In the botanical section, the morphology, genetic diversity, and taxonomy of Phoenix species are explored in depth. The methodologies used to study the classification of the date palm varieties in Morocco focused on key physical characteristics, such as fruit size, color, and consistency. Fruit size refers to the dimensions of the dates, with larger fruits typically being more desirable in both local and international markets. Color is another important feature, as it affects consumer perception of quality, ranging from light to dark brown. Consistency, which refers to the texture of the fruit (soft, semi-soft, or dry), is a critical factor in determining marketability and consumer preference. These traits were assessed through morphological measurements and visual inspection, techniques that are commonly used in studies of plant.

The study also integrates both ecological and cultural analyses to understand the traditional farming practices and the cultural significance of date palm cultivation in Morocco. It examines key practices, such as irrigation methods, soil management techniques, and harvesting practices, and their contributions to the overall sustainability and productivity of date palm farming, particularly in Morocco's arid regions. The cultural analysis focused on understanding traditional farming practices, including irrigation methods, soil management, and harvesting techniques. This analysis, using both qualitative and quantitative data from agricultural organizations, identified constraints such as inefficient water management and the need for modernization of farming practices to ensure sustainability and improve productivity in arid regions.

In the economic analysis, the study utilized production costs, market prices, and yield data provided from international and national statistics to a broader context of the economic landscape to evaluate the economic challenges facing the date palm sector. Market surveys, interviews with farmers, and government reports helped identify constraints like high production costs, limited market access, and competition from imported dates. The study also assessed government policies aimed at improving irrigation techniques, climate adaptation, and market access, offering insights into how these policies could address the economic constraints and improve sustainability.

Together, these analyses offer a comprehensive understanding of the challenges and opportunities facing the Moroccan date palm sector, and how cultural practices can be adapted to enhance its productivity and sustainability in the face of changing environmental conditions.

I. Global Phoeniciculture

I.1. Phoenix

The etymology of the word "phoenix" is as rich and fascinating as the myths and stories surrounding it. In Greek mythology, the Phoenix is a legendary bird that is cyclically regenerated or reborn. Associated with the sun, the nomenclature (Phoenix) also reflects the close cultural and commercial ties between the Greeks and the Phoenicians, skilled navigators and traders of antiquity. It’s also the name given by the Greeks to the palm fronds.

The diversity within the Phoenix genus includes several species, each with its ecological, morphological, and usage peculiarities. Based on the researches some type of Phoenix are more studies and recognized by investigators than others such as Dactylifera, Reclinata, Sylvestris and Canariensis [1]. For the others it could be related to the availability of direct scientific recourse databases or specific botanical works such as those by Barreveld in 1993. The differences between some of the species mentioned are based on the information

available up to the last point of knowledge in April 2023

Phoenix canariensis (Canary Island Date Palm): Native to the Canary Islands, this species is widely used for ornamental purposes due to its majestic appearance.

Phoenix reclinata (Senegal Date Palm): Characterized by an often leaning and sometimes branched trunk. It is native to Africa and appreciated for its aesthetic appeal in landscaping.

Phoenix sylvestris (Indian Date Palm): This species is exploited for the sweet sap it produces, which can be turned into sugar or fermented to produce alcohol.

Phoenix roebelenii (Pygmy Date Palm): This is a small species often used as an indoor plant or in landscaping for small gardens.

Phoenix dactylifera (Date Palm): Cultivated for its fruits, dates.

The varieties of the Phoenix genus hold significant value in their environments, and each is exploited and cultivated for various uses such as the horticultural industry, human and animal food (molasses, jam, coffee) [2]. Our study focuses particularly on those producing date fruits, being the only species of its kind cultivated for its fruits, underscoring their importance not only in terms of biological diversity but also for their substantial contribution to the agricultural economy. These varieties, through their ability to produce fruits sought for their nutritional qualities and their adaptability to arid climatic conditions, play a crucial role in sustaining local communities and perpetuating agricultural traditions in desert regions.

I.2. Phoenix dactylifera

Phoenix dactylifera is a dioecious fruit tree native to the warm and arid regions of the world. Its name is derived from two parts. The term "Phoenix" comes from the name given by the ancient Greeks to the date palm, which they associated with the Phoenicians, as explained in the first part, and "Dactylifera" comes from the Latin "dactylos," itself derived from the Greek "daktulos," meaning finger, in reference to the shape of the date (fruit) [3].

a. Botanical Origin (Taxonomy)

The taxonomy of Phoenix dactylifera has been extensively studied by researchers such as Munier (1973). The taxonomy of Phoenix dactylifera, has been a subject of extensive research over the decades. This species belongs to the Arecaceae family, which is characterized by monocotyledonous flowering plants. The classification of Phoenix dactylifera has evolved over time, reflecting advancements in botanical science and phylogenetic analysis. The traditional classification, as established by Linnaeus in 1734 and Munier in 1973, has undergone various revisions leading to the modern scientific taxonomy. The table 1 below showcasing the current classifications of the date palm.

***Table 1: Classification Phoenix dactylifera* [4]**

Rank	Classification
Kingdom	Plantae
Phylum	Magnoliophyta
Class	Liliopsida
Subclass	Arecidae
Order	Arecales
Family	Arecaceae
Genus	Phoenix
Species	dactylifera

b. Morphological Characteristics

The morphological characteristics of Phoenix Dactylifera, represented in Figure 1, are traditionally studied to predict the uses of the different parts of the date palm on the one hand and more specifically to identify, classify, and evaluate genetic diversity based on morphological. Numerous studies have been conducted that utilize morphological characteristics for cultivar identification, genetic diversity through biochemical or other molecular markers [5].

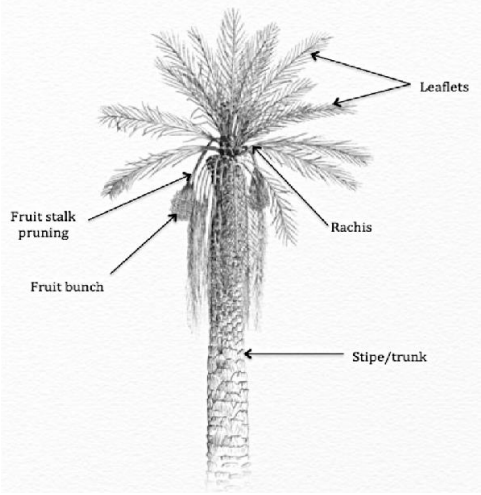

Figure 1: Phoenix dactylifera [6]

The tree can reach up to 30 meters in height, making it a remarkably large species among other Phoenixes. Illustration 1 contains the three essential different parts:

1. **Stipe (Trunk):** Generally straight. It can measure up to 75 cm in diameter at its base. It is marked by diamond-shaped or diamond patterns
2. that are the scars left by the bases of old leaves after they have fallen.
3. **Fruit bunch:** The fruit bunches are large and pendulous, consisting of numerous individual dates. Each bunch can contain hundreds of dates.
4. **Fruits (Dates):** Clustered in heavy bunches that hang under the fronds. Ripe takes a color ranging from golden yellow to dark brown depending on the variety. The fruits can measure from 4 to 8 cm long and contain a single large pit.
5. **Rachis:** The rachis is the central axis or stem from which the leaflets of a compound leaf extend. In the case of the date palm, it is the main support structure for the leaflets, providing both stability and the means for nutrient transport.
6. **Leaflets:** The leaves are large palms that can measure from 3 to 5 meters in length. These fronds are composed of many leaflets (small leaves) arranged pinnately (i.e., on each side of a central axis).

c. Culture and Cultivation

Palms are distinguished by their remarkable resilience and adaptability, allowing them to thrive in a variety of conditions, including those considered limiting. Nevertheless, certain specific climatic conditions optimally promote their growth and development. The cultural requirements of Phoenix dactylifera, as described below, include specific climatic,

hydraulic, agrological, and nutritional factors that are essential to ensure ideal growth and high-quality date production.

Climatic factors: High temperature, almost total absence of rains, and a low hygrometric degree. Rain and cold during fertilization can cause problems, notably the death of organs. Favorable factors for the maturation of dates, prolonged summer heat, and relatively low humidity are crucial.

- High temperatures for flowering vary from 17°C to 22°C depending on the regions [7].
- Sum of temperatures from 5000°C to 6000°C from flowering to maturation for early and late varieties respectively [7].
- Great brightness is paramount for date production. In palm groves, it is observed that the best-lit trees are always the most laden with fruits [7].

Hydraulic factors: Abundant irrigation is crucial; irrigation water volumes vary depending on the regions and the nature of the soil and water used for irrigation (a preference for salinity) [8]. The water consumption of the palm tree varies according to the age of the tree.

- In Morocco, the irrigation dose per tree per month varies from 9 to 16 m^3 in the cold period and from 17 to 25 m^3 in the hot period.
- In Morocco, the submission irrigation is the most used method for palm [9], and groves is watered with waters having between 9 to 10 g/l of salt [10].

Agrological factors: The date palm grows well in various terrains but prefers neutral, deep, light, and normally moist soils. It can tolerate saline soils and supports clay soils if aerated.

- For normal growth, the soil must allow water penetration to a depth of 2 to 2.5 meters [7].

Nutritional factors: The palm has nutrient needs that vary with its age. After harvest, during the formation of fruit buds, during fertilization, and at the beginning of summer, nutrient inputs are necessary for its growth and fruit production. Amendments such as adding organic matter may be necessary to improve soil quality.

- Analyses of date palm leaves show high levels of dry matter, chlorides, and sulfur.

  A study suggests that, the concentration of most mineral elements was increased in date palm leaves as a response to the improvement of soil fertility [11].

d. History

The date palm (Phoenix dactylifera) is among the very first fruits to have been domesticated in the world; it is considered a traditional fruit of the Old World. Along with the olive (Olea europaea L.) in the Mediterranean basin, the common fig (Ficus carica L.), and the wine grape (Vitis vinifera L.) in the Near East [12]. A timeline illustrating the estimated periods of domestication of the date palm relative to other fruits estimates 6300–6800 BP (before the present) [13].

The dates of domestication indicate the estimated periods based on available archaeological and historical data. The beginning of the timeline starts around -9000 BCE, marking the domestication of the common fig and progresses to -6000 BCE where the Date Palm is noted.

Available documents and archaeological (date seeds) sites (caves) show that the cultivation of the date palm is confirmed from the 5th millennium BP [14-15].

The cultivation and geographical origin of the date palm are uncertain and debated. It seems that it was born in Mesopotamia [16], a historical region of the Middle East located between the Tigris and Euphrates. It corresponds for the most part to present-day Iraq and Syria, represented in Figure 2.

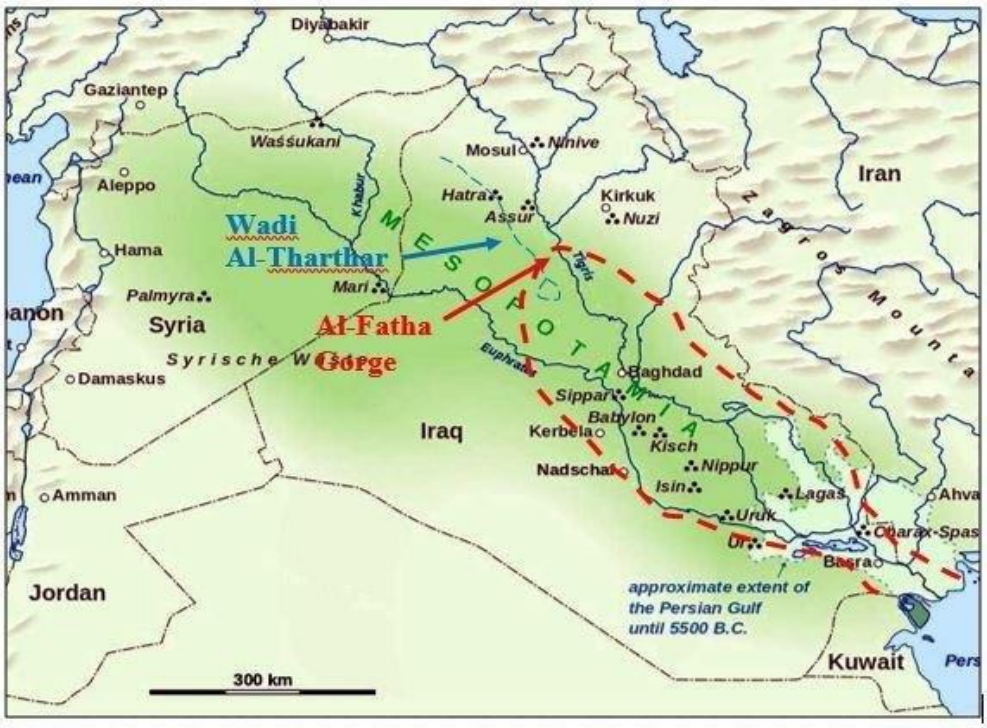

Figure 2: Mesopotamia Zone [17]

The Date Palm has been spread and implanted in the world where the conditions were favorable, and as a result, date palm has wielded a major influence in shaping of the culture and heritage in many countries such as Egypt [18]. The closest expansion theory is that of Munier in 1973, who described the dispersion of the date palm along two major historical routes, the first route from Mesopotamia southward to the Arabian Peninsula and eastward to undivided India, and the second route begins in Egypt (itself the site of an independent domestication of the date palm) across North Africa to Morocco [12].

e. Geographical Distribution

The date palm (Phoenix dactylifera), although specifically associated with arid and semi-arid zones, has been widely distributed in the world from its region of origin thanks to exchanges. Ranging from South-West Asia and North Africa countries [19]. It has spread to regions including Southern Europe, the American continents and Australia [20].

In the North Africa region, dates were first introduced to Spain during the Moorish invasion. The Spaniards later transported date seeds to the Americas, initially to Peru and then to Mexico, which accounts for the presence of date palms in those regions. While historically, transporting young shoots over long distances has been effective, modern plantations of date palms are now being established or developed using carefully selected varieties in several countries. These nations include the United Arab Emirates, Oman, Kuwait, the United States, Mexico, India, Israel, Jordan, Namibia, Australia, and various countries in the Sahelian region of Africa.

f. Global Production and Cultivation

The date palm is extensively cultivated around the world, with the majority of production coming from Asia, particularly the Middle East, which accounts for 56.31% of the global production. Africa, specifically North Africa, contributes to 42.72% of the world's date production [21].

Data on the annual production of the main date-producing countries have been collected from member countries of the United Nations

Food and Agriculture Organization [22] and are mentioned in Table 2.

Table 2: Major date-producing countries in 2021 and 2022 **[22]**

Zone	Value (tons) 2021	Value (tons) 2022
Egypt	1713610	1733432.48
Saudi Arabia	1565830	1610731.00
Algeria	1188803	1247403.75
Islamic Republic of Iran	1168066.45	1030459.72
Pakistan	838251	732935.96
Iraq	750225	715293.32
Sudan	460090.68	442667.08
United Arab Emirates	382988.27	397328.94
Oman	374200	376980.00
Tunisia	345000	369000.00
Libya	180823.28	187870.08
Mainland China	161929.72	161120.80
Morocco	150301.00	137393.77

The majority of the main date producers saw an increase in their production between 2021 and 2022, which can be attributed to improvements in agricultural techniques, favorable climatic conditions, or government efforts to support date production. However, some countries, such as Iran, Pakistan, and Morocco, have experienced a decrease in production. These declines may be due to various factors, including unfavorable weather conditions.

II. Phoenicicultural in Morocco

II.1. Sector in a few figures

a. Area

In the past few years, Morocco has seen significant developments in date palm cultivation. As of 2020, the country has been implementing strategic plans to enhance agricultural productivity, including the cultivation of date palms. Recent data indicate that Morocco has approximately 4.250.200 productive date palms spread across several regions [23]. These efforts are part of broader initiatives to modernize agriculture and enhance the economic impact of key crops such as dates, which are considered a strategic product of Moroccan oases [24].

Phoenicicultural cultivation in Morocco was carried out on a staff of 59.000 hectares according to the Minister of Agriculture and Maritime Fishing [25]. And another source estimates the area of 50.000 ha for a total staff of nearly 5 million feet, which represents 48% of the global phoenicicultural heritage in 2019 [26]. And whose dominant region is that of Drâa-Tafilalet represented in Figure 3, which contains more than 70% of the national global staff of this production and a geographical distribution of 77% according to Minister of Agriculture [25].

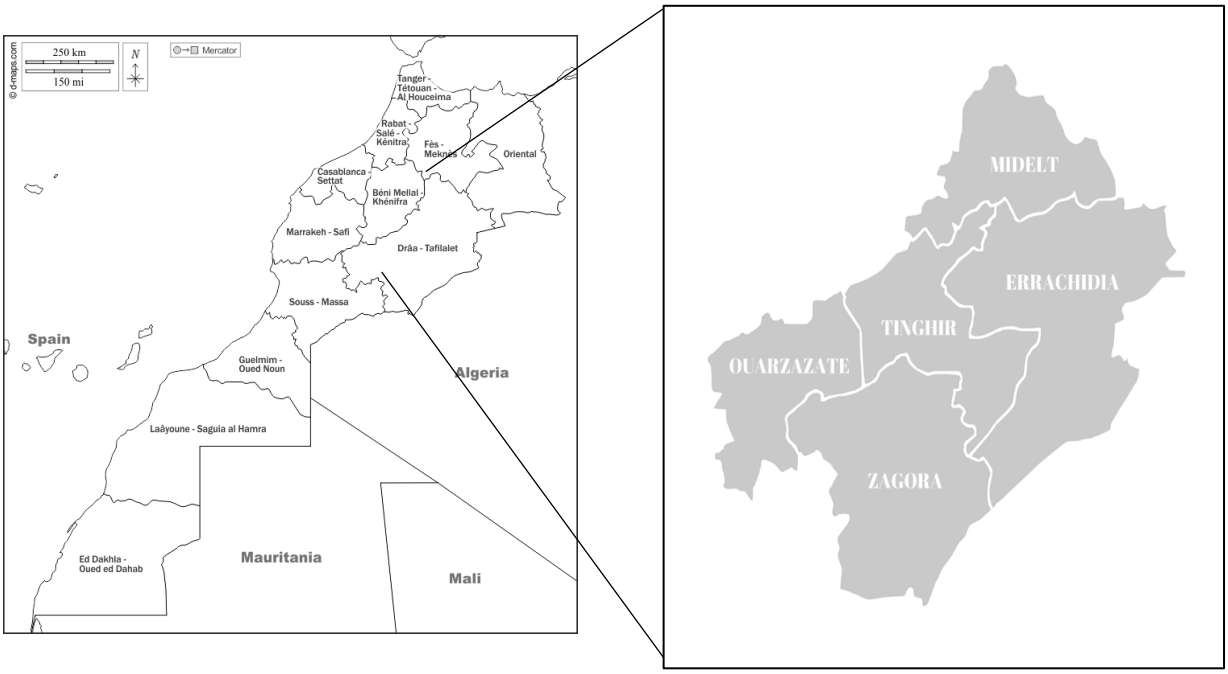

Figure 3: Drâa- Tafilalet Region [27]

The Draa Tafilalet region in southeastern Morocco spans an area of 88.836 km², representing 12.5% of the national territory and accounting for 46% of the country's oasis zones. This region experiences an arid, sub-desert climate with average annual temperature of 20°C [28]. These climatic conditions pose challenges makes it suitable of date cultivation.

b. Production

Date production in Morocco is part of the country's fruit crops, representing an essential pillar of the Saharan agricultural economy [29]. Production in Morocco records an annual average exceeding 112.000 tonnes [30]. The Ministry of Agriculture, Maritime Fishing, Rural Development, and Waters and Forests estimated a forecast production of 115.000 tonnes for the 2023-2024 agricultural campaign. Morocco was ranked the 12th largest date producer with a record of 143.000 tonnes for the 2019-2020 campaign [31]. Currently, according to FAO data, it occupies 13th place globally with a record of 137.393 tonnes in 2022.

c. Import

Morocco produces dates; however, it imports other varieties to meet its national demand. Dates are among the twelve agricultural products that the country imports the most. Table 3 presents the volumes imported for these twelve products during the year 2022 [32].

Table 3: Agricultural commodities most imported by Morocco in 2022 **[32]**

Product	Value	Unit
Wheat	6007646.51	tons
Corn	2097014.89	tons
Raw beet or cane sugar (centrifuged only)	1559937.79	tons
Barley	817291.02	tons
Soybean meal	583802	tons
Soybean oil	524604.56	tons

Sunflower meal	258554	tons
Draffs and brewery or distillery wastes	246125	tons
Wheat bran	225069.35	tons
Beet pulp	200128	tons
Feed plant products n.a.c.	124882	tons
Dates	109092.15	tons

Morocco, despite notable domestic production, positions itself as a major importer of dates according to FAO data, represented in Table 4, due to several socio-economic and agricultural factors that increase its dependence on imports to meet domestic demand. Morocco, which was considered as a major exporter of date palm products, has become an importer

Table 4: Major date-importing countries in 2022 **[33]**

Zone	Value	Unit
India	439477.21	tons
United Arab Emirates	155774.66	tons
Niger	114479.15	tons
Morocco	109092.15	tons

d. The Socio-Economic Role of Date Palm Cultivation in Morocco

The date production and cultivation chain serve as a key economic driver in the southern oasis regions of Morocco. The date palm sector has significant socio-economic importance, deeply intertwined with the cultural and economic fabric of the desert, it represents a sign of presence and endurance in the heart of the vast desert expanses, especially in places like Tafilalet and Drâa-Tafilalet. For many rural families, it's more than just a crop it’s a vital source of income, jobs and social connection in areas where opportunities can be scarce due to harsh environmental conditions. In fact, date palm farming can represent 20–60% of annual agricultural income [24-34]. For local households and the sector helps create over 3 million workdays each year [35], spanning from planting and harvesting to processing and selling dates. Overall, it supports the livelihoods of nearly one million people in these fragile ecosystems [36]. Beyond its economic contributions, it plays a pivotal role in maintaining traditional knowledge and reinforcing cultural identity, especially through practices such as palm weaving, cooperative farming, and local festivals. Dates, a staple consumed during Ramadan, aligning with the religious and significance [37]. The date palm sector is also highlighted through key events such as the Erfoud Date Festival, which has evolved from a local gathering into a prominent trade event, now attracts both national and international participants. Held annually in the third week of October, it combines commercial exhibitions with cultural performances, underscoring the sector’s broader socio-cultural impact, Organized by the Ministry of Agriculture, Maritime Fisheries, Rural Development, Water and Forests, and the Association of the International Date Exhibition in Morocco (ASIDMA). The exhibition showcased the sector's growth and global relevance, with approximately 230 exhibitors and over 90,000 visitors in attendance [38].

e. Date Market in Morocco

Despite the high quality of Moroccan dates, the sector faces significant challenges in competing in international markets, where consistency and quality are crucial for gaining consumer trust. A key barrier is the reliance on multiple intermediaries in the distribution chain, with 53% of dates being sold to collectors, which reduces profitability for producers [39]. Additionally, the seasonal demand for dates, largely driven by religious and festive events, creates market instability, leaving producers with unsold stock during off-peak periods despite large production volumes. High transportation costs, stemming from the geographical dispersion of farms, further limit market access and increase costs. The limited recognition of labelling Protected Geographical Indications (PGI) among consumers [40], combined with growing competition from cheaper imported dates, exacerbates these challenges and restricts the socio-economic potential of this crucial agricultural sector. At the heart of these issues is the fragmented structure of date production, dominated by small-scale producers, which hampers the sector's ability to scale effectively and develop efficiently. This fragmentation, coupled with the lack of standardized techniques (quality) and inadequate marketing efforts, prevents the sector from reaching its full potential on both domestic and international markets.

Several measures can be taken to improve market access, including the creation of cooperatives to consolidate production and increase bargaining power. Additionally, establishing structured marketing channels and distribution networks can bridge the gap between producers and consumers [41]. This includes, training, extension, promotion, transportation [42], using digital innovations typically take the form of smartphone applications (apps), mobile aggregators, online platforms and services, social media, and chat as well where farmers, traders and exporters can collaborate [43], leading to more efficient market access and better price realization. The reluctance of producers to adopt technological innovations and the reliance on traditional agricultural practices hinder productivity and quality improvements [44]. Branding and geographical labeling would also add value by appealing to the growing consumer interest in food origins. These interventions could address the challenges posed by market fragmentation and seasonal demand fluctuations, contributing to more stable and sustainable market dynamics for Moroccan dates.

f. Competitiveness of Morocco's Date Palm Sector

The competitiveness of Morocco's date palm sector plays a crucial role in the country's agricultural economy. Despite the high quality of Moroccan dates, the sector faces numerous challenges that hinder its ability to compete effectively in international markets. Tunisian dates, which dominate the middle to high end market in Morocco, are insufficient to meet this demand [45]. Tunisia has successfully addressed these challenges by implementing cooperative farming models that have improved productivity and market access. Moreover, the success of the Deglet Nour variety, marketed as a Protected Geographical Indication (PGI) [46], has allowed Tunisia to build a strong brand identity, increasing the value of its exports. Morocco could benefit from adopting similar strategies, promoting cooperative farming and branding initiatives, which would enhance the competitiveness of Moroccan dates both nationally and internationally. Algeria, another key player in the date industry, also markets its Deglet Nour variety under PGI, which has preserved traditional farming methods and strengthened Algeria's global presence [47]. However, despite its strong production capacity, Algeria faces challenges in fully capitalizing on its date

production potential, particularly in expanding exports [48]. Morocco could learn from Algeria's export strategies and leverage its rich cultural heritage to attract more international attention and investment. Furthermore, Egypt has made significant strides by investing in post-harvest technologies such as cold storage and advanced packaging, which have extended the shelf life of dates, improved product quality, and enabled Egypt to meet international standards [49], [50]. By investing in similar modern post-harvest technologies and providing training programs for local producers, Morocco could greatly enhance the quality of its dates, improving their competitiveness in the global market.

g. Growth Constraints Impacting Date Palm Cultivation in Morocco

Date palm cultivation in Morocco faces several significant growth constraints that impact both productivity and sustainability. One of the primary challenges is climate change, water scarcity, inefficient irrigation systems, Soil degradation etc…. Climate change plays a central role. Studies utilizing predictive models, such as CLIMEX, have projected that regions currently suitable for date palm cultivation may become less favorable under future climate scenarios. For instance, research indicates that many areas in North Africa, including Morocco, could experience a reduction in climatic suitability for date palms by 2100 due to increased cold and dry stresses [51]. Over the past three decades, Morocco has experienced increased frequency and intensity of droughts, changes in rainfall distribution, and rising temperatures**)**. The projections that indicate that average annual rainfall in Morocco will decrease by 6% by 2015, 13% by 2045, and 19% by 2075, worsening water scarcity issues [52].

Reduced rainfall is expected to exacerbate existing water management challenges in Morocco, where water scarcity and inefficient irrigation systems already pose significant threats to agricultural sustainability. Among the most affected is the traditional khettara irrigation system, which historically channeled water from underground aquifers to oasis fields. This system is now facing serious deterioration due to aging infrastructure and declining water flow, making sustainable water management vital for the continued resilience of date palm cultivation in Morocco's arid and semi-arid zones. However, the deterioration has led to challenges in water management, directly impacting date palm productivity. As a result, inefficient irrigation practices and inconsistent water supply have led to increased soil salinization and degradation, primarily caused by overuse and poor soil management practices such as extensive exploitation of date palm groves, driven by increasing human and livestock populations, has led to reduced soil fertility and compromised tree health, resulting in diminished productivity [53]. In Morocco's oases, soils are typically sandy with high pH levels and poor water and nutrient retention. Together, these growth constraints climate change, water scarcity, and soil degradation directly affect the productivity and sustainability of date palm cultivation in Morocco. Addressing these issues requires innovative solutions, including modern irrigation techniques, sustainable soil management practices, and climate change adaptation strategies to ensure the long-term viability of the sector.

h. Integrative Approaches to Sustainable and Productivity Oasis Agriculture in Morocco

To overcome the growing constraints affecting date palm cultivation in Morocco's

oases, sustainable solutions must bridge traditional practices with modern innovations. Rather than replacing ancestral methods, strategies focus on revitalizing systems like the khettara ancient underground canals through community-based rehabilitation efforts supported by public and non-governmental funding [54]. When combined with modern technologies such as solar-powered pumping, drip irrigation, and aquifer monitoring via remote sensing, these systems can significantly improve water use efficiency while preserving cultural heritage [55], [56]. Integrating these techniques into agricultural extension programs and promoting knowledge-sharing between older and younger farmers helps maintain productivity and safeguard traditional wisdom [57]. In parallel, soil fertility and resource efficiency are addressed through the application of organic fertilizers that enhance soil quality and support sustainable farming [58]. Converting date palm residues into biochar has also proven effective in improving soil structure and promoting plant growth, offering a sustainable solution aligned with circular agriculture principles [59]. Additionally, composting techniques using palm waste further increase organic matter in the soil, reinforcing long term productivity [60]. Traditional systems like the three-tiered cropping model, where date palms provide canopy cover for fruit trees and understory crops, help reduce soil evaporation, foster biodiversity, and optimize land use [61]. However, the erosion of such practices due to modernization and environmental stress has contributed to soil degradation, salinization, and yield decline, weakening both ecological balance and socio-economic resilience. In response, Morocco is advancing adaptive strategies, including the Adaptation to Climate Change in Oasis Zones (PACCZO) project, which combines indigenous practices with institutional support to promote sustainable agriculture. These efforts are reinforced by national policy frameworks like Génération Green 2020–2030, emphasizing climate smart agriculture and resource sustainability [62]. In addition, innovative approaches such as fog and dew harvesting [63]. Together, these integrative solutions reflect a comprehensive strategy for enhancing the long-term sustainability and productivity of Morocco’s oasis agriculture.

II.2. Development of Morocco's Date Palm Sector

To address the challenges posed by climate change, socio-economic issues, and the need for sustainable development, the Ministry of Agriculture, Fisheries, Rural Development, Water and Forests has launched a series of ambitious projects under the “Green Generation 2020-2030” strategy. Several hydro-agricultural and groundwater mobilization projects were launched, particularly in the irrigated perimeters of the Drâa Tafilalet region, aimed at improving the resilience of oasis agriculture to climate change. One of the major goals is to plant 5 million date palms by 2030 [42], with initiatives supporting young agricultural entrepreneurs and promoting the emergence of a new generation of middle-class farmers, offering financial assistance, technical training, and subsidies for modern farming tools to help adopt more efficient agricultural practices. This section outlines the timeline of key events and flagship projects aimed at strengthening Morocco's date palm sector, highlighting both the challenges and opportunities for sustainable development in this critical industry.

The outcomes of these initiatives have been measured through various monitoring and evaluation systems. These systems track improvements in irrigation efficiency, the adoption of modern farming techniques, and market access. One key metric of success has been the improvement of water management, where irrigation systems have been

upgraded, and water use has become more efficient, especially in water-scarce regions. For instance, hydro-agricultural projects have been implemented to optimize the use of groundwater in date-producing areas, reducing dependency on surface water and improving productivity. As a result, the government reports that yield improvements have been observed in areas where modern irrigation techniques have been applied [64]. Additionally, subsidies and technical training have contributed to a gradual increase in date palm productivity, particularly in regions with access to the plan's resources. However, despite these advances, challenges such as water scarcity, climate change, and market fragmentation still present significant barriers to fully achieving the plan's goals, particularly in remote, underdeveloped regions where date farming is highly dependent on traditional practices. The plan's outcomes show that while infrastructure improvements and productivity increases have been achieved, long-term sustainability still requires addressing these remaining challenges [65].

The study's findings suggest several crucial policy recommendations are necessary to overcome environmental constraints to ensure the long-term success of Morocco's date palm industry on both a local and international scale. At the local level, promoting water management through modern irrigation systems and rehabilitating traditional systems is vital. Nationally, investing in climate resilient date palm varieties, post-harvest technologies, and cooperative farming will help address the sector's challenges. At the international level, enhancing market access and branding Moroccan dates through geographical indications (PGI) could significantly improve global competitiveness.

II.3. Genetic Diversity of Date Palms in Morocco

The genetic diversity of Moroccan date palm varieties was analyzed using two complementary molecular markers, SSR (Simple Sequence Repeat) and DAMD (Differentiated Amplified Microsatellite Polymorphism). Both are well established tools in plant genetics and play a vital role in identifying traits like drought resistance, fruit quality, and productivity, all critical factors for selecting varieties suited to Morocco's challenging climate.

Previous studies, such as this one [66] have focused on microsatellite markers to assess genetic variation among Moroccan date palm cultivars. However, this study advances that work by combining SSR and DAMD markers to create more detailed genetic profiles. This dual marker approach offers deeper insights into the genetic relationships between varieties, helping to pinpoint those best adapted to environmental stresses. In addition, [67] highlights how genetic diversity directly influences adaptability to factors like drought, pests, and diseases. By leveraging this diversity, breeders and farmers can select date palm varieties that not only yield higher quality fruit but also perform better under arid and stressful conditions. This is particularly significant for Morocco's date palm industry, where maintaining sustainable and resilient production is essential for long-term economic stability. A rich genetic pool ensures that farmers have access to varieties capable of thriving despite climatic and ecological challenges.

II.4. Varieties

Morocco is renowned for its rich diversity of date palm varieties, each contributing significantly to the agricultural and economic importance of the sector. These varieties are well-regarded for their fruit quality, productivity, and drought resistance, making them suitable for cultivation across Morocco's diverse regions. The country's rich

diversity of approximately 453 date palm varieties, including khalts (hybrids from natural sowing), which constitute about 55% of the total population [68]. Among the most prized varieties, Medjool stands out for its large size, soft texture, and sweet flavor, making it a top export product with significant international demand. Similarly, Boufeggous is highly appreciated for its unique flavor and excellent adaptability to arid conditions, adding great value to both local and international markets. Other varieties such as Aziza, Jihel, and Bouskri contribute notably to Morocco's date production, with Aziza and Jihel playing essential roles in local markets and traditional consumption, while Bouskri is valued for its sweetness and resilience. However, the extent to which these varieties are studied depends on their market potential and adaptability to the environment.

The Table 5 represents the 18 dominant date palm varieties [69], declared by the International Date Salon, including key characteristics such as consistency, color, and size, which directly influence the fruit quality. Consistency refers to the texture of the fruit whether it is soft, semi-dry, or dry impacting its appeal and consumer preference. Color is an important visual factor, with various shades of light brown or dark brown often being desirable depending on the variety. Size also plays a crucial role, as larger fruits are more highly valued, especially in international markets. In addition, Productivity is related to the yield produced by the tree, which is a vital aspect for farmers and commercial growers. Higher productivity translates to a greater number of dates per palm, making the variety more economically viable. The ability of date palms to adapt to environmental conditions, particularly drought resistance, also significantly contributes to their success and overall yield, especially in arid climates.

Table 5: Most dominant date varieties in Morocco

Image						
Name	**Medjool**	**Aguelid**	**Ahardan**	**Azigzao**	**Aziza**	**Boucerdoun**
Consistency	Semi-soft	Semi-soft	Semi-soft	Semi-dry	Semi-soft	Dry
Color	Dark brown	Light brown	Light brown	Light brown	Light brown	Light brown
Size	Large sized, tender, sweet flesh	Medium sized	Medium sized	Medium to large sized	Small to medium sized	Medium sized
Drought Resistance	Moderat, requires regular irrigation to maintain optimal production	High, well suited to dry environments	-	Well adapted to dry conditions, requiring less irrigation	Good, tolerates to dry conditions	High, tolerates dry conditions well
Productivity	High	Moderate	Low	Good	Moderate	Moderate

Appreciation	Excellent high commercial value highly demanded on the international market	Relatively good valued in trade for its earliness	Medium trade for its earliness	Low appreciated locally	Appreciated in its cradle	Medium trade, less known
Image						
Name	**Bouijjou**	**Bouittob**	**Bourar**	**Boslikheene**	**Bouskri**	**Bouzeggar**
Consistency	Dry	Dry	Semi-dry	Semi-dry	Dry	Semi-soft
Color	Light brown	Light brown	Dark brown	Light brown	Dark brown	Black
Size	Medium to large sized	Small sized	Medium sized		Long sized	Small sized
Drought Resistance	Resilience to dry conditions	-	Resistance to dry conditions are limited	Highly resistant to drought, well-suited to arid conditions with minimal irrigation requirements	Moderate, benefits from adequate irrigation	-
Productivity	Moderate	Moderate	Moderate	Moderate	Good	
Appreciation	Medium rade	Medium highly appreciated in its cradle in Bani	Good highly appreciated in the Drâa Valley	Low but commercially appreciated in the Tafialet region	Medium highly appreciated for its sugar content and great ability to be preserved at the national level	Low appreciated in the Drâa Valley
Image						

Name	Otoukdim	Iklan	Racelahmer	Jihel	Boufeggous	Bousthammi
Consistency	Dry	Semi-soft	Dry	Semi-soft	Soft	Soft
Color	Light brown	Black	Light brown	Light brown	Dark brown	Black
Size	Small sized	Small sized	-	Medium sized	Medium to large sized	Small sized
Drought Resistance	-	-	-	Moderate, benefits from regular irrigation	High, well adapted to dry climates and requires less irrigation	Moderate , suggesting a degree of drought tolerance
Productivity	Moderate	High	Moderate	High	Good	High
Appreciation	Medium cultivated at a relatively medium altitude in the mountains	Low trade	Medium trade, less known	Low trade	Good trade	Low but appreciated for self-consumption excellent taste

Varieties that demonstrate a balance of excellent fruit quality and high productivity are more extensively studied and valued for both local consumption and export, such as Medjool and Boufeggous, have been extensively researched, particularly due to their large fruit sizes, high productivity, and strong market demand. These varieties are well-known for their exceptional commercial value, which drives significant research efforts focused on improving their productivity and drought resistance. Similarly, varieties like Aziza and Aguelid, appreciated for their early fruiting and adaptability to dry conditions, have also garnered attention in trade and research. The larger fruit sizes and the higher market demand for these varieties lead to more extensive studies aimed at enhancing their agricultural performance. On the other hand, varieties such as Ahardan, Bouittob, Bouzeggar, Otoukdim, Iklan, and Racelahmer, which are less commercially recognized or have lower trade value, receive considerably less research attention. These varieties typically feature smaller fruit sizes, lower productivity, and limited market appeal, making them less prioritized in breeding programs. Their relatively lower commercial demand and trade value result in less focus on improving their resilience and productivity. Ultimately, the level of study and recognition these varieties receive is directly linked to their potential for market success and their ability to thrive in arid climates, where adaptability is crucial for sustained agricultural production. That's why the choice of varieties, along with the investments play a significant role in shaping the economic success and agricultural efficiency of the date palm industry in Morocco that support long-term growth and profitability, ensuring a steady income for farmers in the country.

At the final stage of their maturation, dates undergo significant evaporation of their water content, resulting in an increased concentration of sugars. This transformation directly influences several essential attributes

of the fruit. Indeed, at this critical stage, dates fully develop their distinctive taste profile as well as their characteristic color and texture. And on this basis, the classification of dates is done.

a. Classification

The analysis of the data in Table 5 reveals that the varieties of dates in Morocco are divided into four main categories according to their consistency: soft, semi-dry, dry, and semi-soft. These classifications rely on a specific indicator known as the quality or hardness index (r), calculated by dividing the total sugar content (in g total sugars /100 g dry matter) by the water content (in g of water/100 g dry matter) [70]. And whose value ranges from 15 to 63.

Internationally, three consistency categories have been established to classify dates: soft for an r index below 2, semi-soft for an r index between 2 and 35, and dry for an r index above 35 [71]. This same classification is adopted by The UNECE DDP-08 standard in 2010, which establishes detailed criteria for the marketing of dates, including quality requirements at the time of export after packaging and conditioning at the international level [72]. On a national level, the Ministry of Agriculture and Maritime Fishing of Morocco introduced in 2012 a standard called General Standards for the Marketing of Moroccan Dates (NGCDM). This standard incorporates the principles of the UNECE DDP-08 standard while integrating specificities related to local varieties. Figure 4 represents the distribution of consistency of the 18 Moroccan varieties (Table 5).

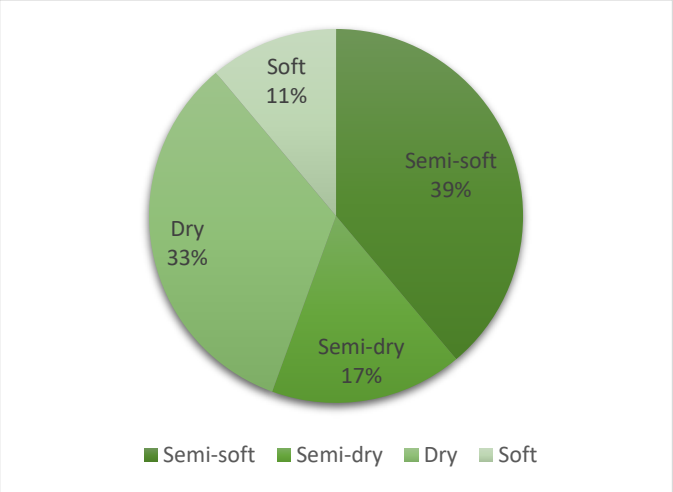

Figure 4: Approximate distribution of dominant date types by consistency in Morocco

Semi-soft dates represent the largest part of the diagram with 39%. This suggests that almost half of the varieties of dates available on the market have a texture neither too hard nor too soft. Dry dates follow with 33%, indicating that a third of the varieties are of firm consistency and contain less moisture. They may be ideal for long-term conservation. Semi-dry dates, which represent a non-standardized class like the others, account for 17%, showing that a notable portion of the production offers an intermediate texture. And finally, soft dates represent 11% of the total, indicating that this variety is the least common. Soft dates are often sought for their succulent texture and are generally consumed fresh. This distribution could reflect the climatic and cultivation conditions favoring the

production of certain consistencies of dates more than others. The diversity of textures also shows a certain variety in the offering of dates, allowing to satisfy a range of tastes and technological uses. The color of dates can vary considerably, reflecting the diversity of cultivated varieties. When they reach maturity and are ready for harvest or consumption, dates can present a palette of colors ranging from light brown to dark brown and sometimes almost black.

Figure 5 allows a quick and easy visualization of the distribution of different color categories within the entire data set (Table 5). It is particularly useful for comparing proportions and for making quick deductions about the relative frequency of each category.

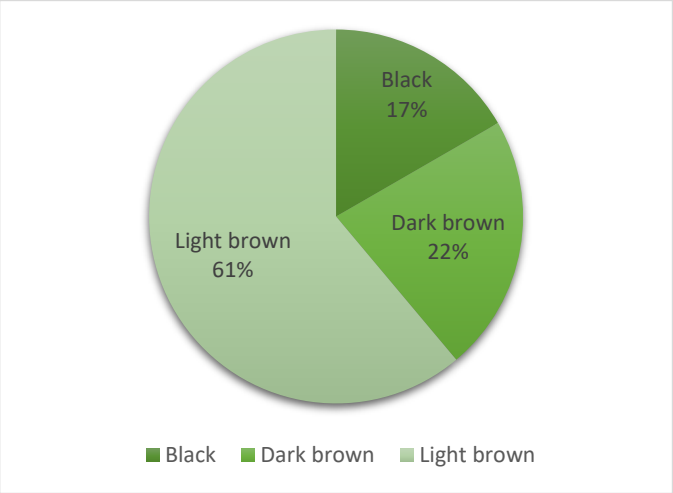

Figure 5: Approximate distribution of dominant date types by color in Morocco

Light brown represents the majority of the ensemble with 61% of the data classified in this category. This indicates that the light brown color is the most common or most prevalent in the observed group. The second most frequent color is dark brown, accounting for 22% of the data. This means that just over a fifth of the varieties exhibit a dark brown color. And finally, the black color represents 17% of the ensemble, making it the least widespread of the three categories present.

One can establish a relationship between consistency and color, as shown in Figure 6, which represents the distribution of colors according to consistency. Dry dates exhibit a bright color and a hard flesh while soft dates take on a dark black color and a soft flesh. Then the colors could be a matter of quality analysis where the colors represent grades or specific characteristics associated with the texture or consistency of the product. Black dates, potentially being softer or more mature, might have less of this component than light brown dates, which could be less mature or drier. These suggestions are hypothetical based on the distribution performed.

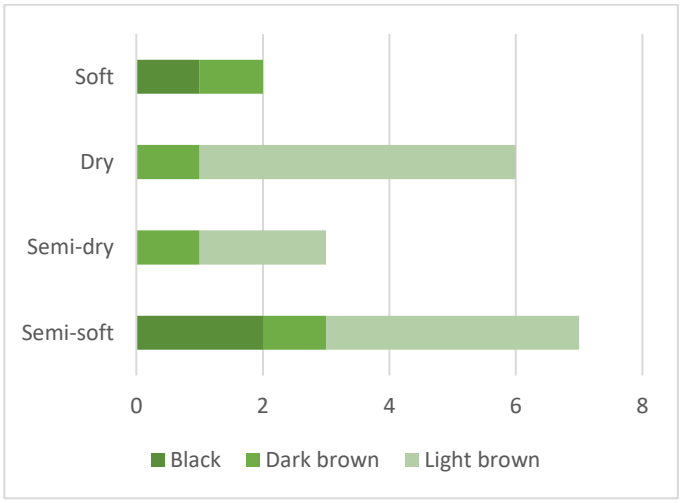

Figure 6: Approximate distribution of the distribution of dominant dates by consistency according to color in Morocco

Soft dates show a balanced distribution between the two colors (Black and Dark brown). This may indicate that soft dates tend to have a darker color in general, dry dates seem to be predominantly light brown with lesser amounts in dark brown, while the semi-dry category light brown dominates with lesser presences of dark brown. This may suggest that semi-dry dates retain a lighter hue similar to dried dates and the semi-soft dates show a more balanced distribution between the three colors compared to the others with a slight predominance of light brown followed closely by black and finally by dark brown. This trend could indicate that soft dates have a propensity to become darker in color and lighter for dried varieties. Here is a summarized representation of Table 6, which reviews the distribution of color and consistency across the dominant date varieties, with semi-dry and dry types showing a noticeable difference in the presence of lighter colors compared to softer varieties.

Table 6: Approximate review of the distribution of color and consistency of the dominant date varieties in Morocco

	Black	**Dark brown**	**Light brown**
Soft and Semi-soft	Presence	Moderate presence	Moderate presence
Semi-dry and Dry	Absence	Moderate presence	More present and perceptible

Color variations may be due to biological causes; each variety of date has its genetic characteristics that include consistency and color at maturity. The coloration of dates can be influenced by the ratio between sugar and water (r index) inside the fruit, especially during ripening, and with the loss of water, the sugars present in dates are concentrated, giving a sweet appearance for dried dates. The presence of a high-water rate can cause the Maillard reaction and oxidation, which can cause a darker coloration. Without chemical analyses and studies of the composition of dates, it is difficult to provide a definitive explanation of color variations in relation to consistency.

To better understand the dispersion of the 453 date palm varieties, and not just the 18 studied, future research should actively involve stakeholders, particularly local farmers and cooperatives. Their engagement is crucial to ensure that the findings reflect the practical realities and challenges faced by producers. Farmers' feedback on agricultural practices, cooperatives' insights into market challenges, and policy input can help develop more targeted and actionable solutions. Incorporating local perspectives will be essential for designing sustainable policies and technological innovations that enhance productivity and improve market access for Moroccan date farmers. This inclusive approach will ensure that recommendations address the sector's needs effectively, supporting both local livelihoods and the broader economic growth of the date palm industry.

3-CONCLUSION

Date palm cultivation, especially in Morocco, stands for a kind of agricultural tradition. Among the main ones, some cultivars, which stand out due to quality, are Medjool, Boufgouss, and Jihel. In spite of this, it is nevertheless an economic sector plagued by persistent problems that cumulatively stymie it: climatic constraints, water scarcity, and market fragmentation-all deterrents to fully realizing the economic potential.

Varieties range from soft to semi-dry to dry dates, and diversity depends not only on adaptation to specific environmental conditions but also on different uses in local and international markets. In this respect, the future challenges for this sector will be higher competition from imported varieties and the need for foreign markets to realize the true quality of Moroccan dates.

Despite this leading position in global production, Morocco should focus on several key areas to improve productivity, sustainability, and market access. Firstly, optimizing water management systems through innovative technologies such as solar powered irrigation and rehabilitating traditional systems like khettara could enhance water use efficiency in Morocco's arid regions. Additionally, research on climate-resilient date palm varieties is essential to mitigate the impacts of climate change and ensure long-term production sustainability. Another important area for future research is improving post-harvest handling and storage technologies, including cold storage and modified atmosphere packaging, to reduce spoilage and extend shelf life, thereby increasing marketability. Finally, enhancing market access by establishing more efficient supply chains, promoting the formation of cooperatives, and exploring value-added products could provide greater economic opportunities for farmers and improve Morocco's competitiveness in the global market. These research directions will contribute to a more sustainable, resilient, and profitable date palm industry in Morocco. These priorities reflect the most pressing issues for improving the sustainability, productivity, and economic viability of the date palm sector in Morocco, aligning with global challenges such as climate change and the need for innovative agricultural practices. After all, the future of the Moroccan date palm sector rests in how it will be able to balance tradition with modernization so that its contribution, both economic and cultural, is assured to be perpetual while capitalizing on the wide variety of date palms that the country has.

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Scientific Research

Antioxidative effect of Maillard reaction products of spermine–sugar system on partially purified plum polyphenol oxidase

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ABSTRACT

One economic issue that arises from the procedures used to produce dyes from naturally occurring phenolic chemicals that enzymes have oxidized is called "enzymatic browning". Polyphenol oxidase's existence in most fruits and vegetables is in charge of this problem. Adding antioxidants is one of the most crucial strategies for halting this process. We used spectrophotometry to examine the antioxidant effects of Maillard reaction products on this enzyme. Five hours of heating at 100 °C was used to study the properties and antioxidant activity of model systems including spermine and monosaccharides (glucose, fructose, and ribose). Vitamin C was used as a control to compare the DPPH radical scavenging activity of the MRP spermine-sugar model systems. Intermediate products at A_{294} and a browning intensity at A_{420} nm were increased with heating time. This increase was associated with a decrease in reduced sugar and free amino group contents. Polyphenol oxidase from Mirabelle plum (*Prunus domestica subsp. syriaca*) was fractionated by ammonium sulfate precipitation, dialysis, and ion exchange chromatography. A single peak was obtained with specific activity 11292.6 U/mg protein. The MRPs exhibited inhibitory effects on purified Mirabelle plum PPO from (42.6- 70.2%) compared with vitamin C which was 57.9%. Furthermore, the Lineweaver-Burk plots discovered the inhibition modes noncompetitive by the strongest system, spermine-glucose.

1- Introduction

Commonly, plum fruit (*Prunus domestica*) is a stone fruit that belongs to the family Rosaceae of the genus *Prunus.* They have a variety of sizes, colors, tastes, and nutritional value[1, 2]. Due to their essential nutritional value, plums are a valuable food in our diet. Plumps are a rich source of the health-promoting compounds carotenoids, anthocyanins, and phenolic acids. Plums also have a high quantity of pectin, organic acids, sugars, tannins, aromatic compounds, and enzymes, making them low in calories and comparatively rich in nutritional value[3, 4]. Plums are available in different cultivars: Damson, Prune, Pershore, Greengage, Myrobalan, Victoria, and Mirabelle[5]. The Mirabelle plum (*Prunus domestica subsp. syriaca*) is thought to have been domesticated from a wild Anatolian fruit. It can be recognized by its oval shape, flesh with a smooth texture, and most importantly, its flecked yellow color (Figure 1). It is renowned for being flavorful and sweet. Although this fruit is usually used to make juice, it is also frequently fermented to make wine or distilled to create plum brandy. They are also widely utilized in the production of ja[6-8].

Figure 1: Mirabelle plum Fruit

The principal result of the Maillard reaction (MR), commonly referred to as "nonenzymatic browning," is Amadori chemicals, which are produced when carbohydrates react with protein N-terminal amino groups[9]. The reaction depends on temperature, pH, and reactant's kind[10]. This is typically documented that the Maillard reaction products (MRPs) affect the antioxidant capacity of foods[11, 12]. Antioxidant properties of MRPs are assumed to differ broadly and are powerfully influenced by the circumstances of the reaction and the procedures used for evaluation[13, 14]. They are frequently created in food products during thermal treatment or storage and utilized as inhibitors directly[15].

In numerous consumable plant products, polyphenol oxidase (PPO) catalyzes the oxidation of phenolic compounds in the attendance of oxygen throughout post-harvest handling and processing. Enzymatic browning products are dark-colored pigments that are produced by the fast

condensing and polymerization of O-quinones, the principal oxidation products that are produced. Due to the degradation of the product's nutritional, functional, and organoleptic qualities, brown pigmentation is typically regarded as harmful to food quality[16]. Several studies have indicated that polyphenol oxidase is inhibited by MRPs, for example, MRP inhibited PPO isolated from potatoes and apples[17] mushrooms, and eggplant[18]. However, the inhibitory effects varied depending on the enzyme origin and nature of the reactant type of MRP[19]. The study aimed to examine the ability of MRPs to inhibit partially purified PPO activity from mirabelle plum and prevent enzymatic browning.

Experimental part

Materials:

Spermine, gallic acid, and vitamin C were obtained from Sigma-Aldrich Co. Catechol (o-dihydroxybenzene) and polyethylene glycol are purchased from BDH Co. Glucose, Fructose, and Ribose from Fluka Co.

2-Methods

Preparation of MRPs:

Spermine-sugar model systems were produced according to [20]. Each system was created by mixing 0.05 M ribose, fructose, and glucose individually with 1% spermine. After that, each mixture was transferred to screw-sealed tubes, tightly capped, and heated in a water bath at 100 C. After heating for 0, 1, 2, 3, 4, and 5 hours, the samples were taken. The heated samples were properly immersed in ice water to cool. Each MRP sample was kept at 4°C until it was analyzed and used.

Antioxidant activity measurement using the DPPH radical scavenging technique

Three milliliters of DPPH solution (5 mg/10 mL) were combined with two milliliters of MRP solution, and 25 milliliters of methanol were added. The mix was vigorously shaken before being left in the dark for 45 minutes. At 515 nm, the decrease in absorbance was monitored. The results were expressed as milligram gallic acid equivalents per gram of MRPs[21]. Vitamin C was used as a control antioxidant.

Analyses

Colorless intermediate and browning intensity measurement:

Spectrophotometrically, a colorless intermediate was detected at A_{294} nm and the intensity of the brown color was estimated by following A_{420} nm[22].

Reducing sugar measurement:

To determine the reducing sugar concentration, 1 ml of the samples and 1 ml of dinitrosalicylic acid were added to a test tube. The mixture was heated for five minutes at 100°C. Then cool at room temperature and add 8 ml of distilled water. At 540 nm, absorbance has been determined. Using glucose (10–100 mg/ml), a standard curve was created[23].

Free amino group measurement:

To determine free amino acids, 0.2 ml of ninhydrin reagent is added to 1 ml of sample. The mixture was heated at 100 °C for 10 minutes and then cooled at room temperature. Absorbance was measured at 570 nm. The standard curve was done using lysine (5-50 mg/ml)[24].

Reducing power measurement:

Within a glass tube, 1 ml $K_4[Fe(CN)_6]$ (1%) was added together with 1 mL of the sample and 0.5 mL sodium phosphate buffer (0.2 M,

pH 6.6). Following a 20-minute incubation period at 50 °C, 1 milliliter of 10% TCA had been added to the mixture. The mixture was subsequently centrifuged for 10 minutes at 805 xg. Once 1 ml of supernatant was obtained, 200 µL of 0.1% FeCl3 and 1 ml of D.W. were added to it. The absorbance was estimated at 700 nm. Absorbance increase served as the indicator of reducing power[25].

Determination of protein content:

Total protein conc. was estimated, using BSA as a standard at 650 nm[26].

Enzyme assay:

Using catechol as a substrate, PPO activity was measured spectrophotometrically. One milliliter of 0.01 M catechol, 0.9 ml buffer (pH 7.2), and 0.1 ml of purified enzyme were mixed. The absorbance was determined at 420 nm with a 10-second interval for 5 minutes after the enzyme was added. One unit of PPO was used as the enzyme quantity, resulting in a 0.001 unit increase in absorbance per minute[27].

Enzyme extraction:

Fresh mirabelle plums purchased from a local market were used to extract PPO. It was cleaned and peeled. In the presence of 2% PEG, 40 grams was homogenized with 100 ml of phosphate buffer (0.1M, pH 7.2) using a blender for 2 min. Enzyme-containing juice was centrifuged for 20 minutes at 10000 xg after being filtered. The resulting extract was subjected to 80% of $(NH_4)_2SO_4$ precipitation. Then the pellet was dissolved in a small volume of the same buffer[28].

Dialysis:

Dialysis was carried out by placing an enzyme-containing solution in a dialysis tube against phosphate buffer (0.1 M, pH 7.2). The solution was stirred at 4˚C with six-time changes of buffer.

Ion exchange chromatography:

The dialyzed solution was placed on a column containing a CM-cellulose cation exchanger. Phosphate buffer was used to elute a protein at a flow rate of 0.7ml/min (each fraction 3 ml). The protein was followed using absorbance at 280nm. The fractions containing PPO activity were detected, pooled, and lyophilized then used in the following experiments[29].

Determination of PPO molecular mass

The PPO molecular mass was estimated by using slap-electrophoresis which was applied to the concentrated protein extract obtained from the former separation step. Sodium dodecyl sulfate gel electrophoresis was used. The protein bands were visible when applying the Coomassie Blue staining method[24]. A standard curve was adopted to evaluate the molecular mass of PPO.

Enzyme inhibition:

The inhibitory effect of PPO was studied by adding 0.2 ml MRPs to the reaction mixture of the PPO assay that measured as described above from a range of 0-5 hours.

Inhibition mode of PPO:

The inhibition of purified PPO was studied by using the spermine-glucose system as an inhibitor. The activity was followed at 420 nm using (0.25-3mM) of substrate after

incubation of 0.2 ml enzyme with 0.1 ml of inhibitor for 30 minutes[30].

3-Results and Discussion

MRPs with varying browning and color appearances are anticipated to form in each system, aiding in comprehending their free radical scavenging properties. The system is permitted to react with DPPH to evaluate the produced MRPs' ability to scavenge free radicals. One type of chromogenic radical that can interact with antioxidants directly is DPPH. DPPH, a stabilized radical, is frequently employed to assess primary antioxidant activity[31, 32]. Table 1 demonstrates the scavenging influence of MRPs on DPPH radicals. All MRPs were discovered to have antioxidant properties. Among them, the system glucose- spermine, showed higher scavenging effects (0.49 mg. gm^{-1}) besides vitamin C.

Table 1: The scavenging effect of MRP (mg gm^{-1}) to quench DPPH radicals

MRP product	(mg.gm^{-1})
Glucose- spermine	0.49
Fructose- spermine	0.45
Ribose- spermine	0.34
Vitamin C	0.61

MRPs have excellent antioxidant properties in many food products due to metal ion chelation, radical chain and H_2O_2 breakdown, and reactive oxygen species scavenging[33]. MRPs produced by chitosan-sugar systems showed increased DPPH radical scavenging activity. The most effective ones were those derived from chitosan-glucose[34]. The MR generated by an amino-sugar system was linked to the generation of compounds with high antioxidant activity. The MRPs produced from glucose and amino acids (lysine, histidine, and methionine) revealed significant scavenging properties in addition to vitamin C and catechin[18].

On the other hand, spermine is one of the polyamine compounds, which is characterized by its small molecular weight and alkali behavior because it contains four amine groups[35]. It is synthesized from L-ornithine or by the decarboxylation of amino acids. This compound has a scavenging ability against various ROS. Its antioxidant properties depend mainly on chelating minerals[36-38].

Variations in UV (A_{294}) and browning intensity (A_{420})

According to Ajandouz et al., absorbance at A294 nm and A420 nm indicates the presence of colorless intermediate molecules and ending browning compounds[39]. A slow variation in A_{294} nm of entirely MRPs derived from spermine-sugar model systems was initiated with an increasing heating interval of up to 2 hr. Subsequently, a severe increase, specifically with glucose was observed as the heating period enhanced up to 5 hr. as illustrated in Figure 1.

This rise coincided with the findings of Phistut and Jiraporn (2013), who reported that MRPs produced from glucose and chitosan exhibited the greatest increase in A_{294} nm, followed by those derived from lactose, fructose, and maltose, respectively[34]. A rise of A_{294} nm was observed by Lerici et al. (1990) when the glucose-glycine combination was heated. According to Benjakul et al. (2005), the heating period of the porcine plasma protein

reducing sugar model system was found to cause a constant increase in A_{294} nm[40].

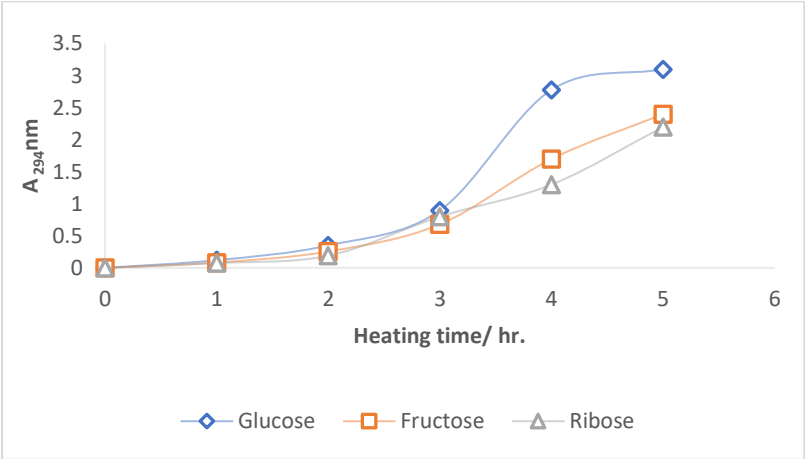

Figure 2: Variations in A_{294}nm with different heating periods

The easiest observable consequence of the MR is brown color development (A_{420} nm), which can be assumed visually. Its intensity indicates the advanced state of MR and frequently serves the extent to which MR is present in meals. As proved by the increase in browning intensity through heating, some intermediate products appear to be converted to the final brown compounds, while others appear to be generated by both reactants. Browning intensity at A_{420} of all MRPs gradually increased to 5 hr. of heating. The highest absorption was clear for the Spermine-glucose model system compared to other sugars (Figure 3).

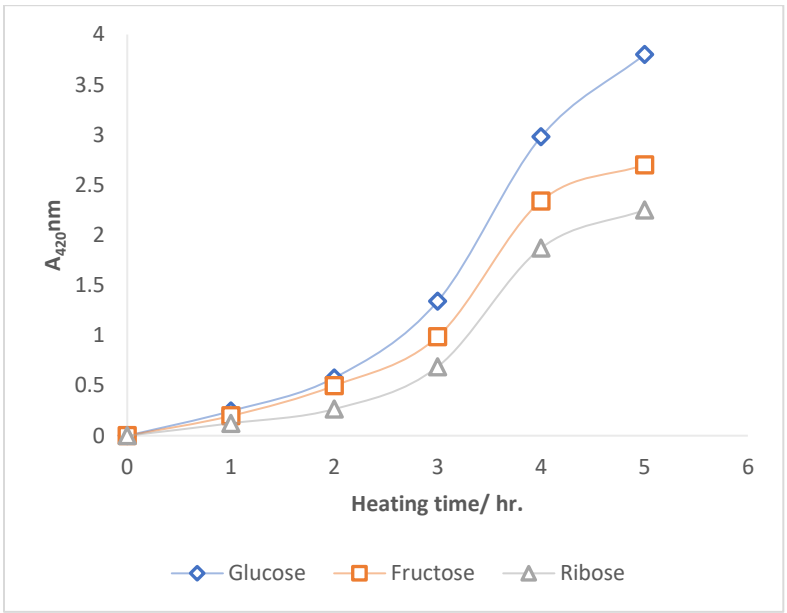

Figure 3: Variations in A_{420}nm with different heating periods

For MRPs derived from Chitosan-Glucose, fructose, and lactose, A_{420} nm increased distinctly after heating[34]. Conversely, some researchers discovered that fructose contributes more to browning than glucose[41]. This indicated that the formation of brown pigments was directly proportional to the creation of intermediate products.

Variations in free amino group content

All MRPs developed from spermine-sugar systems have their reactive amino groups modified, as seen in Figure 4. The amount of amino groups in all MRP samples decreased with increasing heating time, and it increased when spermine and glucose reacted. According to this discovery, spermine's amino group is covalently linked with sugar to generate a more glycated product, especially as the heating time increases.

Phisut and Jiraporn (2013) revealed that the free amino groups' chitosan declined little by little as a result of the MR with monosaccharides glucose, lactose, and fructose respectively [34]. During continued heating of a casein–sugar system, lysine decline was demonstrated as mentioned by [42].

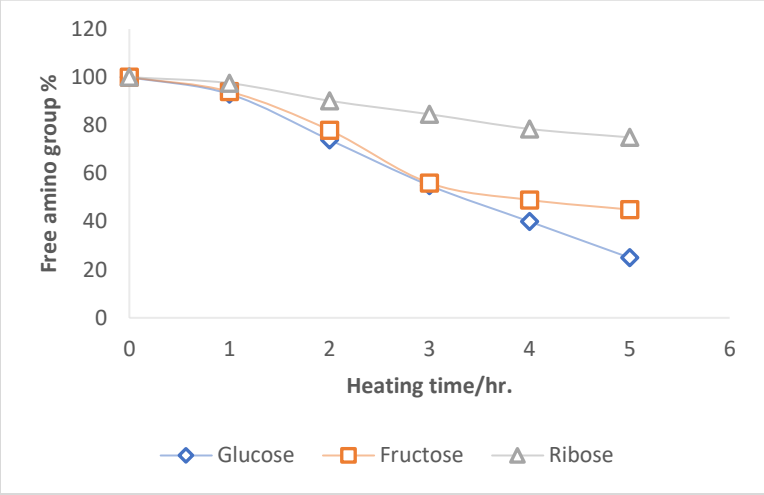

Figure 4: Variations in free amino group content with different heating periods

Variations in reducing sugar content

Figure 5 illustrates a progressive decline in the reduced sugar content of all MRPs made from spermine-sugar solutions. The MRPs obtained from the spermine-glucose model system demonstrated a dramatic drop in decreased sugar content. This outcome demonstrated the use of glucose as a reactant in MR, demonstrating a quicker rate of reaction than other sugars.

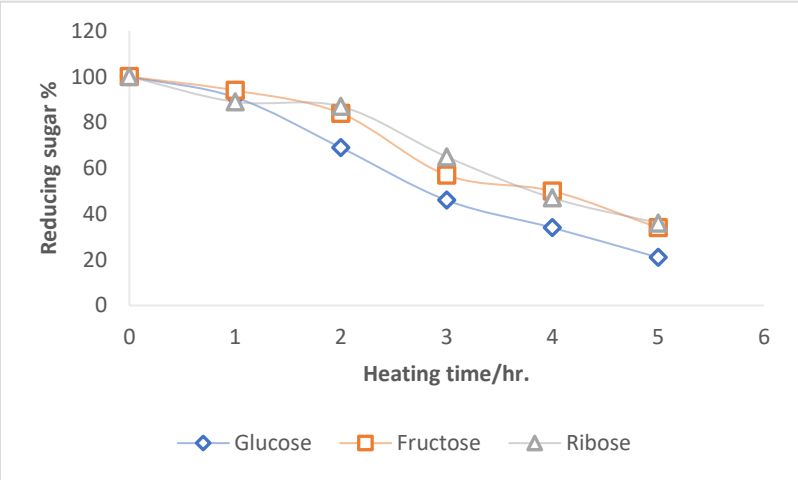

Figure 5: Variations in reducing sugar with different heating periods

When heat-induced MR is applied to chitosan-glucose, casein-glucose, and fructose-lysine, the reduced sugar content decreases[20, 34]. According to the findings, the decrease in the free amino group and reduced sugar content corresponded to an increase in browning intensity A_{420} and A_{294}. This revealed that prolonged heating catalyzed the interface of amino groups in spermine with reducing sugar through glycation development. As a result, glucose was more reactive than other sugars in the formation of glycated spermine, as evidenced by the greatest decrease in free amino groups with a concurrent increase in browning. The reaction rate of glycation may depend on the acyclic formula and the carbonyl groups' electrophilicity[42].

Enzyme Purification:

When sedimentation with 80% $(NH_4)_2SO_4$, the sp. activity of the enzyme has given rise to 234.79 U/mg protein with a purification fold of 0.54 related to the crude. These results indicate that Mirabelle plum PPO was magnificently purified to 26.31 fold. As can be seen, the protein content diminished afterward-successive purification steps but the specific activity value increased from 343.28 to 11292.6 U/mg protein (Table 2). The elution profile of PPO by column chromatography exposed a lone peak (Figure 6).

Table (2): Purification steps of PPO from Mirabelle plum (*Prunus domestica subsp. syriaca*)

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Sp. Ac. (U/mg protein)	Yield %	Purification Fold
Crude extract	94.4	27.74	11903.64	343.28	100	1
Ammonium sulfate	16.8	18.09	5311.09	234.79	35.69	0.54
Dialysis	12	6.41	3371.37	420.73	22.65	0.97

CM-Cellulose	34.5	0.192	2721.52	11292.6	18.28	26.31

* A unit is defined as the amount of enzyme that oxidizes one micromole of substrate per minute at 25 °C.

PPO purification from Sapodilla plums (*Achras sapota*) illustrates increased activity after precipitating by 80% of ammonium sulfate with a specific activity of 762.7 UE. One peak of the enzyme was separated by using sephacrylS-200 chromatography molecular mass was approximately 66 kDa[43]. PPO was purified from Damson plum using ammonium sulfate precipitation, dialysis, and finally, affinity purification by Sepharose 4B-L-Tyrosine-p-aminobenzoic acid with purification-fold 93.88[44]. The enzyme was extracted from Stanley plums (*Prunus domestica L.*) with 36-fold via $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and Sephadex G-100[45]. On the other hand, this enzyme was purified from different sources including, Hemsin Apple[46], peaches[47], apricot[48] by using various techniques.

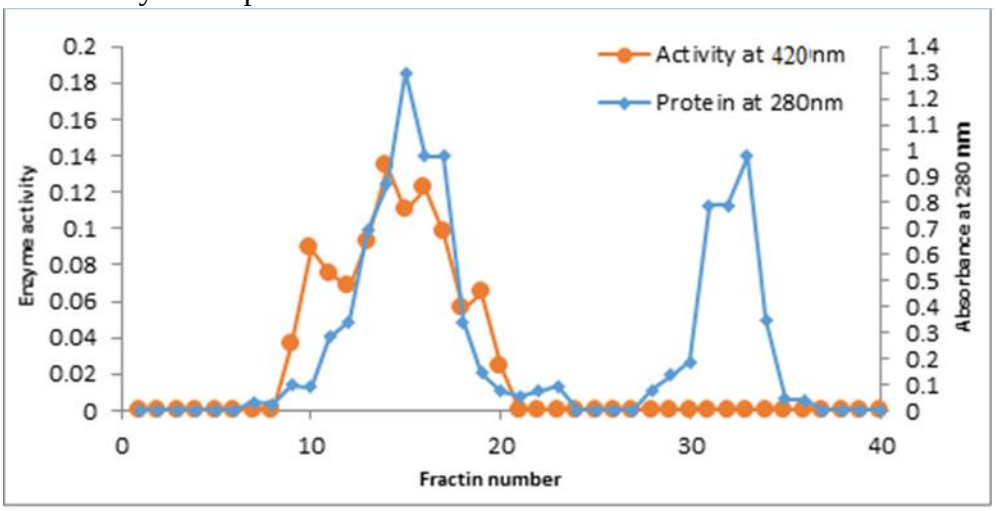

Figure 6: Elution profile of Mirabelle plum PPO by CM-cellulose column (2×25 cm)

Molecular weight:

A single clear band of enzyme was revealed after applying the SDS-PAGE electrophoresis technique. The protein band migrated, close to a molecular mass of pepsin (24 kDa). The molecular mass of purified Mirabelle plum PPO was approximately calculated to be 22 kDa (Figure 7). Also, by using SDS-PAGE gel electrophoresis, Das et al., (1997) found a single polypeptide band with 25 kDa of pineapple's PPO. A single protein band of purified PPO from sapodilla plum and damson plum was estimated to be about 29 and 50 kDa respectively [43]. On the other hand, different molecular masses of PPO have been described before such as in banana 41 kDa [49], and atemoya fruit 82 kDa [50].

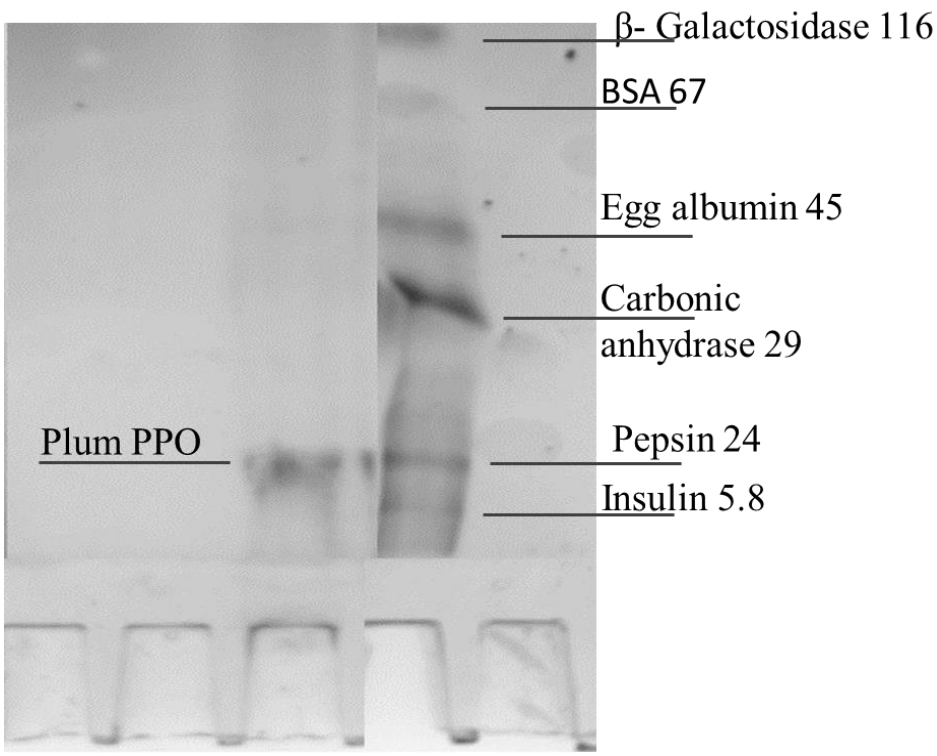

Figure 7: SDS-PAGE electrophoresis of PPO purified from Mirabelle plum with standard proteins.

PPO inhibition:

MRPs, such as Amadori rearrangement products, have the potential to scavenge oxygen radicals and chelate metals. Another distinguishing feature is the PPO inhibitory effect, which may help to prevent enzymatic browning in vegetables and fruits[51]. It was observed that MRPs inhibited the purified PPO from plum. The impact of diverse MRPs of sugars and spermine on enzyme activity is conveyed in Figure 8.

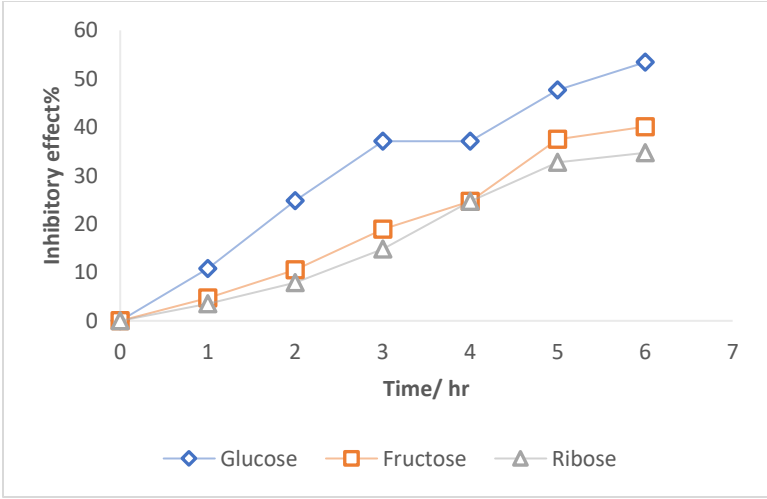

Figure 8: Inhibitory effect of spermine-sugar system on purified PPO from Mirabelle plum

Many studies have shown that MRPs are capable of inhibiting the PPO activity in foods. Billaud et al. (2005) investigated the MRP effect of glutathione or cysteine with different monosaccharides and disaccharides as antibrowning agents on PPO activity in mushrooms, apples, and eggplant[17]. On the other hand, the MRPs synthesized from glucose with tyrosine, proline, and glumatic acid individually, exposed inhibitory effects on enzymes isolated from potatoes and apples[18]. The presence of MRPs caused the noodles to be significantly less dark in color, which was attributed to MRPs' inhibitory effect on PPO[52]. Further, Figure 9 shows an evaluation of MRP and vitamin C inhibitory effects on PPO compared to the control.

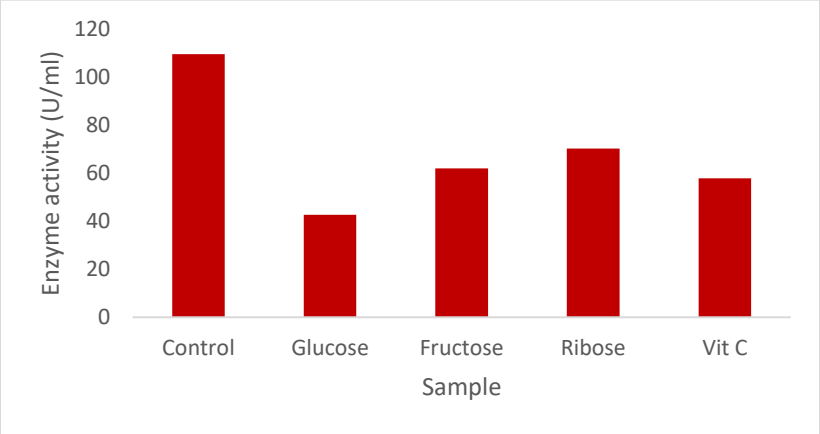

Figure 9: Comparison between the inhibitory effects of MRPs solutions and vitamin C) on PPO extracted from Mirabelle plum

It was shown that spermine–glucose had extra inhibitory effects than other MRPs and vitamin C, however, the inhibition of rest MRPs was significant (42.6- 70.2%). According to the literature, MRPs derived from glucose with glycine initiated a significant decline in enzymatic browning in apples and mushrooms[53]. Certain fractions generated during the MR have been shown to have the ability to either inactivate enzyme activity or form PPO inhibitors. These compounds' ability to suppress oxidation processes is based on several mechanisms, including reducing activity, free radical scavenging, and metal-ion chelating[54].

Inhibition mode of PPO

The inhibition of the purified plum PPO activity was investigated with the existence of a spermine-glucose system. The inhibition mode was verified by drawing a Lineweaver-Burk plot (Figure 10) using different concentrations of catechol as substrate. The results exhibited a non-competitive inhibition mode. The value of Vmax was reduced from 0.212 units /ml/ min) without inhibitor to 0.101 units/ml/ min) in the presence of inhibitor, while the Km value remained approximately constant at 2.56 mM. This enzyme has been non-competitively inhibited in previous studies after being purified from different plant sources such as quince[55], purslane[56], and potato[57].

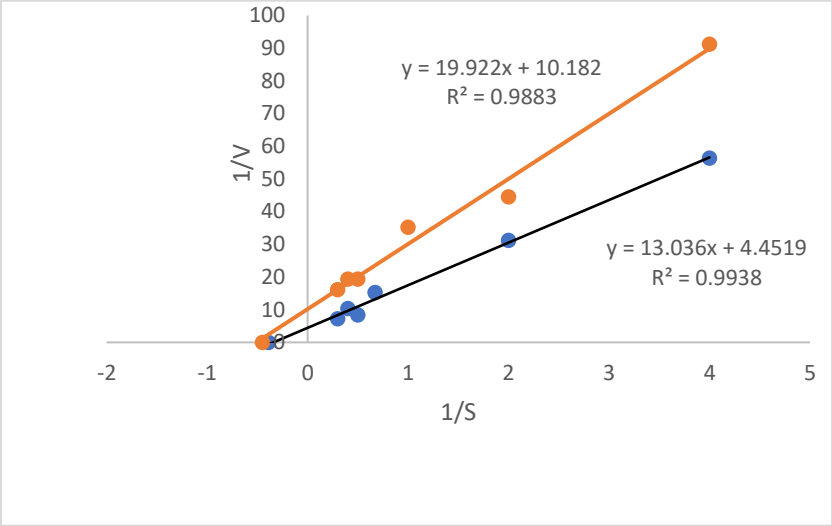

Figure 10: The inhibition mode of purified plum PPO by the spermine-glucose system

4-Conclusion

Enzymatic browning is an economic problem that happens as a result of processes complicated in the production of dyes from enzymatically oxidized phenolic compounds of natural origin. PPO present in fruits and vegetables, is responsible for this phenomenon. One of the most important methods used to stop this reaction is adding antioxidants**.** The antioxidant properties of MRPs are recognized in the formation of reductone structures that have both reducing and metal complexing properties. Numerous model systems were used for this purpose. Additionally, MRPs were revealed to have powerful radical scavenging activity, which was significant when compared to the known antioxidant vitamin C. MRPs from spermine-sugar systems exhibit high antioxidant properties, with glucose being the most effective reactant for MR with spermine's amino groups, resulting in highest browning pigment formation. The strongest inhibitory effect of MRPs on PPO extracted from Mirabelle plum was found with the spermine-glucose system. We conclude that enzyme inhibition by compounds may be due to their antioxidant capacity or chelation with the copper ion, a cofactor of PPO.

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Scientific Research

Comparative Analysis of Biochemical Compositions and Quality Attributes of Green Tea and Black Tea from Prominent Bangladeshi Brands

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ABSTRACT

Tea is the most widely consumed non-alcoholic beverage in the world, valued for its economic significance and health benefits, largely attributed to its polyphenolic compounds with potent antioxidant properties. The purpose of this research is to assess the chemical composition and quality of green and black tea products available in the Bangladeshi market. Samples were collected from five leading brands of each type: black tea brands included Ispahani, Kazi & Kazi, Jafflong, Halda Valley, and Seylon, while green tea brands comprised Ispahani, Kazi & Kazi, Jafflong, Halda Valley, and Lipton. The analysis focused on proximate compositions, total phenols, flavonoids, DPPH radical scavenging activity, caffeine levels, theaflavins (TF), thearubigins (TR), high polymerized substances (HPS), and total liquor color (TLC). A comparative analysis of green tea and black tea reveals significant differences in their chemical compositions and potential health benefits. Green tea boasts a higher phenolic content, ranging from 72.94 to 75.68 mg GAE/g, and exhibits greater antioxidant activity, with DPPH (2,2-diphenyl-1-picrylhydrazyl) values between 91.29 to 96.55 ml Trolox/g, compared to black tea, which has phenolic content of 66.21 to 67.32 mg GAE/g and DPPH values of 79.84 to 87.73 ml Trolox/g. Conversely, black tea contains higher levels of caffeine (31.95 to 37.36 ppm), flavonoids (63.70 to 67.78 mg QE/g), and tannins (14.02 to 17.36 mg TAE/g) than green tea, which has caffeine levels of 25.18 to 28.41 ppm, flavonoid content of 27.17 to 37.80 mg QE/g, and tannin content of 5.87 to 7.21 mg TAE/g. The analysis indicates that black tea has higher concentrations of theaflavins, thearubigins, highly polymerized substances, and total liquor color compared to green tea, contributing to its distinctive flavor and appearance. While the moisture, ash, and lipid levels in both tea varieties are comparable, showing only minor differences, the research emphasizes the unique biochemical profiles of each type. Green tea is noted for its greater levels of polyphenols and antioxidants, which enhance its health benefits, whereas black tea is characterized by its higher caffeine content, making it a more stimulating beverage. These findings highlight the distinct qualities of green and black tea, suggesting that each may appeal to different consumer preferences and health needs.

1- Introduction

Tea (*Camellia sinensis* L.) is one of the most ancient beverages and enjoys worldwide popularity. Because tea plant farming requires a warm and humid environment, it primarily occurs in tropical and subtropical areas [1]. As a result, most tea is grown on large plantations in East Africa and Southeast Asia [2]. With its rich aroma and diverse flavors, tea consumption is deeply ingrained in the daily lives of millions, contributing significantly to the nation's economy and heritage. The collection of leaves marks the start of tea production, followed by their processing. During the stages of transformation, tea leaves undergo oxidative and hydrolysis reactions triggered by intrinsic enzymes present in the leaf cells (including polyphenol oxidase and peroxidase) [3]. Depending on the level of fermentation and processing methods, tea can be divided into six main categories: yellow, white, and green tea (not fermented), oolong tea (partially fermented), black tea (fully fermented), and dark tea (post-fermented) [4-5]. The production of black tea involves the oxidative polymerization of flavan-3-ols, facilitated by the enzyme polyphenol oxidase. This process results in the creation of compounds such as bisflavanols, theaflavins, thearubigins, and other oligomers. In contrast, green tea is made by quickly steaming or hot air-drying freshly picked leaves to deactivate polyphenol oxidase, thereby preventing fermentation. This method yields a dry and stable product while also contributing to the tea's vibrant green color due to the blanching effect from the steam or hot air exposure [6]. The quality of tea, influenced by a myriad of factors such as geographical origin, climate, soil conditions, cultivation practices, and processing methods, plays a crucial role in determining consumer satisfaction and market competitiveness [7].

Catechins are the most prominent and biologically active compounds in green tea, almost all of which are extracted during the tea brewing process. Catechins and flavonol glycosides, the main contributors to the bitterness and astringency, displayed similar distribution patterns as polyphenols. It offers a range of health benefits, such as antioxidant, anti-inflammatory, and potential anticancer properties [8]. On the other hand, black tea, distinguished by the presence of theaflavins and thearubigens, enhances cardiovascular health and supports the immune system. More than 75% of catechins are converted into complex polymers like theaflavins (TFs), thearubigins (TRs), and asinensins (TSs) during the fermentation process, which is the primary step in the manufacturing of black tea [9]. With their distinct compositions and health-promoting qualities, both types enhance general well-being. Approximately 4000 bioactive chemicals have been found in tea, of which 33% are polyphenols, with catechins making up the majority of these compounds. Additionally, there are other chemical components such as volatile organic molecules, proteins, carbohydrates, proteins, alkaloids, and trace elements [10]. Teas and tisanes differ significantly in their polyphenol profiles, which could help to explain some of their various potential biological activities. These include effects that are anti-obesity, antiviral, antimutagenic, antimicrobial, anticarcinogenic, antiosteoporotic, antioxidant, antiatherosclerotic, antiallergic, antifibrotic, hypolipidemic, and hypocholesterolemia) [11-17].

Tea was transformed into different products or ingredients utilized in food processing. Tea-flavored foods satisfy consumer demand for green products while also providing

combined benefits for nutrition enhancement and overall human health. Tea powder or ground tea can be directly made into tea bags, whether for instant use or as ready-to-drink (RTD) options. Additionally, numerous studies have indicated that tea extracts or powder are commonly utilized in bread, noodles, biscuits, ice cream, bars, and even in animal feed [18-22]. Tea-based food products, being a significant application, have contributed greatly to enhancing food safety and diversity.

Understanding the biochemical composition and quality attributes of commercially available green and black tea in Bangladesh is vital for ensuring consumer confidence and promoting trade. As tea holds significant cultural importance in the country, consumers must trust that the products they purchase meet quality standards and offer the associated health benefits. Additionally, a thorough understanding of the specific compounds in Bangladeshi teas, such as catechins and flavonoids, can enhance their positioning in the global market, attracting both domestic and international investment. However, there is a notable lack of comprehensive studies assessing the biochemical composition and quality parameters of these teas, which hinders informed decision-making among stakeholders. Therefore, conducting detailed scientific research to evaluate the biochemical makeup and qualitative characteristics of green and black tea in Bangladesh is essential. This study aims to fill the existing information gap, ultimately supporting the development of high-quality tea products that align with both local and international standards and contribute to the sustainable growth of the tea industry.

2-Materials and Methods

2.1 Chemical and reagents

For several experiments, the analytical grade reagents listed below such as Sodium Carbonate (Na_2CO_3), Gallic Acid ($C_7H_6O_5$), Hydrochloric Acid (HCl), Folin-Ciocalteau Reagent, Sodium Hydroxide (NaOH), Sodium Nitrite ($NaNO_2$), Aluminum Chloride ($AlCl_3$), Vanillin ($C_8H_8O_3$), DPPH ($C_{18}H_{18}N_5O_6$), Sodium Dihydrogen Phosphate Dihydrate ($NaH_2PO_4 \cdot 2H_2O$), Potassium Ferricyanide ($K_3[Fe(CN)_6]$), Ferric Chloride ($FeCl_3$), Disodium Hydrogen Phosphate Dihydrate ($Na_2HPO_4 \cdot 2H_2O$), Trichloroacetic Acid (CCl_3COOH), Ferrous Sulfate Heptahydrate ($FeSO_4 \cdot 7H_2O$), Sulfuric Acid (H_2SO_4), 3,5-Dinitrosalicylic Acid ($C_7H_4N_2O_5$), Phenol (C_6H_5OH), Sodium Potassium Tartrate ($KNaC_4H_4O_6$), and Ethyl Alcohol (Ethanol, C_2H_5OH) were purchased from Merck (Darmstadt, Germany).

2.2 Samples collection

Different brands of tea samples (5 green tea and 5 black tea samples) were collected from the markets of Dhaka & Chittagong, Bangladesh. The selection was done based on brand popularity and overall availability across the country. The names of the collected samples have been given below:

Table 1: Black Tea and Green Tea samples

Black Tea Samples	Green Tea Samples
Ispahani	Ispahani
Kazi and Kazi	Kazi and Kazi
Jafflong	Jafflong
Halda Valley	Halda Valley
Seylon	Lipton

2.3 Preparing a solvent extract for the assessment of antioxidant activity and polyphenol content

Halim *et al.* [23] investigated an approach to extracting phenolic compounds from samples involving modifications. 19 mL of 100% ethanol was used to dissolve 1 gram of each sample. 1 hour of stirring at room temperature was performed using a magnetic stirrer (VS-130 SH, Korea) at a rotational speed of 1000 rpm. The filtered slurries were then passed through Whatman filter paper (No. 41) samples were preserved at -4°C for further use.

2.4 Determination of proximate composition

The proximate composition of the samples was determined using the methods described by Akhter *et al*. [24]. The moisture level was determined by putting 5 g of the sample in an oven (Model, ED 56, Tuttlingen, Germany) at 105°C for 24 hours or more and recording the weight loss as a percentage. The ash content was determined by incinerating at 550°C in a muffle furnace. The ash content was expressed as the percentage ratio of the sample weight to the ash weight. The lipid content in all analyzed samples was assessed using the method described by [25].

2.5 Estimation of theaflavin (TF), thearubigin (TR), highly polymerized substances (HPS), and total liquor color (TLC)

A hot plate was used to steep 2 grams of untreated tea samples in 90 mL of boiling water for 10 minutes. The brew was filtered and then transferred to a 100 ml flask, with hot water added. The study examined the quality of tea using spectrophotometry analyzing parameters like theaflavins (TF), thearubigins (TR), highly polymerized substances (HPS), and total liquor color (TLC) [26].

2.6 Determination of total phenolic content (TPC)

The total phenolic content (TPC) was determined using the modified Folin-Ciocalteu method [27]. The absorbance of the supernatant was read at 725 nm (UV-1800 UV/Vis, Shimadzu, Japan). The TPC was expressed using milligrams of gallic acid equivalents (mg GAE) per g of sample.

2.7 Determination of total flavonoid content (TFC)

TFC was determined using the colorimetric method with some modifications as described by *Halim et al.* [28]. The absorbance was read at 510 nm (UV-1800 UV/Vis, Shimadzu, Japan). The TFC was expressed as mg Quercetin equivalents (mg QCE) per g of sample calculated from a standard curve for Quercetin.

2.8 Determination of tannin content

Using the Vanillin Hydrochloride Method, the samples' tannin content was ascertained [23]. Twenty minutes were spent incubating

1 ml of the extracted material and 5 ml of vanillin hydrochloride. Next, at 500 nm, the absorbance was measured using a spectrophotometer. In milligrams of catechin equivalent per gram of dry extract, the tannin concentration was reported.

2.9 Determination of antioxidant activity

The free radical scavenging activity of the samples was determined using the Anika *et al.* [29] technique. Initially, 40 minutes were spent stirring a solution of 1 mM DPPH in 80% (v/v) methanol. 80% (v/v) methanol was used to adjust the solution's absorbance to 0.650 nm at 515 nm. Following the appropriate vortexing, 50 µl of the extracted sample was combined with 1.90 ml of 0.1 mM DPPH. For thirty minutes, the mixture was then left in the dark. At 515 nm, the absorbance was then measured. The blank in this case was methanol. Percentage inhibition of the DPPH radical was used to express the antioxidant activity. The following equation was used to calculate it:

DPPH scavenging capacity (%) = (A control-A sample)/ (A control) ×100 [An absorbance at 515 nm]

2.10 Statistical analysis

Every experiment was repeated three times, obtaining mean values and standard deviations. The experimental results were statistically analyzed using a one-way analysis of variance to ascertain the statistically significant differences between various formulations at a 95% confidence level (ANOVA). Duncan's multiple range test ($p<0.05$) was employed to assess the significance of the mean differences. The statistical analyses were all performed with SPSS 22.0.

3-Results and Discussion

3.1 Moisture, dry matter, lipid, and ash content of black tea and green tea samples

Controlling the moisture content of materials from the introduction of the raw materials to the packing of the finished product is essential for maximizing efficiency, optimizing yield, and producing a high-quality, consistent output [30-32]. In comparison to the other samples of black tea (Seylon) revealed that it had a higher moisture content while green tea (Lipton) had a noticeably higher moisture content. Based on Table 2's data, that suggests an average stable phenomenon. Since more polyphenols that help retain moisture content are destroyed during the fermentation process, which is excluded when processing black tea, this may be the cause of the higher moisture content in green tea samples. Our results are similar to those of Makanjuola [33], who found that black tea had a moisture content of 3.9-9.5%. Every tea sampled for this study had a moisture content of less than 7.35-10.61%, making it appropriate for a longer shelf life [34]. The use of packing materials to keep commercial tea samples at a consistent moisture level while they are being stored is another crucial element; hence, the moisture content of commercial tea is a crucial indicator of quality. The lower water content in all samples suggested enhanced stability and longer shelf-life due to the lowered possibility for the growth of microbes as well as better stability towards chemical and physical reactions [35].

The ash content of tea significantly impacts its quality. There is a correlation between the mineral and moisture content of a sample and the ash content of tea. According to Faramayuda *et al.* [36], mineral content quantifies the amount of physiological ash, which comes from the plant tissue itself, and non-physiological ash, which is the leftover material that sticks to the plant surface. Table 2 presents the ash content percentages of

various black and green tea samples, indicating the mineral content and overall quality of each tea. For black tea, the highest ash content is found in the Jafflong sample (6.36%), while the lowest is in Halda Valley (5.48%), with other samples like Ispahani, and Kazi and Kazi showing similar values of around 5.9%. In the green tea category, Jafflong again has the highest ash content (6.22%), and Halda Valley has the lowest (4.73%), with Ispahani, Kazi and Kazi, and Lipton showing lower values between 4.83% and 5.20% respectively. Tea may have less moisture, which would explain the increased ash content. A lower ash percentage in tea could be because tea is produced by adulterating extracted raw materials, resulting in a lower-quality tea. As suggested by earlier studies, ash levels should be kept below 5.54% to preserve tea quality while it is being stored. These findings also showed a favorable correlation between ash content and maintaining tea quality [37].

Lipid is recognized as a significant field in biochemical research since it serves as both a structural and storage element in plant tissues. Neutral lipids, glycolipids, and phospholipids, which form the lamellae fractions or various cell membranes in chloroplasts of leaves, are broken down by acyl hydrolases into free fatty acids. Variations in fatty acids across different molecular species could significantly influence the assessment of cultivars with enhanced tea-making capabilities. Due to differences in withering intensity, rolling method, and particle size in orthodox processing [38]. Lipid constitutes a significant part of the fresh tea leaf. Table 2 shows the lipid levels in black tea samples, which ranged from 4.43±0.74% to 4.73±0.97%. Jafflong black tea exhibited the highest lipid level at 4.73%, whereas Jafflong green tea leaves displayed the most significant lipid content among green teas at 4.84%. These findings correspond with prior research by [39], which discovered that teas in Pakistan had fat content ranging from 4% to 7%, and also stated that teas from Bangladesh had fat levels between 6% and 9%. Additionally, Kodagoda and Wickramasinghe [40] noted that both green and black teas contain lipid 3%. Furthermore, Hosen *et al.* [41] supported these results by indicating that the lipid content of protoplasm may represent 3% to 7% of its dry weight, highlighting the importance of fat content in evaluating tea quality.

Table 2: Moisture, dry matter, lipid, and ash content in black tea samples

Sample	Moisture (%)	Dry matter (%)	Lipid (%)	Ash (%)
Black tea				
Lipton	10.61±0.72^{a}	90.40±0.72^{b}	4.274±0.89^{a}	4.85±0.41^{b}
Ispahani	7.35±0.23^{c}	92.64±0.23^{a}	4.62±1.24^{a}	5.97±0.17^{a}
Kazi and Kazi	7.20±0.46^{c}	92.79±0.46^{a}	4.43±0.74^{b}	5.81±0.24^{a}
Jafflong	9.05±0.82^{a}	90.94±0.82^{b}	4.73±0.97^{a}	6.36±0.31^{a}
Halda Valley	8.00±0.37^{b}	92.02±0.37^{a}	4.50±0.83^{b}	5.48±0.17^{a}
Seylon	9.16±0.92^{a}	90.83±0.92^{b}	4.58±1.12^{b}	5.604±0.53^{a}
Green tea				
Ispahani	7.50±0.51^{b}	92.50±0.51^{b}	4.40±0.87^{a}	5.20±0.12^{b}
Kazi and Kazi	10.47±0.21^{a}	89.52±0.21^{b}	4.79±0.91^{a}	4.83±0.17^{b}
Jafflong	7.60±0.37^{b}	92.40±0.37^{a}	4.84±1.05^{a}	6.22±0.21^{a}
Halda Valley	10.01±0.41^{a}	90.00±0.41^{b}	4.621±0.97^{a}	4.73±0.37^{b}

Values are the Mean ± standard deviation of three replicates. [a-c] Different superscript alphabets indicate significant differences among the black tea and green tea samples ($p < 0.05$).

3.2 Caffeine content in black tea and green tea samples

The amount of caffeine in tea is quite significant due to its potential health implications and its effect on the sensory experience associated with drinking tea. Additionally, caffeine content is a crucial factor in evaluating commercial tea products. This research aims to investigate the caffeine levels in different samples of green and black tea to better understand the variations in caffeine concentration across various tea types. The caffeine levels in the black tea samples ranged from 31.95±1.28 ppm to 37.36±1.89 ppm, while the green tea samples exhibited caffeine concentrations between 25.18±1.21 ppm and 28.41±0.73 ppm (Table 3). Importantly, the caffeine levels in black tea are associated with its quality, as they contribute to the formation of vibrant precipitates during the brewing process. The highest caffeine level was observed in green tea sourced from Halda Valley, measured at 37.36±1.89 ppm. The results of this study are consistent with previous research conducted by Shokrzadeh *et al.* [42]. Furthermore, the findings align with those of Adnan et al. (2013), who suggested that to ensure superior product quality, the caffeine level in commercial tea should be limited to below 4%. Various factors influence the caffeine levels in black teas, including the specific tea variety, the timing of leaf harvesting, the season, and the geographical region. Additionally, harvesting later and using older leaves in commercial tea production may also affect caffeine content [37].

Table 3: Caffeine content in black tea and green tea samples

Black tea samples	Caffeine (ppm)	Green tea samples	Caffeine (ppm)
Ispahani	31.95±1.28[b]	Ispahani	26.37±1.37[b]
Kazi and Kazi	35.51±1.32[b]	Kazi and Kazi	27.91±1.89[b]
Jafflong	32.86±2.40[b]	Jafflong	26.67±1.94[b]
Halda Valley	37.36±1.89[a]	Halda Valley	28.41±0.73[a]
Seylon	33.72±2.97[b]	Lipton	25.18±1.21[b]

Values are the Mean ± standard deviation of three replicates. [a-c] Different superscript alphabets indicate significant differences among the black tea and green tea samples ($p < 0.05$).

3.3 TF, TR, HPS, and TLC content in black tea and green tea samples.

Different samples of black and green tea showed differences in the concentrations of the following: total liquor color (TLC), highly polymerized substances (HPS), theaflavin (TF), and thearubigin (TR). Among the black tea samples, the highest TF content was detected in Kazi and Kazi (1.029%), while the lowest TF content was found in Jafflong (0.875%). Similarly, among the green tea samples, Halda Valley exhibited the highest TF content (0.520%), whereas Ispahani had the lowest (0.22%) (Table 4). These discrepancies in TF levels are likely influenced by factors such as tea variety, elevation, local cultivation practices, and processing methods. Chy *et al.* [43] noted that TF content in black tea typically ranges from 0.3% to rarely exceeding 2%, aligning with our findings. The attractive color of tea infusion is linked to TF, which acts as a vital quality measure of black tea and significantly contributes to producing premium tea. Hazra *et al*. [44] noted that the hue of tea is an important factor that can attract and increase value for consumers. Moreover, the TR level

was notably higher in the black tea sample from Kazi and Kazi (5.452%) compared to others. Increased TR content enhances the richness, intensity, and mouthfeel of the tea liquor, as supported by Khan *et al*. [26], who reported TR concentrations ranging from 2.32% to 6.25% in black tea samples. TR lowers the brightness of the tea liquid. The larger quantity of thearubigin mainly adds to the ashy flavor of the beverage, with a slight enhancement in astringency. TR affects the texture (thickness) and brownish color of the tea [19].

Highly polymerized substances (HPS) play a significant role in defining the body, strength, and color of tea beverages. In our study, the range of HPS content was 6.34% to 3.79% in black tea and 3.44% to 1.72% in green tea. This is consistent with the findings of Khan *et al.* [26], who reported HPS levels in black tea samples ranging from 3.70% to 7.21%. A highly polymerized substance is very effective in evaluating and classifying the quality of tea. Together with HPS, TR in tea liquor contributes to the color and mouth feel. Total liquor color (TLC), which encompasses TR, TF, and HPS, was highest in the black tea sample from Kazi and Kazi (4.61%). Alam *et al*. [45] assessed different tea brands in Bangladesh and found results consistent with our study. The total liquor color in Indian black tea was observed by Someswararao *et al.* [46] to range between 3.89% and 5.7%, which is in good agreement with our results. Furthermore, as particle size increased, concentrations of TF, TR, HPS, and TLC also increased. This phenomenon can be attributed to finer tea grades having a larger surface area, facilitating greater oxidation of catechins and the production of TF, TR, HPS, and TLC, thereby enhancing extraction during brewing. TF, TR, HPS, and TLC exhibit a multitude of health advantages, such as aiding in fat reduction and blood sugar regulation, as well as offering protection against lifestyle-related illnesses including obesity, cancer, atherosclerosis, inflammation, viral and bacterial infections, osteoporosis, and dental caries [47-49].

Table 4:TF, TR, HPS, and TLC content in black tea and green tea samples

Black Tea Samples	TF (%)	TR (%)	HPS (%)	TLC (%)
Ispahani	0.92 ± 0.27^{b}	5.11 ± 1.31^{a}	4.25 ± 1.43^{b}	3.02 ± 0.62^{b}
Kazi and Kazi	1.02 ± 0.19^{a}	5.45 ± 1.08^{a}	4.36 ± 1.29^{b}	4.61 ± 0.48^{a}
Jafflong	0.87 ± 0.32^{b}	4.84 ± 2.19^{a}	6.34 ± 1.72^{a}	2.56 ± 1.07^{b}
Halda Valley	1.01 ± 0.17^{a}	4.95 ± 0.89^{a}	5.23 ± 0.98^{b}	3.21 ± 0.91^{b}
Seylon	0.98 ± 0.51^{b}	3.21 ± 1.51^{b}	3.79 ± 1.86^{b}	2.95 ± 1.13^{b}
Green Tea Samples	**TF (%)**	**TR (%)**	**HPS (%)**	**TLC (%)**
Ispahani	0.22 ± 0.15^{a}	2.07 ± 1.81^{a}	2.21 ± 1.21^{b}	4.13 ± 0.44^{b}
Kazi and Kazi	0.51 ± 0.18^{a}	2.73 ± 1.05^{a}	2.33 ± 1.22^{b}	5.22 ± 0.36^{a}
Jafflong	0.22 ± 0.41^{a}	1.76 ± 2.12^{b}	3.44 ± 1.77^{a}	1.78 ± 1.00^{c}
Halda Valley	0.52 ± 0.11^{a}	1.95 ± 0.51^{a}	2.28 ± 0.60^{b}	3.25 ± 0.80^{b}
Lipton	0.29 ± 0.11^{a}	1.84 ± 2.50^{b}	1.72 ± 1.82^{c}	2.51 ± 1.19^{b}

Values are the Mean ± standard deviation of three replicates. $^{a\text{-}c}$ Different superscript alphabets indicate significant differences among the black tea and green tea samples ($p < 0.05$)

3.4 Bioactive compounds and antioxidant activity of black tea and green tea samples

Tea is a popular beverage to drink all around the world, and its nutritional worth is being researched as a possible way to enhance other

nutritional attributes. Therefore, knowledge regarding its nutritional evaluation is practically necessary for both tea promoters and final consumers. A few of these have been taken into consideration in the current effort. Tea's antioxidant properties are attributed to several bioactive secondary metabolites, making it a potential source of antioxidant supplements. The antioxidant characteristics of tea polyphenols are responsible for their beneficial benefits. Table 5 displays the total phenol and flavonoid, tannin, and DPPH concentrations of green and black tea types.

The relationship between phenolic content and antioxidant activity in plants is strong and significant. Phenolic compounds can scavenge a variety of oxidizing free radicals, such as hydrogen peroxide, hydroxyl radicals, singlet oxygen, and more, since they are efficient reducing agents and hydrogen donors. Phenolic compounds are useful tools in the battle against oxidative stress and its harmful effects on the body because of their capacity to neutralize and combat oxidative free radicals, which greatly increases their antioxidant activity [50]. The total phenolic content in black tea samples varied from 63.64±0.81 to 67.32±0.58 mg GAE/g, while in green tea samples, it varied from 72.94±0.47 to 75.68±0.37 mg GAE/g. The present values are less than those mentioned by Zhao et al. (2019). They assessed the total phenol content in black tea, which varied from 37.23 ± 0.28 to 101.29 ± 1.58 mg GAE/g, and in green tea, it ranged from 148.16 ± 2.72 to 252.65 ± 4.74 mg GAE/g. This result was consistent with the study conducted by Abdullah & Mazlan [51], which showed that all green tea samples had greater phenolic content than those of herbal teas. Additionally, factors affecting catechin levels include the cultivation area, the surrounding environment, and the initial processing of the leaves before drying. Furthermore, the general composition of the teas is greatly influenced by the season, climatic conditions, and the maturity of the tea leaves.

The flavonoid content in tea is a key indicator of its antioxidant capacity, with significant variations observed between black and green tea samples. In black tea, flavonoid content ranged from 63.60 ± 1.03 to 67.78 ± 0.53 mg QE/g in Halda Valley. Specifically, Ispahani contained 63.60 ± 1.03 mg QE/g, Kazi and Kazi 65.14 ± 0.23 mg QE/g, Jafflong 64.53 ± 0.40 mg QE/g, and Halda Valley had the highest at 67.78 ± 0.53 mg QE/g. In contrast, the flavonoid content in green tea samples showed more variability. Ispahani green tea contained 31.87 ± 1.38 mg QE/g, Kazi and Kazi 32.67 ± 1.44 mg QE/g, Jafflong 33.67 ± 1.22 mg QE/g, Halda Valley the highest at 37.80 ± 1.28 mg QE/g, and Lipton the lowest at 27.17 ± 1.52 mg QE/g. These results indicate that black tea generally has higher flavonoid content, contributing to its antioxidant properties. Our findings partially corroborate Nhu-Trang *et al.* [52], who reported that black tea extracts have the highest total flavonoid content, followed by green tea. The variations in flavonoid content between black and green tea samples underscore the influence of processing techniques and intrinsic differences in the tea leaves. The higher flavonoid content in black tea may be attributed to the fermentation process, which enhances certain polyphenolic compounds. In contrast, the lower flavonoid content in green tea is likely due to the minimal oxidation it undergoes, preserving different sets of polyphenols. Ullah *et al.* [53] noted that flavonoids are believed to be responsible for antioxidant activity, anticarcinogenic effects, and anti-arteriosclerosis benefits.

The tannin content in tea plays a significant role in determining its taste profile and potential health benefits, with notable

variations observed among different tea samples. In our analysis, the tannin content was measured in milligrams of tannic acid equivalents per gram (mg TAE/g). Among the black tea samples, Ispahani exhibited a moderate tannin content of 14.02 ± 1.03 mg TAE/g, while Kazi and Kazi showcased a slightly higher level at 14.37 ± 1.03 mg TAE/g. Jafflong tea, on the other hand, displayed a lower tannin content of 13.16 ± 1.43 mg TAE/g, providing a potentially smoother taste experience. Halda Valley black tea stood out with the highest tannin concentration at 17.36 ± 0.02 mg TAE/g. In green teas, Ispahani featured a lower tannin content of 5.87 ± 0.01 mg TAE/g compared to its black tea counterpart. Kazi and Kazi's green tea displayed a slightly higher tannin concentration at 6.26 ± 1.10 mg TAE/g, while Jaflfong's offering mirrored this profile at 6.04 ± 0.02 mg TAE/g. Halda Valley's green tea had the highest tannin content among the green teas at 7.21 ± 0.01 mg TAE/g. Lipton green tea had a moderate tannin content at 6.70 ± 0.12 mg TAE/g. As such, tea's color is significantly influenced by the amount of tannin in it. The quantity of tannins in tea is directly correlated with its blackness, as mentioned by Piyasena *et al.* [54]. To explain it simply, darker-colored teas have more tannin in them. Many plant-based meals and beverages, including tea, include tannins, which are polyphenolic chemicals. The unique bitterness and astringency of black tea are attributed to tannins. The industrial processes used to produce black tea are mostly responsible for its greater tannin content. Tea leaves are subjected to rolling, oxidation, and withering to produce black tea. Tea leaves develop and accumulate tannins as a result of various processes, most notably oxidation. According to these findings, the flavor and possible health advantages of various tea kinds are influenced by the varied tannin profiles found in them.

An antioxidant molecule is characterized as a compound that can prevent or reduce the oxidation of biomolecules, even in minimal amounts. These antioxidants are essential in protecting food and plants from deterioration caused by oxidation and shielding the body's biomacromolecules from oxidative damage. Evaluating the antioxidant capacity in food and plants is highly important [55-56]. To evaluate the antioxidants' capacity to scavenge free radicals, DPPH assays are frequently used as methods. Rahman *et al.* [32] point out that the DPPH radical's ability to scavenge free radicals stems from its propensity to decolorize in the presence of antioxidants. The results have shown that the antioxidant activity levels were significantly higher in green tea (91.29±0.75 to 95.47±0.94 ml Trolox/g) compared to those of black tea (79.84±0.93 to 87.73±1.04 ml Trolox/g). Similar results were reported in earlier studies [57]. The higher antioxidant activity is due to the potent antioxidant activities of catechins in green tea which are due to their three adjacent hydroxyl (OH) groups on the β-ring as in epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epigallocatechin (EGC) and gallocatechin (GC) which are more effective in scavenging free radicals than the two adjacent OH groups as in catechin gallate (CG) and epicatechin (EC). The content of EGCG and EGC in green tea is much higher than in black tea [58]. Nevertheless, the antioxidant effectiveness of catechins relies not only on their chemical structure but also on the surrounding environmental conditions [59].

In the case of black tea, antioxidants found in black tea include catechins and their derivatives, along with theaflavin and thearubigin, which are produced through enzymatic oxidation and impart color to the

infusion of black tea. The aromatic component additionally acts as an antioxidant in black tea. Carotenoids, fatty acids, glycosides, and amino acids act as fundamental components in the formation of aroma compounds in tea, and their concentrations are affected by the processing techniques. Some examples of aroma compounds with amino acid or carbohydrate precursors are (E)-2-Hexenal, Hexanal, Hexanoic acid, (Z)-3-Hexen-1-ol, (E)-Linalool oxide (furanoid), and phenylacetaldehyde. All of these components exist in larger amounts in black tea compared to green tea [60-61].

Table 5: Bioactive compounds and antioxidant activity of black te and green tea samples

Black tea sample	Total Phenol (mg GAE/g)	Total Flavonoid (mg QE/g)	Tannin (mg TAE/g)	DPPH (ml Trolox/g)
Ispahani	63.64±0.81^{b}	63.60±1.03^{b}	14.02±0.01^{b}	80.29±0.73^{c}
Kazi and Kazi	67.32±0.58^{a}	65.14±0.23^{b}	14.37±0.03^{b}	85.43±0.92^{b}
Jaflong	64.21±0.95^{b}	64.53 ± 0.40^{b}	13.16±1.43^{b}	87.73±1.04^{a}
Halda valley	66.30±0.37^{a}	67.78 ± 0.53^{a}	17.36±0.03^{a}	84.13±0.87^{b}
Seylon	66.22±1.2^{a}	63.70±1.19^{b}	15.30±0.02^{b}	79.84±0.93^{c}
Green tea sample				
Ispahani	72.63±0.49^{b}	31.87±1.38^{b}	5.87±0.01^{c}	93.32±1.39^{b}
Kazi and Kazi	74.48±0.86^{a}	32.67±1.44^{b}	6.26±1.10^{b}	96.55±1.02^{a}
Jafflong	73.08±1.07^{b}	33.67±1.22^{b}	6.04±0.02^{b}	92.46±0.98^{b}
Halda valley	75.68±0.37^{a}	37.80±1.28^{a}	7.21±0.01^{a}	95.47±0.94^{a}
Lipton	72.94±0.47^{b}	27.17±1.52^{c}	6.70±0.12^{ab}	91.29±0.75^{b}

Values are the Mean ± standard deviation of three replicates. $^{a\text{-}c}$ Different superscript alphabets indicate significant differences among the black tea and green tea samples ($p < 0.05$).

4-Conclusion

This study investigated the antioxidant potential and active component profiles of five marketed brands of green and black tea in Bangladesh. The results revealed significant differences in the concentrations of key bioactive compounds among the brands analyzed. Notably, the levels of total phenols, DPPH radical scavenging activity, theaflavins, thearubigins, and highly polymerized substances were assessed, revealing that Kazi and Kazi tea exhibited the highest overall quality among the brands analyzed. This suggests that Kazi and Kazi tea may offer superior health benefits compared to other brands. In contrast, Halda Valley tea was noted for its highest caffeine content, which may cater to consumers seeking a stronger stimulant effect. Other brands, including Jafflong, Ispahani, and Lipton, also demonstrated adequate nutritional value, indicating a diverse range of options available to consumers in the Bangladeshi tea market. These findings emphasize the importance of brand selection in optimizing the health benefits associated with tea consumption. The notable variations in antioxidant properties and active components highlight the necessity for consumers to be informed about the quality of the tea they choose. Future research should continue to explore the health implications of these findings and the potential for enhancing the antioxidant profiles of tea through processing methods.

5-Data Availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

6-Conflict of interest

The authors have no conflicts of interest to disclose that are compatible with the subject matter of this article.

7-Consent to participate

All authors have expressed their authorization to engage in this publication.

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9-Author Contributions

Md. Ashraful Islam: Formal analysis, Investigation, Supervision, Methodology, Software, writing – original draft. **Fahriha Nur A Kabir:** Writing – review & editing. **Sourav Biswas Nayan:** Writing – review & editing. **Anwara Akter Khatun:** Writing – review & editing, **Adrita Afrin:** Writing – review & editing, **Md. Shohel Rana Palleb:** Software, writing – review & editing & **Md. Abdul Halim:** Conceptualization, Formal analysis, Investigation, Supervision, Methodology, Software, writing – original draft, Writing – review & editing.

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Scientific Research

Traces and fate of *lactobacillus plantarum* isolated from indigenous Iranian dairy product: a comprehensive review.

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ABSTRACT

In Iran, a country characterized by diverse ecosystems and abundant biodiversity, both dairy and non-dairy local products are produced. These products and native plants include numerous lactic acid bacteria that can be isolated and identified. Among these, dairy products stand out as a rich source of *Lactobacillus Plantarum*. This bacterium plays a vital role in inhibiting pathogen growth, tolerating acidic and bile conditions, and producing exopolysaccharides. As a probiotic, *Lactobacillus Plantarum* offers health benefits to consumers. Our research focused on indigenous *Lactobacillus plantarum* strains isolated from local Iranian dairy products, revealing the country's genetic reservoir of this bacterium and other *Lactobacillaceae* family members. These findings pave the way for further exploration, including isolation, identification, and industrial applications. According to previous studies, it can be concluded that indigenous *Lactobacillus Plantarum* strains of different parts of Iran are probiotics and have a starter role that we can use on an industrial scale to produce probiotic products and Starter applications according to the characteristics of each isolated strain. However, *Lactobacillus plantarum* strains isolated from some nstive foods are not fit for commercial applications due to their poor technical resilience, low competitiveness, and weak antibacterial characteristics. As a result, both researchers and industry professionals must carefully assess the type and origin of native *Lactobacillus plantarum* strains before using them in industrial processes. This review study showed that each native *Lactobacillus plantarum* isolate has distinct characteristics, which will make their unique properties extremely valuable for developing co-starters, single or mixed starter cultures, and future superfoods.

1-Introduction

Nowadays, the rising consumption of dairy products like yogurt and doogh has led countries to prepare a large quantity of necessary starters to produce these products. On the other hand, efforts to enhance production efficiency and industrialize these products have involved the manipulation of starters. However, the lack of precise information about these engineered strains poses a significant challenge in the realm of food hygiene and consumer health. Currently, two strains of *Lactobacillus delbrueckii* ssp *bulgaricus* and *Streptococcus thermophilus* are used to produce yogurt. In the not-far past, indigenous mixed starters were employed to produce dairy products. These traditional products exhibit a diverse range of aroma and taste profiles, which can be attributed to the rich microbial flora present in them. Over centuries, indigenous starters have remarkably adapted to the preferences and physiological conditions of inhabitants across various climates. The selection of these bacteria has occurred naturally over thousands of years, influenced by factors such as taste preferences, food culture, climatic conditions, and other aspects of body microbiomes. [1-3].

Due to industrialization, many of the diverse bacteria present in traditional dairy products have been lost. Nowadays, yogurt production primarily relies on just two bacterial strains. However, understanding the natural combination of starter and non-starter microbial flora from traditional products allows us to create a starter that produces a healthy and standardized product while preserving the essential characteristics of these foods. According to this discussed subject and the studies conducted by researchers, it seems that non-starter flora isolated from dairy and even non-dairy products can be used to produce indigenous starters on an industrial scale. In this review study, an attempt has been made to investigate the potential of *Lactobacillus Plantarum* bacteria isolated from indigenous Iranian dairy products to use in the production of industrial mix starters. Lactic Acid Bacteria (LAB) are used as starters in the production of various fermented dairy and non-dairy products. These bacteria exhibit saccharolytic activity and possess a fermentative metabolism and Most of them produce Lactic Acid as their final product [1].

Driven by the demands of various countries and the significant economic potential of lactic acid bacteria (LAB), particularly in developing nations, extensive endeavors have been undertaken to discover and isolate indigenous LAB. For nearly a century, researchers have diligently worked to identify and use these bacteria for industrial applications [4].

Considering Iran's vast territory and diverse climates, there is substantial potential to identify industrially valuable Lactic Acid Bacteria (LAB). Recent studies on local products across various provinces in the country revealed a rich biodiversity of these bacteria, particularly in dairy products. Consequently, these products serve as valuable sources for isolating and identifying LAB . [5, 6] Notably, studies show that local yogurt is the most important source for isolating these bacteria [4, 7].

Throughout history, humanity has greatly benefited from the extensive family of Lactic Acid Bacteria (LAB) in the production of fermented products. This diverse group includes 224 species and 29 subspecies. Among them, *Enterococci, Leuconostoc, Pediococci, Lactococci, Streptococci,* and notably, *Lactobacillus*, stand out. *Lactobacillus plantarum*, in particular, holds a significant place in nature due to its presence in various plants across diverse habitats and climates. Lactic Acid Bacteria (LAB) have significantly influenced the production of various fermented foods, whether

intentionally or unintentionally. However, their impact extends beyond food production. These bacteria also play a vital role in shaping the microbiome of the digestive system. *Lactobacillus plantarum* exhibits heterofermentative behavior. When fermenting both hexose and pentose sugars, it generates carbon dioxide, ethanol or acetate, and lactic acid [1, 8-10].

Also, Thanks to its diverse antimicrobial metabolites, *Lactobacillus plantarum* possesses several beneficial features. These include inhibiting and preventing the growth of undesirable bacteria, such as pathogens and spoilage bacteria. Additionally, it contributes to reducing the risk of digestive system infections and the development of inflammatory bowel disease. [10-13].

research indicates that this bacterium has been successfully isolated from various Iranian local foods, including yogurt, milk, olives, honey, kefir, vinegar, tarragon, and chal. Notably, it is also utilized in probiotic products, both in dairy and non-dairy food items [14]. studies conducted on various local and traditional products including various dairy items from different regions in Iran, have consistently identified abundant indigenous Lactic Acid Bacteria (LAB). For instance, the research conducted by Leshni et al. in 2016 revealed that *Lactobacillus plantarum*, isolated from honey across 13 Iranian provinces, possesses probiotic abilities and can effectively inhibit *Staphylococcus aureus* bacteria. [15].

Numerous studies have investigated the strains found in Iran's indigenous dairy products over recent years. These findings are summarized in Table 1.

through our examination, we relized that *Lactobacillus Plantarum* plays a crucial role in indigenous Iranian dairy products. Interestingly, this bacterium is rarely used in industrial dairy starters. Consequently, its presence highlights the importance of exploring how it affects the technological characteristics of diverse dairy items. In this article, an attempt has been made to uncover the secret of *Lactobacillus Plantarum's* presence with a comprehensive review of indigenous Iranian dairy products.

Table 1. Isolation and identification of *Lactobacillus plantarum* present in some indigenous products of Iran.

author	**year**	**isolation source**	**isolation location**	**isolated strains**	**Characteristics**
[15]	2018	Honey	88 honey samples from 13 provinces	4 isolates of *Lactobacillus Plantarum*	**It was shown that Iranian honey has species of *lactobacillus paracasia* and *lactobacillus Plantarum*, which have a good inhibitory effect on pathogen bacteria, including *Staphylococcus aureus*.**
[16]	2016	Yogurt	Goat, cow, and sheep yogurt of Yazd province	12 isolates, which were 7 strains of *Pediococcus* and 5 *lactobacillus* from *Lactobacillus Plantarum*, *L.*	**The resistance to acidic conditions in the *lactobacillus* was better than *Pediococcus*. (pH= 2.5)**

author	year	isolation source	isolation location	isolated strains	Characteristics
				Fermentom, and *Lactobacillus Kefiry*.	
[17]	2012	Cheese	Koozeh cheese of East Azerbaijan province	28 strains were isolated that belonged to three species of *Lactobacillus: Lactobacillus Plantarum, Lactobacillus delbruki* and *Lactobacillus casei*.	**among these strains, it has been found that some can tolerate pH=3.**
[18]	2020	Doogh, curd, cheese, and butter	A sampling of traditional workshops in East Azerbaijan province (doogh, curd, cheese, and butter) was performed.	In this study, in addition to *Lactobacillus Plantarum*, other *lactobacillus* were identified and isolated, such as *Lactobacillus casei* and *L.acidophilus*.	**these bacteria can tolerate acidic and alkaline conditions. In this study, it is also recommended that these isolated bacteria can be used as starters**
[19]	2018	traditional cheese	24 samples collected from 8 different traditional cheeses in West Azerbaijan province	A total of 118 *lactobacillus* strains were isolated, including *Lactobacillus Plantarum*	**The results of this study showed that *Lactobacillus Plantarum*, *Lactobacillus Casei,* and *Lactobacillus helveticus* are good compounds of starters with acceptable Permanence. These strains can also be used in the production of industrial cheese to obtain the exclusive properties of traditional cheese**.
[20]	2015	yogurt and traditional cow milk	A sampling of traditional yogurt and traditional cow milk	A total of 14 bacterial strains from the area were identified.	**In addition to having probiotic properties, these strains also had the potential to be used in the dairy**

author	year	isolation source	isolation location	isolated strains	Characteristics
			available in Khoy City		**industry as a starter, as well as tolerance of the acidic conditions of the stomach and bile salts**.
[21]	2017	Yogurt	Kerman province yogurt sampling Yogurt and Doogh and cheese and fermented milk	The isolation of 47 lactic acid bacteria, of which 12 were probiotic. Among these 6 were *Padiococcus acidilactici* and 6 other cases *lactobacillus plantarum*, *lactobacillus fermentum*, *lactobacillus brevis*, *lactobacillus casei*	**The isolated bacteria can tolerate the acidic conditions of the stomach and bile salts and have probiotic potential. These bacteria have the potential of probiotics and can be used in functional products.**
[22]	2018	Horre	Horre, Khuzestan Province	Isolation of *Lactobacillus plantarum* and *Lactobacillus fermentum*	**The two isolated strains can tolerate bile salts and acidic pH, so we can use them in the industry as probiotics**.
[23]	2011	Milk, doogh, and curd	Sampling of Iranian milk, Doogh, and curd	Isolation of *Lactobacillus* species *Lactobacillus plantarum* *Lactobacillus casei* *Lactobacillus brevis*	**The isolated bacteria had high cholesterol absorption potential and 18 strains could tolerate pH = 2.5, so it was suggested that this strain could be used as a probiotic in the industry. Also, these bacteria had an antagonistic effect on indicator pathogen bacteria**.

[24]	**2017**	**Motal cheese**	**Unidentified**	**19 isolates were of the *Lactobacillus* genus, of which 4 *Lactobacillus plantarum* isolates were identified**	**Probiotic and technological features were not evaluated.**
[25]	2021	milk and yogurt	10 samples of traditional milk and yogurt from 5 rural areas of Mianeh city, Azerbaijan province	58 isolates of acid tolerant LAB. *Lactobacillus plantarum, Lactobacillus casei.*	**These two bacteria (*Lactobacillus plantarum, Lactobacillus casei*) can prevent biofilm formation by *Pseudomonas aeruginosa*.**
[26]	2009	Local Yogurt	18 examples of yogurt from the tribes of Fars province	22.3% of isolated *Lactobacillus* bacteria were *Lactobacillus plantarum* strains (124 isolates)	**In this study, *L. plantarum* bacteria were not dominant in local yogurt**. **The isolated bacteria can be used as a starter in the dairy industry and more studies should be done on them in the future**.
[27]	2018	dairy products	Eastern regions of the country (isolated strains were taken from the laboratory)	*Lactobacillus plantarum*	**This *L. plantarum* can produce gamma-amino acid**.
[28]	2018	local Cheese, yogurt, and Doogh made from sheep and cow milk.	90 samples of dairy products from the villages around Mahabad city	*Lactobacillus plantarum* was isolated, 46% of the strains were from cheese, 13% of the strains were from yogurt, and 36% of the strains were from Doogh.	**According to the upcoming study, cheese is a more suitable environment for the growth and purification of lactobacilli than yogurt and doogh. The dominant microflora in milk was the *Lactobacillus plantarum* bacteria.**
[29]	2021	Cow and sheep milk. and vegetables. Jug cheese	Bookan city and Selmas Cow and sheep milk and vegetables	Three strains isolated *Lactobacillus plantarum* KMJC4	**In this study, *Lactobacillus brevis* and *Lactobacillus plantarum* were introduced as probiotic strains.**

	(vegetable + cow milk) *L. Brevis* KMJC1 (cow and sheep milk) *L. curvatus* KMJC3 (cow and sheep milk) *Lactobacillus acidophilus* KMJC2 (cow milk, sheep milk).	***Lactobacillus Plantarum* had the highest antibiotic power against pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella enterica* subsp. *Enterica* serovar *Typhimurium* (*S. Typhimurium*).** ***L. plantarum* had the highest adhesion strength** **All strains except *L. curvatus* could tolerate the simulated environment of the gastrointestinal tract**. **These bacteria can be used in the pharmaceutical and food industries as probiotics.**

[30]	**2009**	**Traditional yogurt**	**194 samples of local Qashqai and Bakhtiari tribe yogurts**	**102 species of lactobacilli were identified. Some of these lactobacilli included: *L. casei, L. gasseri, L. acidophilus, L. salivarius, L. delbrueckii* and *L. plantarum***	**These strains have antimicrobial effects on enteropathogenic bacteria such as *salmonella typhi* and *E. coli*, and *L. casei* had the most effect. These isolated bacteria have probiotic properties**.
[31]	2011	traditional dairy products yoghurt, cheese	Gorout and shour, Different regions of Ardabil province (Moghan and Meshkin Shahr)	38 species of lactic acid were identified. 12 species were *Lactobacillus* and 26 species were Enterococci. In this study, *L. plantarum* was also part of these bacteria.	**The isolated strains can grow at low pH. They can tolerate pH=2.5 and bile salt of 0.3%. at pH=4 all isolates have inhibitory ability against pathogens such as *E. coli* PTCC 1399, *yersinia enterocolitica* ATCC 1159, and *Listeria innocua* DSMZ 20649. some of these isolates can also have this ability at pH=6.5.**
[32]	2016	(cheese, yogurt, curd, and tarkhineh)	100 samples from rural areas of Kermanshah (20 samples of each product)	9 types of LAB bacteria were isolated, and *L. plantarum*15HN was also a part of them	**This isolated *Lactobacillus plantarum* can grow at low pH. It can also prevent the growth of 13 pathogen-indicator bacteria**. **It prevents the adhesion of pathogens to the cells of the digestive system (the lowest adhesion of *E. coli* was when indigenous *L. plantarum* was used.)**
[24]	2017	Raw milk motal cheese	6 samples from the rural areas of Mughan Plain, Ardabil	19 strains of *Lactobacillus* were identified, of which 4 were *Lactobacillus plantarum*.	**In this study, it was found that these *lactobacillus plantarum* strains are capable of producing plantarisin A and plantarisin EF, which can have an antimicrobial effect on bacteria such as**

					***Escherichia coli* ATCC 25922, *Listeria innocua* ATCC 33090, and *Staphylococcus aureus* ATCC 25923. It was also found that those strains which produce bacteriocin have the potential to be used as starters and co-cultures.**
[33]	2012	traditional yogurt and cheese	30 samples of Chaharmahal and Bakhtiari province yogurt and cheese	43 isolates were identified	**Lactic acid production was higher at 37 C° than at 25 C°. Optimal production of lactic acid.** **proper tolerance of acidity and salt**. **in case of conducting additional studies, we can use them in the food industry.**
[34]	2021	7 yogurt samples from different parts of Behbahan city	Traditional yogurt	4 *Lactobacillus* bacteria were isolated. *L. plantarum Lactobacillus buchneri Lactobacillus casei*, *Lactobacillus acidophilus*	**All the isolated lactobacilli could produce bacteriocin that in this study, *L. plantarum* had the greatest inhibitory effect on pathogenic bacteria *Shigella dysenteriae Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus* due to the production of bacteriocin.** **The produced bacteriocins were resistant to the effects of pH and heat, but were sensitive to proteolytic enzymes and became inactive**. **The produced bacteriocins can be used as biological preservatives in the food industry**

[35]	**2017**	**cheese, whey, yogurt, and yogurt drinks (Doogh)**	**37 samples of dairy products from villages around Khorram Abad, Lorestan province**	**7 strains of *Lactobacillus casei* and 5 strains of *L. Plantarum* Two strains of *Saccharomyces cerevisiae* and 2 strains of *Bacillus subtilis* were identified**	**The strains obtained in this study are most sensitive to antibiotics and can hydrolyze fatty acid and casein. In this study, two strains of *L. plantarum* SYL5 (one strain from yogurt and the other from cheese) a strain of *S. Cerevisia*e DDy2, and two strains of *L. casei* AKL2, DDL2 had the most technological features and they can be used as starters**.
[36]	2006	Traditional Iranian Liqvan cheese	8 samples of Liqvan cheese from Liqvan village	215 strains were isolated and identified after fermentation as *Lactobacillus* (46%), *Enterococcus* (42%), and *Pediococcus* (12%). Lactobacilli were very similar to *Lactobacillus plantarum*.	**Production of CO2 from glucose. Production of CO2 from gluconate. Fermentation of ribose, mannitol, sucrose, lactose, sorbitol, and melibiose. Growth at 15 °C.**
.[37]	2016	kashk-e zard and tarkhineh	23 samples of kashk-e zard(Sistan and Balochistan province) And 27 sampels of tarkhineh from kordestan province.	One of the isolated strains was *Lactobacillus plantarum* (other strains were also isolated)	**8 samples of Liqvan cheese from Liqvan village The isolated strains had antimicrobial ability against indicator microbes, and their cell extracts also had an inhibitory effect on pathogenic bacteria.**
[38]	2014	Cheese	Cheese 24 samples of 8 types of cheese in the west of Iran	118 strains were isolated and identified.18 % of isolate was *Lactobacillus plantarum*.	**We can use the isolated strains to produce starters on an industrial scale. Starter species including *Lactobacillus agilis, Lactobacillus plantarum,* and**

					Lactobacillus casei **can be used and exploited on an industrial scale.**
[39]	2019	khiki cheese	14 samples of khiki cheese from Semnan city	105 isolates from the family of lactic acid bacteria were identified, of which 52 were *Lactobacillus* strains, 53.6% were *Lactobacillus plantarum*, 32.7% were *Lactobacillus paracasei*, and 13.7% were *L. casei*	**The isolated strains can be used for food starters.**
[40].	2017	yogurt, doogh, curd Shiraz Cheese, and Tarkhineh	200 samples of yogurt, doogh, curd Shiraz Cheese, and Tarkhineh	A total of 92 strains were isolated, including *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, of which *L. Plantarum* was also a part of them.	**among the isolated strains *L. plantarum* 15HN, *Lactococcus lactis subsp, cre moris* 44L and *E. mundtii* 50H had tolerance to low pH and bile salts, desirable antimicrobial activity, and acceptable antibiotic sensitivity, and they can be introduced as new probiotics to the food industry**
[41]	2021	Caw's and sheep's Koomeh	5 samples of Caw's and 3 samples of sheep's Koomeh from Naein City	In total, 15 bacterial isolates were identified	**6 out of 15 isolates had characteristics such as good resistance to pH=2.5. 60 ٪ of the isolated strains were sensitive to bile salts, the isolates had good antimicrobial effects and had high antibiotic resistance. The isolates could reduce 70% of cholesterol in the environment.**

[42]	2016	Chal (fermented camel milk)	Chal samples prepared from Turkmen Sahara in Golestan province	*Lactobacillus* strains: (*L. plantarum*, *L.paraplantarum*, *L. kefiri,* *L. gasseri,* *L. paracasei)* *Leuconostoc*: (*Leu. Lactis*) *Weissella*: (*W. cibaria*) *Enterococcus*: (*E. faecium*)	**Some strains showed antioxidant ability. In this study, camel's milk and cow's milk (Tehran Kaleh Company) were fermented by these strains, and *Leucnostoc lactic* bacteria had the highest radical scavenging activity compared to other strains. Finally, it was suggested that we can use the fermented milk of camels and cows as new functional foods.**
[43-45]	2016 2009 2010	Liqvan cheese	Iranian traditional Lighvan cheese over manufacturing and ripening	125 isolates were isolated, 36 of them were *Lactobacillus* isolates, and *Lactobacillus plantarum* and *L.Paraplantarum* were also present.	**in this study, *Lactobacillus paracasei* was the strain that can be used as an industrial food starter or co-culture.**
[46]	2013	Milk, Cheese, Yogurt, Doogh And Curd	10 samples of each product were collected from nearby villages and Jahrom city	In total, 50 *Lactobacillus* strains were isolated, 9 of them were *Lactobacillus plantarum* strains. Three *L. plantarum* isolates can inhibit *E. coli*, *Salmonella typhimurium,* and *H. pylori* pathogens. *L. Plantarum* strains were isolated from milk and yogurt.	**19 of these bacteria have a growth-inhibiting effect on pathogenic bacteria such as *Salmonella typhimurium*, *E. coli,* and *Helicobacter pylori*. Purification, identification, and use of *Lactobacillus* bacteria from native foods and use as probiotics in dairy products are useful for the prevention and treatment of gastrointestinal infections.**
[47]	2015	Sheep yogurt and ewe colostrum	In total, 100 samples were prepared.	125 isolates of lactic acid bacteria were isolated. 17	***L. plantarum* 17C and *L. plantarum* 13C isolated from colostrum**

				strains belonged to *Lactobacillus*, among these strains, there was *Lactobacillus plantarum*. 3 of the strains were *L. Plantarum*	**They have the ability to tolerate low pH and bile salts and have favorable antimicrobial activity, on the other hand, they also have acceptable antibiotic sensitivity.** ***L. plantarum* 17C has antiproliferative effects on the HT-29 human colon cancer cell line.**
[48]	2013	Yogurt	50 yogurt samples from northern Iran, Guilan province	*L. plantarum* was isolated along with other lactobacilli	**The isolated lactobacilli are resistant to acidic conditions and bile salts** **They have a good antimicrobial effect on the studied pathogens** **These isolates can be used on an industrial scale.**
[49]	2014	Yogurt	60 yogurt samples from Khuzestan, Khorasan, Yazd, Fars, Guilan, Mazandaran, Kerman, Shahrekord	137 isolates of *Lactobacillus* were isolated, among which *Lactobacillus plantarum* was also present	**The isolates are ready to produce volatile compounds and check their starter properties, and more studies should be done on them**.
[50]	2016	Milk and yogurt	5 samples of Khorasan Razavi goat and ewe yogurt and 1 sample of milk	102 strains were isolated, among which *Lactobacillus plantarum* was also present	**The majority of identified strains could be used as starters**. **There is a need to investigate volatile compounds for the use of strains on an industrial scale Indigenous starter culture may help preserve and enhance the properties and authenticity of traditional yogurts.**

[51]	**2012**	**yogurt and cheese**	**yogurt and cheese**	**In this study, five species of *Lactobacillus plantarum*, two species of *Lactobacillus brevis,* and one species of *Lactobacillus casei* were accurately identified from 22 isolates that were isolated from traditional dairy products.**	**They had probiotic properties.**
[38]	2014	Cheese	24 samples of traditional cheese in the West Azerbaijan region	118 *Lactobacillus* strains were isolated, and most of the isolated bacteria were *Lactobacillus plantarum*, *Lactobacillus casei, Lactobacillus delbrueckii,* and *Lactobacillus agilis.*	**They had probiotic properties and pH tolerance**
[52]	2010	Liqvan cheese	1 kg traditional Liqvan cheese (with a shelf life of 4 months	The most abundant lactobacilli isolated and identified based on biochemical and morphological tests were facultative heterofermentative lactobacilli,	**These bacteria are responsible for creating the special flavor and taste of Liqvan cheese by using the activities of lipolysis and proteolysis**.

				especially *Lactobacillus plantarum* and *Lactobacillus casei*. The dominant species isolated from Liqvan cheese were *Lactobacillus plantarum*, *Lactobacillus casei,* and *Lactobacillus paracasei*	
[53]	2019	traditional Poosti cheese	Iranian traditional Poosti cheese	The isolated species included *Lactobacillus plantarum* (S12E, S7B, S14D, S8D, S32E and S8C) and other species included *Lactobacillus paracasei* (S5D), *Lactobacillus acidophilus* (S37A), *Lactobacillus brevis* (S15B) and *Lactobacillus L. buchneri* (S15C).	***Lactobacillus plantarum* S32E bacterium is the most stable against the simulated conditions of the stomach and intestine. Also, *Lactobacillus plantarum* S12E and *Lactobacillus acidophilus* S37A were more sensitive to antibiotics and compared to the reference strain, they showed a significant ability to reduce cholesterol**.

[54]	**2015**	**Yogurt, curd and cheese**	**Yogurt, curd and cheese**	**9 Lactobacillus strains were isolated**	• **2 *L. Plantarum* strains along with one *Lactobacillus* *Lactobacillus pentosus* strain had the highest biosurfactant production capacity. The biosurfactants produced by these two bacteria showed a suitable ability in the emulsifying cation process.** **The highest amount of biosurfactant production for *Lactobacillus plantarum* strain is at 37°C and for *Lactobacillus pentoses* strain is at 30°C. The production of this biosurfactant in both strains is at neutral pH.**
[55]	2014	traditional dairy products cheese, yogurt, curd, and tarkhineh	in total, 200 samples of traditional dairy products	Molecular identification showed that the isolated strains were *Lactobacillus plantarum* 15HN (yogurt) and *Lactococcus lactis* ssp *Lactis* 44Lac (cheese)	**Among the isolates, these two strains showed significant probiotic properties. They were also resistant to bile salt and low pH. *Lactobacillus plantarum* strain 15HN showed high antagonistic activity against *Salmonella typhimurium*, *S. marcesens*, *Staphylococcus aureus*, *L. monocytogenes*, *Klebsiella pneumoniae*, *S. flexneri*, *Pseudomonas aeruginosa*, *S. mutans*, *S. sapriphyticus* subsp. *saprophytic* and indigenous *Escherichia coli*. Also, this strain was sensitive and semi-sensitive to antibiotics chloramphenicol, tetracycline, erythromycin, ampicillin, gentamicin,**

					clindamycin, sulfamethoxazole, and penicillin and resistant to vancomycin. The prescreening results showed that *L. plantarum* 15HN secretion metabolites can significantly enhance the growth of human cancer cells with no significant effects on human normal cells at in vitro condition
[56]	2013	Milk, cottage cheese, cheese (one day old), ripened cheese (three months old) Liqvan.	10 samples of milk, cottage cheese, cheese (one day old), and ripened cheese (three months old) Liqvan were studied as 4 stages of the cheese production process.	in total, 95 strains of the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus,* and *Leuconostoc* were identified and isolated in all production stages.	**The most species in all stages of production were: *Lactococcus lactis* subspecies *lactis* (25.26%), *Lactobacillus plantarum* (20%), *Enterococcus faecium* (15.78%), and *Enterococcus faecalis* (15.78%). According to the obtained results, it seems that these dominant species play an important role in the ripening and production process of Liqvan cheese and it is possible to use these species on an industrial scale**.
[57]	2020	Yogurt, cheese, Kaymak, doogh, curd and milk	50 traditional dairy products (yogurt, cheese, Kaymak, doogh, curd and milk)	16 *Lactobacillus* strains were isolated: Of which seven strains were *Lactobacillus plantarum*.	**Blood sugar levels and diabetes symptoms were significantly reduced in the diabetic rats treated with *Lactobacillus plantarum* and *Lactobacillus reuteri*.** **Also, the results showed that the rats that were affected by *lactobacillus* had the maximum weight in the fourth week and the minimum weight in the first week**

[58]	**2021**	**traditional cheese**	**6 Samples of traditional cheese**	**in total, 7 *Lactobacillus* strains were identified and isolated. The results of sequences statistical analysis showed that *Lactobacillus* species belonged to *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus acidophilus,* and *Lactobacillus casei*.**	**It was also found that these strains have good probiotic and technological potential. The results of safety and health aspects showed that these strains can be used for human consumption. In this research, the highest self-aggregation and coagulation were observed in *Lactobacillus acidophilus* strain B14 (51.3%) and *Lactobacillus plantarum* strain B20 (43.6%). *Lactobacillus plantarum* strain B20 and *Lactobacillus acidophilus* strain B14 showed the highest probiotic activity.**
[59]	2021	Yogurt, cheese, milk and whey	10 samples of Yogurt, 5 samples of cheese, 8 samples of milk, and 5 samples of whey	24 strains of LAB, including *Lactobacillus plantarum*	**The results of this study show that the new C1 strains of *Lactobacillus plantarum* together with chitosan nanoparticles can have synergistic effects in reducing aflatoxin type B1 in food products. Also, the results showed that this strain has extensive microbial activity and good probiotic activity, and it is also resistant to erythromycin, Fusidic acid, gentamicin, kanamycin, nalidixic acid, neomycin, Ofloxacin, and vancomycin antibiotics**
[7]	2017	Koozeh Paneer (Iranian Koozeh	five samples of Koozeh cheese were randomly collected from the rural areas of Mazandaran	8 strains of LAB including 4 *Lactobacillus plantarum*	**Antagonistic activity against some pathogens (highly inhibiting the activity of *E. coli*). ((According to the analyses, *L. fermentum***

	traditional cheese)	province including Babol, Cherat, Alasht, Shirgah,and Firouzkouh.	**(MT.ZH893 and MT.ZH993) and *L. plantarum* MT.ZH593 are the best probiotics among the tested ones. Results showed that all the LAB strains were potetial probiotics to develop new formulations for designing functional food products with health-promoting properties)).**

2- background

In Iran, research on lactobacilli primarily focuses on their application in the food industry, particularly in enhancing dairy product quality [1]. Due to Iran's diverse local products, much research has been conducted to identify and isolate indigenous bacterial strains. For instance, a study by Salimi et al in 2013 isolated *Lactobacillus* strains from raw milk in Ardabil province. These indigenous strains include *Lactobacillus plantarum*, *Lactobacillus. acidophilus*, *Lactobacillus. curvatus*, and *Lactobacillus. casei*. Similarly, *Lactobacillus. plantarum* and *Lactobacillus. brevis* were found in raw milk from Sarab City, Ardabil and their acidic activity and performance were confirmed suggesting their potential as starter in the industry.[6]. This topic is so important that other countries also isolate indigenous bacteria for their commercial use in industry because indigenous bacteria have the potential to produce regional local products on an industrial scale. In Iran, most research on isolating and identifying lactobacilli in food has focused primarily on dairy products. Fewer studies have explored lactobacilli in other types of food [60]. According to the studies, the most numerous bacteria in ripened cow and sheep cheeses are *Lactobacillus plantarum* and *Lactobacillus casei*[52]. Also, according to the research conducted by Ahmadi et al., *Lactobacillus agilis* and *Lactobacillus plantarum* were the dominant species isolated from Liqvan cheese made from sheep's milk[61].

2-1- some researches on indigenous products

In the study conducted by Qadiri Afshar et al. in 2018, it was shown that *Lactobacillus* bacteria can be isolated from olives. In this research, 4 strains of *Lactobacillus plantarum* and 1 strain of *Lactobacillus acidophilus* were isolated from Iranian olive samples [62].

In 2015, Emami et al. showed that *Lactobacillus plantarum* isolated from local Iranian olives has antimicrobial ability against *Shigella dysentery* (PTCC1188) and *Escherichia coli* (PTCC1399). In this research, 57% of the isolated strains were *Lactobacillus plantarum*[63].

Also, this bacterium has been isolated from the raw sources of indigenous Iranian plants, such as The rhizosphere of Lenjan rice roots in Isfahan province. this isolated bacterium has probiotic properties [64].

furthermore, in the study conducted at Lahijan Azad University, researchers identified 23 isolates of *Lactobacillus* on 80 samples of fresh vegetables (cabbage, silage, and cucumber) in spring. Among these, three isolates of *Lactobacillus plantarum*, two isolates of *Lactobacillus casei*, and one isolate of *Lactobacillus brevis* demonstrated the highest capacity to withstand acidic conditions and bile salts within the digestive system. Also, these isolated strains had antimicrobial properties

against pathogenic bacteria, *Staphylococcus aureus* PTCC 1431, *Salmonella typhimurium* PTCC 1639, and *Escherichia coli* PTCC 1399 [65].

Lactobacillus plantarum is commonly found in isolates from local products in Iran and other countries, and it exhibits unique efficiency. Notably, research by Antara NS et al. in 2004 demonstrated that using multiple starters have advantages on microbial and physicochemical characteristics of orutan (an Indonesian fermented sausage). In fact the use of Multiple starters effectively neutralizes the activity of *Enterobacteriaceae.* In contrast, the use of single starters results in delayed neutralization. *Lactobacillus plantarum and Pedicoccus acidolactis* are particularly effective as multiple starters. [66].

In 2007, Scherlink and Vandami employed 19 different sugars for the biochemical identification of lactobacilli. These sugars included glucose, galactose, fructose, mannose, arabinose, rhamnose, xylose, lactose, sucrose, trehalose, cellobiose, maltose, mannitol, raffinose, inositol, sorbitol, salicin, gluconate, and squalene. Notably, during this test, strain IL 127 exhibited similar behavior to *Lactobacillus plantarum*, fermenting rhamnose, while *Lactobacillus plantarum* itself did not[1].

Through dipeptidase, aminopeptidase, endopeptidase and proteinase activities in *Lactobacillus plantarum* and other local mesophilic lactobacilli isolated from Parsabad traditional metal cheese, the production of small peptides and volatile amino acids was observed. These Component contribute to the creation of aromatic compounds and a pleasant taste in these local cheeses. Research indicates that mesophilic *lactobacillus* bacteria, including *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lactobacillus paracasei*, play a crucial role in enhancing the flavor profile of traditional Metal Cheese. [2]

On the other hand, *Lactobacillus plantarum* bacteria isolated from indigenous Iranian dairy products can be used as a probiotic in the industry [20, 21, 67].

In a research conducted in 2013 on isolating *Lactobacillus plantarum* from cheese and exploring its potential as a probiotic, researchers discovered that *Lactobacillus plantarum* CJLP55 possesses unique probiotic properties. Notably, this strain exhibits high resistance to conditions resembling the digestive system and is also resilient against antibiotics like vancomycin and polymyxin B. Furthermore, it demonstrates an antibacterial effect against pathogenic strains, making it valuable for various applications in the dairy industry [67].

In a study by Hirano et al., the cell-free supernatant from *Lactobacillus crispatus* and *Lactobacillus plantarum* exhibited inhibitory effects on various pathogenic bacteria, including *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Staphylococcus aureus*. These effects were observed through the creation of inhibition zones [68].

also In the study of Pour Shaker et al., the antimicrobial effect of indigenous *Lactobacillus Plantarum* isolated from local dairy products in the Nadushan region of Yazd was observed [69]. *Lactobacillus plantarum* bacterium was also isolated from Iranian Liqvan cheese, which has applicable and important characteristics. this bacterium exhibits high efficiency in terms of both acid production and proteolytic activity. It should be said that Besides *Lactobacillus plantarum*, other lactobacilli demonstrate similar characteristics. For instance, indigenous *Lactobacillus casei*, isolated from Liqvan traditional cheese, exhibits properties comparable to those of indigenous *Lactobacillus plantarum* [36].

In 2008 Kubota et al. investigated biofilm formation by LAB and their resistance to environmental stresses and it was discovered that

among the three examined *Lactobacillus* strains, only *Lactobacillus plantarum* and *Lactobacillus brevis* were capable of forming stable and concentrated biofilm surfaces [70]. This highlights the significance of this bacterium in biofilm formation and its practical applications in the industrial context.

On the other hand, the *Lactobacillus.plantarum* strain isolated from Liqvan cheese has a high ability to produce exopolysaccharides and has been able to significantly reduce inflammation and heal skin wounds in rats [71].

The results of another study by Nasrabadi et al. demonstrated that *Lactobacillus plantarum* isolated from Iranian traditional cheese has a significant effect on stomach ulcers caused by acetic acid in rats [72].

In research which is conducted by Hernando et al., molecular analysis showed that two strains isolated from dairy products (traditional cheese and yogurt) belonged to *Lactobacillus plantarum* strain HBM-IAUF-1 and *Lactococcus lactis* HBM-IAUF-8. In this study, *Lactobacillus plantarum* HBM-IAUF-1 exhibited the highest cytotoxicity against SK-BR3 cancer cells after 72 hours, with a concentration of 1000 µl/ml. Similarly, *Lactococcus lactis* HBM-IAUF-8 demonstrated the highest cytotoxicity under the same conditions, but at a concentration of 500 µl/ml. The findings indicated that incorporating traditional and organic dairy products, along with increasing utilization of probiotics in the food industry, may contribute to cancer prevention [73].

"Nasrabadi et al. isolated lactobacilli strains from Babol and Semnan cheeses. These strains demonstrated in vitro tolerance to acidic and bile salt conditions, as well as the ability to bind to Caco-2 cells, which was variable. Furthermore these strains could potentially colonize the human intestine, although further studies are needed to confirm this [74].

In a 2016 study by Ghafourian et al., exopolysaccharide-producing bacteria, including *Lactobacillus plantarum* and two other *Lactobacillus* strains, were inoculated into camel milk. As a result, lactose fermentation products such as lactic acid and extracellular polysaccharides were produced. These modifications enhance the acceptability of the final product for consumers. Additionally, the antioxidant activity of the product increased, making it suitable as a prebiotic offering. On the other hand, keeping this product at the temperature of the refrigerator after 14 days, increased its antioxidant properties, which means that we can consider a shelf life of 14 days in the refrigerator for such a product [75].

In another study focused on the ripening process of a specific type of Dutch cheese, it was found that *Lactobacillus plantarum* and *Lactobacillus casei* convert citrate into several compounds, including CO_2, acetone, diacetyl, acetate, and 2,3-butanediol. Notably, CO_2 gas contributes to creating pores within the cheese, while the other mentioned compounds enhance the cheese's favorable aroma and taste. [76].

"In a study by Abdolahi et al., it was demonstrated that *Lactobacillus plantarum* can inhibit the growth of *Helicobacter pylori* in mixed culture under in vitro conditions. This inhibition is attributed to the production of lactic acid and bacteriocin. furthermore it could be used as a probiotic for in vivo research [77].

In a separate study, *Lactobacillus plantarum* isolated from camel milk also exhibited this capability and caused the largest diameter in the inhibition zone of *Helicobacter pylori* bacterium. As a result, the consumption of probiotic foods has been recommended to the public and people who suffer from digestive diseases [78].

In another study, *lactobacillus* bacteria have inhibited the growth of this pathogen and an inhibition zone has been formed [46].

Research shows that this bacterium can be used to produce functional drinks with health benefits for consumers. Rahim Abadi et al. demenstrated that it is possible to produce a synbiotic malt

drink with suitable nutritional and biochemical characteristics by using three probiotic strains of *Lactobacillus plantarum*, *Lactobacillus casei,* and *Lactobacillus acidophilus*. On the other hand, synbiotic samples with probiotic microorganisms had better antioxidant activity than the control sample.in this case, *Lactobacillus plantarum* bacterium had less antioxidant activity than the other two bacteria [79].

Lactobacillus plantarum has the ability to produce plantaricin bacteriocin, which finds application in the food industry as a preservative. Additionally, the optimal growth conditions for *Lactobacillus plantarum* isolated from Iranian Tarkhineh under in vitro conditions(table 2) make it a potential candidate for producing probiotic foods [80].

Table 2. The optimal condition for the growth of *Lactobacillus plantarum* T5jq301796.1 bacterium isolated from Iranian Tarkhineh under invitro condition.

Measured parameters	**The optimum condition of each parameter**
pH	**7.26**
Glucose	**25.96 gl-1**
Yeast extract	**1.82%**
Stirring	**40rpm**
Temperature	**37-40°C**
maximum viable cell in the batch fermentation	**10^{10} CFU ml-1**

Iranian food-derived *Lactobacillus plantarum* bacteria, in addition to the mentioned characteristics, have the ability to mitigate the harmful effects of aflatoxin B1 in humans. Their use represents a suitable biological solution for reducing this toxin and enhancing food safety [81].

According to the studies, the role of mesophilic lactobacilli in the production of aromatic substances and the aroma and flavor of traditional cheeses has been confirmed. For example, it has been demonstrated in a research on Fossa (pit) cheese that *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lactobacillus paracasei* bacteria generate free amino acids and small peptides through their aminopeptidase, dipeptidase, proteinase, and endopeptidase activities. These compounds ultimately contribute to the delightful aroma and flavor of the cheeses. on the other hand, *Lactobacillus plantarum* and *Lactobacillus casei* bacteria produce co2, which plays a role in creating voids in cheese [82].

In 2019, Gandomi et al. investigated the adhesion properties of 5 strains of *Lactobacillus plantarum* isolated from traditional Siahmazgi cheese. The results showed that these 5 strains have favorable coagulation, hydrophobicity, and cell adhesion. These isolates could be good candidates as promising probiotics for use in functional foods including dairy products [83].

Lactobacilli isolated from Metal Cheese have shown different technological characteristics. Strains of *Lactobacillus brevis* (m4, m9) and *Lactobacillus plantarum* (m16) exhibit significant proteolytic activity. Additionally, *Lactobacillus. plantarum* m19 demonstrates the highest acid production activity. These strains hold promise as potential candidates for use in the industry as starters or pseudo starters [84].

On the other hand, Yazdi et al. identified and isolated 28 strains of LAB using the 16S rRNA method from 7 samples of Zaboli yellow curd. The majority of these isolates were classified

within the genus *Lactobacillus*, accounting for 24.09% of the strains. They can be used on an industrial scale [85].

In another study conducted by Behbahani et al. *Lactobacillus plantarum* L15 which was isolated from Hureh Khuzestan, exhibits an antagonistic impact on *E. coli* bacteria. This particular bacterium demonstrates anti-adhesion properties and competes effectively against the pathogen and making it a viable alternative for combating this pathogen [86].

In a research studied by Farhang Far et al., they investigated 22 isolates of *Lactobacillus plantarum* obtained from Siahmazgi cheese. Their findings revealed that 5 strains exhibited favorable characteristics, including tolerance to acidic conditions and bile salts, robust growth kinetics at low pH, survival in simulated digestive conditions, and antibacterial activity. Notably, strains F2 and F7 demonstrated even greater resistance. Consequently, these specific strains hold promise as potential new probiotic candidates for future investigations [87].

On the other hand, Traditional Iranian yogurts are a valuable source of LAB. so it is necessary to develop more quantitative and qualitative research on the isolation and identification of new LAB isolates from local yogurts to introduce dairy starters and probiotic microorganisms [88].

In the study conducted by Joghatai et al. on *Lactobacillus plantarum* isolated from Liqvan cheese and human feces, they concluded that due to the favorable probiotic and anticancer properties, they could be used for industrial and clinical applications [89].

In a 2020 study by Shahrampour et al, they demonstrated that pectin alginate edible film significantly enhances the survival of *Lactobacillus plantarum* KMC45 bacterium. These edible films could serve as a promising medium for delivering probiotics and creating functional foods [90].

In a study conducted by Abutaleb et al. in 2020, they demonstrated the feasibility of isolating and identifying Lactic Acid Bacteria (LAB), particularly *Lactobacillus*, from raw camel milk. The findings revealed that these isolated strains not only exhibit protective and functional effects for consumers but also hold potential applications in both the dairy and pharmaceutical industries. [91].

in their 2015 study, Gandomi et al. isolated 71 strains of *Lactobacillus* from Siahmazgi cheese. To assess bacterial growth profiles, they employed a modified liquid culture medium under specific conditions similar to those found in fermented sausages. Among these strains, 5 *Lactobacillus plantarum* strains exhibited high acid activity and pH reduction capabilities when cultured in MRS medium at 30°C for 24 hours and 40°C for 48 hours, resulting in an average pH of approximately 4. Remarkably, all 5 strains (except LSCD11) demonstrated growth tolerance in a 10% salt. Additionally, these strains displayed antimicrobial properties against pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7. Notably, the LSCD7 and LSCD14 strains stood out for their excellent technological attributes, making them suitable candidates as starters in the production of fermented meat products [83].

In a 2018 study conducted by Adeli et al. they isolated and identified *Lactobacillus plantarum* from raw camel milk sourced from different regions of Kerman. Alongside other bacteria such as *Weissella confusa*, *Leuconostoc mesenteroides*, and *Weissella paramesenteroides*, *Lactobacillus plantarum* exhibited significant antibacterial activity against two pathogens: *Staphylococcus aureus* subsp *aureus* PTCC1431 and *E. coli* ATCC25922. Notably, these isolated probiotics demonstrated a stronger antimicrobial effect against *Staphylococcus aureus* bacteria compared to *E. coli* [92].

In a 2020 study conducted by Mousavizadeh et al. in Mazandaran province, researchers identified 5 strains of *Lactobacillus plantarum* bacteria. These strains were investigated and found to exhibit inhibitory effects against the *Pseudomonas aeruginosa* bacterium.[93].

In their research on the antifungal properties of *Lactobacillus plantarum* and *Lactobacillus brevis* bacteria isolated from Iranian Liqvan cheese and Metal cheese, Afzali et al. found that *Lactobacillus plantarum* exhibited the highest inhibitory effect or clear zone after *Lactobacillus. brevis*. This effect was compared to the indicator yeasts *Rhodotorula mucilaginosa PTCC 5257*, *Saccharomyces cerevisiae PTCC 5269*, and *Kluyveromyces lactis PTCC 5185*[94].

In the study, researchers isolated 16 strains from Metal Cheese, out of which 6 strains were identified as *Lactobacillus plantarum*. Additionally, it was demonstrated that *Lactobacillus brevis* exhibited the highest antimicrobial ability against the specified yeasts [95].

In 2014, Vasei et al. isolated 54 strains of *Lactobacillus* from Tarkhineh, a traditional Iranian fermented cereal-based food. Among these isolates, there were 19 strains of *Lactobacillus plantarum*, 17 strains of *Lactobacillus fermentum*, 8 strains of *Lactobacillus brevis*, 9 strains of *Lactobacillus pentosus*, and 1 strain of *Lactobacillus diolivorans*. The rep-PCR profiles revealed that *Lactobacillus plantarum* exhibits high intraspecies diversity. Notably, three of these strains demonstrated significant probiotic potential, including two strains of *Lactobacillus plantarum* and one strain of *Lactobacillus fermentum* [97]. In another study conducted by Nami et al. in 2019, *Lactobacillus plantarum* YS5 was isolated from homemade yogurt in different regions of the country. This bacterium exhibits the ability to reduce high cholesterol and has a strong impact on lowering serum cholesterol levels. Notably, it lacks dangerous properties such as BSH activity, antibiotic sensitivity, and hemolytic activity. On the other hand, the specific strain demonstrates significant anti-pathogenic effects, along with a high tolerance to acid and bile salts, hydrophobicity, and self-aggregation. Further research is recommended to explore the functional effects of this bacterium for potential use as a probiotic strain in the industry. The study findings highlight its high probiotic potential, suggesting that *Lactobacillus plantarum YS5* could be employed in the food industry to create low-cholesterol products for individuals with hypercholesterolemia [98].

In their study, Bahadori et al. examined 131 *Lactobacillus* bacteria from 123 samples of pasteurized and local milk and cheese. The primary objective was to identify and investigate the presence of the beta-galactosidase enzyme gene in *Lactobacillus plantarum* strains. Notably, this gene was observed in the standard strain *Lactobacillus plantarum RITCC 1273* and 29 other samples of *Lactobacillus plantarum* band bp 399. The findings suggest that utilizing probiotic bacteria that produce beta-galactosidase can enhance the nutritional value and digestibility of dairy products, benefiting individuals with lactose intolerance [99].

In a 2011 study focused on isolating lactic acid bacteria (LAB) from local dairy products, researchers obtained 20 isolates from *Lactobacillus* subspecies. it was found that most of the strains absorbed over 75% of cholesterol from their environment and demonstrated strong antimicrobial effects against pathogens. Additionally, these bacteria displayed resilience in the acidic pH of the stomach and intestinal environment. Notably, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus casei* isolated from local

yogurt and cheese also exhibited antimicrobial properties and efficient cholesterol absorption. Based on these findings, the lactobacilli isolated from local products hold promise for use in functional food production and as starters [23].

In a 2020 study by Mousavizadeh et al. focusing on local Iranian dairy products (yogurt, curd, and kefir), researchers isolated 5 strains of *Lactobacillus plantarum* bacteria with antimicrobial abilities. Notably, these isolated strains demonstrated the ability to inhibit the growth of Escherichia coli. Furthermore, increasing the dosage of this bacterium plays a nutritional role in promoting overall health. As result it is recommended to utilize these bacteria for the production of starter cultures [100].

In a study conducted in Iran on curd, pickles, and tuna, researchers successfully isolated lactic acid bacteria (LAB) from these foods. The dominant bacterium identified in this study was *Lactobacillus plantarum*. Notably, the isolated strain exhibited significant effectiveness in preventing pathogenic bacteria associated with foodborne diseases. Importantly, this strain did not produce biogenic amines and demonstrated the ability to produce lipase enzymes. However, it was not resistant to common antibiotics. These LAB strains hold potential for use as starter cultures in the food industry and can also use as preservatives to extend shelf life. Additionally, *Lactobacillus plantarum* strains (LBC1, LBP1, and LBT1) isolated from fermented foods showed inhibition against pathogens such as *Vibrio parahaemolyticus*, *Salmonella Typhimurium*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Escherichia coli*. [101]. Moreover, In the study conducted by Hamzelu et al. *lactobacillus* bacteria were isolated from local yogurt in Tehran, Sanandaj, and Golpayegan, Iran. Among these isolates, certain lactobacilli strains were found to have the capability to produce group B vitamins (B2, B3, B6, B9). it is recommended that these bacteria can be used in the production of functional foods [102].

In a 2014 study, researchers investigated 18 samples of gelatinous cheese and 15 samples of yogurt prepared from Chahar Mahal and Bakhtiari provinces. During this study, *Lactobacillus plantarum* was identified. Specifically, two strains were found in yogurt, and five strains were detected in cheese [103].

In another study by Jafarei et al. in 2019, researchers isolated *Lactobacillus plantarum* bacteria from 60 samples of local Iranian dairy products, including yogurt, doogh, dry curd, and semi-dry curd (15 samples of each product). These samples were collected from villages in the Fars province. [104].

3- Results

Numerous research studies conducted by various researchers have focused on local products such as Khiki cheese, Olive Raw milk, Doogh, Yogurt, Horreh, and Tarkhineh. These products are prepared from domesticated animals and plants found in different regions. The investigations have revealed the presence of several lactic acid bacteria, including *Pediococci* such as *Pediococcus acidulactis*, *Lactococci* sach as *Lactococcus lactis* subspecies *lactis*, *Enterococci* sach as *Enterococcus faecium*, *Bifidobacteria* and Lactobacilli including *Lactobacillus casei* and *Lactobacillus fermentum*, *Lactobacillus delbrueckii*, *Lactobacillus brevic*, *L.buchneri* and *Lactobacillus acidophilus* and specially *Lactobacillus plantarum*. these isolated bacteria have capabilities such as inhibiting the growth of pathogens, tolerating acidic and alkaline conditions, probiotic properties, and the ability to produce exopolysaccharides. in addition to these features, they have industrial and commercial potential and can be used in the industry [15, 17, 21, 105].

The studied sources show that the bacterium *Lactobacillus plantarum* as a potential local

probiotic has a high ability to produce functional fermented dairy products because it brings many benefits from different aspects of health and functional properties for the consumer. On the other hand, Recent research on Iranian non-dairy products, including olive, has demonstrated the efficacy of this bacterium in preventing and treating infections caused by pathogens such as *Shigella dysentery* and *Escherichia coli* as a practical and important method [63]. Studies also show that soil rhizosphere bacteria, especially *Lactobacillus*, play a great role in plant health and growth in different ways [106].

Given the growing preference for industrial dairy products over traditional ones and the high cost of imported commercial starters, there is an opportunity to use this bacterium for commercial purposes. By doing so, we can produce dairy products with an authentic Iranian flavor profile while also preserving this valuable national genetic resource. Moreover, there exists an opportunity to decrease reliance on imported commercial starter cultures from foreign nations. This approach not only yields economic benefits but also mitigates foreign exchange outflows from the country. It is recommended that more studies are needed for the exploitation of this probiotic bacteria and the properties of biofilm formation by them and other indigenous bacteria. Furthermore, there is a need for in-depth investigation into the feasibility of developing novel functional products using these bacteria in the form of multi-strain probiotics and single, double, or multiple Starters.

4- CONCLUSION

Research on Iranian local dairy and non-dairy products indicates that *Lactobacillus* bacteria are commonly found in most dairy foods. Notably, no adverse effects have been reported, and these bacteria exhibit several positive effects. Based on findings, some native strains of *Lactobacillus plantarum* can withstand low pH (2.5) and bile salts, grow at 15 °C, and produce postbiotics like γ-amino acids, lactic acid, and plantaricins A and EF; they inhibit a range of pathogens (e.g., *E. coli*, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*, *Shigella dysenteriae*, *Listeria innocua*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Helicobacter pylori*, *Klebsiella pneumoniae*, and *Salmonella flexneri*), help lower environmental cholesterol, work synergistically with chitosan nanoparticles to reduce aflatoxin B_1 in food products, Production ability CO_2 from glucose and galactose, and ferment sugars such as ribose, mannitol, sucrose, and sorbitol.

These findings have cleared the path for future research with the aim of isolating, identifying, and industrially utilizing these bacterial strains. According to previous studies, certain *Lactobacillus plantarum* native strains from various regions of Iran have potential probiotic characteristics and, depending on the unique features of each isolated strain, could be used as starter culture applications and production of industrial probiotic foods. However, *Lactobacillus plantarum* strains isolated from some native foods are not fit for commercial applications due to their poor technical resilience, low competitiveness, and weak antibacterial characteristics. As a result, both researchers and industry professionals must carefully assess the type and origin of native *Lactobacillus plantarum* strains before using them in industrial processes. This review study showed that each native *Lactobacillus plantarum* isolate has distinct characteristics, which will make their unique properties extremely valuable for developing co-starters, single or mixed starter cultures, and future superfoods (including pro-, pre-, post-, para and metabiotic products). Their presence contributes to unique organoleptic properties in

certain dairy products. These results show that we can use this indigenous bacterium in the production of mixed or multi-strain starters to improve the aroma and taste properties of industrial dairy products.

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ردیابی و سرنوشت *لاکتوباسیلوس پلانتاروم* جدا شده از لبنیات بومی ایران: مروری جامع

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چکیده

ایران کشوری پهناور و وسیع با تنوع اکوسیستمی و زیستی بالایی است؛ که در آن محصولات بومی خواه لبنی و غیر لبنی زیادی تولید می‌شوند. این محصولات و گیاهان بومی دارای باکتری‌های اسیدلاکتیک متنوعی هستند که می توان آن‌ها را جداسازی و شناسایی نمود. گروهی از این محصولات، فراورده‌های لبنی بوده و منبع غنی از باکتری *لاکتوباسیلوس پلانتاروم* هستند. این باکتری نقش اساسی در جلوگیری از رشد پاتوژن‌ها داشته و همچنین قادر به تحمل شرایط اسیدی، قلیایی و تولید اگزو پلی‌ساکاریدها می‌باشد. به عنوان یک پروبیوتیک، *لاکتوباسیلوس پلانتاروم* می تواند برای مصرف کنندگان سلامت محور باشد. مطالعه ما به ویژگی‌های باکتری *لاکتوباسیلوس پلانتاروم* بومی جداشده از فراورده‌های لبنی ایران، ذخایر ژنتیکی بومی این باکتری و دیگر باکتری‌های خانواده *لاکتوباسیلاسه* پرداخته است. این موضوع زمینه را برای مطالعات بعدی در راستای جداسازی، شناسایی و کاربرد صنعتی این باکتری فراهم نموده است. از نتایج مطالعات پژوهشگران پیشین می‌توان این‌چنین استنتاج کرد که از یک سو سویه‌های *لاکتوباسیلوس پلانتاروم* بومی نقاط مختلف ایران پروبیوتیک بوده و نقش استارتری دارند؛ لذا می‌توان با توجه به ویژگی‌های هر سویه جداسازی شده، آن‌ها را در مقیاس صنعتی برای تولید فراورده‌های پروبیوتیکی و کاربردهای استارتری مورداستفاده قرارداد. و از سوی دیگر باید توجه داشت که سویه های *لاکتوباسیلوس پلانتاروم* جداسازی شده از برخی از غذاهای بومی به دلیل ضعف تکنولوژیکی و پتانسیل رقابتی و آنتی میکروبی پایین توانایی صنعتی شدن ندارند. در نتیجه محققان و صنایع در صورت بهره برداری صنعتی از *لاکتوباسیلوس پلانتاروم* بومی باید در انتخاب نوع و منبع این میکروارگانیسم دقت نمایند. در این مطالعه مروری مطابق با اطلاعات بدست آمده مشخص شد که هر سویه جداسازی شده، دارای ویژگی های متفاوتی با دیگر سویه های جداسازی شده‌ی *لاکتوباسیلوس پلانتاروم* بومی می باشد و بهره برداری از این باکتری، چشم اندازی شگرف در تولید کمک استارترها، سینگل و میکس استارترها و سوپرفودهای آینده خواهد بود.

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The effect of adding apple peel powder on the physical and sensory properties of biscuits

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ABSTRACT

This study examined the viability of using APP as an initial raw material to make nutritional fiber powders and cakes targeted at those with diabetes. The investigation into using apple peel powder (APP) in biscuits. Apple peel powder was added to wheat flour at percentages of 1%, 2%, and 4% to make the biscuits. The sensory qualities and physical characteristics of the biscuits were examined. When the powder's chemical and water solubility index were 210±5.77350%, 216±12.01850%, and 0.34±.00577g/mL and 7.33±.66667%, composition was examined, apple powder had high values for its fiber, protein, and ash contents (13.66±.88192%, 3.03±.31798%, and 2.89±.67185, respectively). The functional qualities of the powdered apple peels showed that the peels' capacity to absorb water or oil respectively. The ability to absorb fat and water, as well as to biscuits' diameter, thickness, and spread ratio increased with the addition of apple peel powder, and the value increased with the degree of substitution. increase from 12.3333±.33333 to 13.5000±.28868 cm, 5.0300±.03512 to 5.3000±.05774 cm at level 2%APP, and 2.4333±.00882 to 2.6700±.00577 % at level 4%APP, respectively. The biscuits that had 1% APP had acceptableensory quality. According to this study, adding APP can result in fiber-rich cakes and bread with s superb texture qualities, low water content, and good sensory quality

1-Introduction

Most people consider biscuits to be among the most delectable baked goods, as they are leavened. Among bakery products, biscuits make up the largest group of snack items. These foods are steady and come with benefits such as being ready to eat, being widely consumed, having a long shelf life, and having good eating quality. Since practically all consumer types accept and consume biscuits in many different nations, they provide a useful vehicle for nutritional enhancement (Sadal *et al*., 2018).

These wastes represent a major environmental risk because of their great susceptibility to microbial deterioration. It is necessary to control and make useful use of the food waste.

Although Although fruit peels are usually thrown out into the environment as trash, they are a vital source of organic antioxidants. and ant nutrients due to the various phytochemicals present in them, just like in any other plant (Agbaje *et al., 2020*). A biscuit's nutritional quality and acceptability are enhanced by the presence of fiber, which can be achieved by varying the proportion of whole grains other than wheat in the raw ingredients or by adjusting the proportion of fiber in simple recipes. Apple fiber is mostly composed of carbohydrates and dietary fiber, with trace levels of protein, fat, and ash. It is produced from apple pulp (apple pomace) by drying and grinding it into a powder without any bleaching or refining. Additionally, apple fiber is a strong source of phytochemicals, particularly flavonoids and phenolic acids, which have been linked to antioxidant capabilities (Alsuhaibani, 2015).

Phenolic chemicals are abundant in apples; the total extractable phenolic content of fresh apples varies between 110 and 357 mg/100 g. It is well known that apples' peels have a significantly higher concentration of total phenolic chemicals than their flesh. Additionally, there are differences in the distribution and type of these phytochemicals between the apple's peel and flesh (Kelly *et al.,* 2003).

2-Materials and Methods

We bought yellow apples(*Malus domestica)*, wheat flour (72% extraction), and other supplies for making biscuits from a Basrah local store

Apple peel powder preparation.

To get rid of any superfluous foreign contaminants, the apple was first cleaned with tap water and then with distilled water. Using kitchen knives, the thoroughly cleaned fruit was peeled by hand. After being oven-dried for 72 hours at 37°C, the peels were milled into an acceptable powder (Agbaje et al., 2020).

Analyses of apple peel powder.

After oven drying to a constant weight at 105°C, the moisture content was measured.

The Kjeldahl method was used to measure the protein content of APP (N = 6).

, ash was measured two grams of each sample was put in a temperature-controlled furnace that had been warmed to 600 degrees Celsius. For two hours, the sample was kept at this temperature. This technique uses ether extraction followed by solvent evaporation to estimate the fat content of the APP. A

percentage of the initial sample weight is used to express the fat content. The crude fiber content is then ascertained by filtering, washing, and drying the sample. A proportion of the initial sample weight is used to represent crude fiber. and carbohydrate contents were measured By difference, the total carbohydrate was obtained l.

Physico-chemical characteristics of apple peel powder

Bulk Density

A 10 mL tar-coated cylinder was gently filled with APP. Up until the sample level stopped changing, the cylinder's bottom was gently tapped against the surface. Bulk density (g/mL) was used to record the sample weight per unit volume (Weng *et al.*, 2020).

Water and oil absorption capacities

The Dhankhar *et al*. (2019) approach was used to evaluate the water and oil holding capacities. Each powder sample was precisely weighed (1 g) in a weighing dish, and it was then mixed with 10 ml (V1) of either refined vegetable oil or distilled water to create suspensions. After 30 minutes of aging, the suspensions were centrifuged for 10 minutes at 2200 g. supernatant was transferred into a 10 ml graduated cylinder following centrifugation, and volume was recorded as V2. The amount of water absorbed by the flour sample was represented as a percentage .

Water solubility index and swelling capacity

A method for figuring out a sample's swelling power was established by Dhankhar et al. (2019) using the water solubility index (WSI) and swelling capacity (SC). One gram of each flour sample was mixed with fifteen milliliters of distilled water and shook to create a suspension. After thatthe suspensions were maintained in an 80ºC water bath and centrifuged for 10 minutes at 3000 rpm.

SC = wt of sediment x 100/wt of powder −wt of dried solids in supernatent

Making biscuits

With a few minor adjustments, biscuits were made according to a standardized recipe (Dhankhar *et al., 2019*). The primary ingredient was wheat flour was mixed. at1%, 2%, and 4%, compared the control . To prepare the dough, wheat flour (100 g) and(3g) baking powder. It was combined with sugar 30g and 20 g fat , water 16 ml and then the necessary ,added the appropriate quantity of water to make the dough smooth. . The proportionate amount of sugar was replaced with date powder in the recipe for the partially substituted biscuits. The dough was formed into a circular shape with a diameter of 5 cm using a cutter after being smoothed out with a rolling pin to create a flat foundation with a thickness of 10 mm. The biscuits were baked for thirty minutes at

180°C in a laboratory oven. The biscuits were then removed from the oven, allowed to cool, and stored for further research in an airtight container. constant across all batches

Analytical physical

Three biscuits were arranged side by side and rotated ninety degrees to determine the diameter. After measuring the diameter (in

centimeters) of three biscuits once more, the mean value was found. To measure thickness, three biscuits were stacked one on top of the other,. By splitting up dividing the average biscuit diameter by the average biscuit thickness, the spread ratio was found. (Dhankhar *et al., 2019).*

Sensory evaluation

Utilizing a nine-point hedonic rating system as per Dhankhar et al. (2019), a panel consisting of eight members from the Food Science Department in Iraq. individuals conducted sensory analysis of the biscuits. The biscuits', color, flavor , appearance, texture, and overall acceptability were all evaluated.

Analytical statistics

The results were statistically analyzed using a one-way analysis of variance (ANOVA) with three replications in a randomized block design. The program was used to perform the Least Significant Difference test (LSD) at $p<0.05$ The SPSS (2019) was used to do statistical analysis.

3-Results and Discussion

The proximate analysis of the apple peel powder .

The proximate analysis of the apple powder used in this investigation is compiled in Table 1. The apple peel powder had a higher quantity of fiber (13.66±.88192%), ash (2.89±.67185%), and a lower content of fat (2.57735%), protein (3.03±.31798), and carbohydrates (71.40%). These findings are lower than those of Agbaje et al. (2020), who had previously reported that apple peel contains 2.60% protein, 14% fiber, and 1.68 ash, respectively.

Table 2 : Proximate analysis of apple peels powder

properties	Amount%
Moisture	7±.57735
Protein	3.03±.31798
fat	2±.57735
ash	2.89±.67185
fiber	13.66±.88192
Carbohydrate	71.40±.67234

These findings concurred with those of Kamaljit *et al.* (2011) and Reis et al. (2012). The highest potential for integration in the bakery sector to produce high-fiber biscuits is found in the fiber content of apple peels. Apple peels contain between 14 and 30 percent crude fiber by dry weight.

The study utilized apple peels that had significant dietary fiber and ash content. Every one of the APP's chemical properties agrees with findings from earlier research of a similar kind. Apple fiber, the main by-product of The apple juice industry is rich in cell wall material and an intriguing source of pectins. It has a greater total dietary fiber content than wheat and oat bran; therefore, it has good water-holding ability in some food products (Alsuhaibani,. 2015).

Physico-chemical parameters of apple peel powder

Table 4 displays APP's bulk density and functional characteristics. Apple peel powder had a bulk density of 0.34±.00577 g/ml, which is similar to values published by Alalor et al. (2014). the bulk density, which affects the quantity and strength of packing materials, energy density, texture, and mouthfeel (Udensi, 2006). The solubility value of apple peel powder was 54.33 ±2.96273%, which is consistent with the findings of Njintang et al. (2014), who stated that the mucilage's solubility was 60%.

Table 3 : Psycho-chemical parameters of apple peels powder

Physico -chemical parameters	values
Bulk density (g/ml)	0.34±.00577
water holding capacity%	210±5.77350
Oil holding capacity%	216±12.01850
water solubility index%	54.33 ±2.96273
Swelling capacity%	7.33±.66667

Additionally, the water and oil holding capacities were 210±5.77350 and 216±12.01850%, respectively. These values are completely consistent with the findings of Zaker *et al*. (2016), who reported that the water and oil absorption capacities of orange peel powder were 5.9 and 9.5 gram per gram, respectively. that, in comparison to the other attributes,

Foods with high OHC and WHC content can serve as useful ingredients. Adding components with a high water holding capacity can modify the viscosity and texture of prepared food; these modifications are attributed to the gelling, bulking, and thickening effects (Adriana and Man, 2014).. Conversely, substances with high OHC can function as emulsifiers and are crucial in stabilizing food systems with high fat content. APP's swelling power was 7.33±.66667%, which is lower than Adeyanju et al.'s published (2014) value of 9.50% of ipin (ficus elastica) gum. With the exception of bulk density and water solubility index, all examined characteristics showed

higher values than those reported by *Dhankhar et al. (2*019).

Biscuits' physical characteristics

Table 1 shows the impact of different levels of apple peel powder incorporation on the biscuts' physical characteristics. The biscuits' diameter gradually increased as the level of proportion of APP increased, from 12.3333±.33333 for control without APP to 13.5000±.28868 in biscuits containing 2% APP. The potential cause of the diameter increase could be the APP's ability to bind water. As the proportion of APP in the flour blend increased, the biscuits' thickness and spread ratio gradually increased. This poor spreading was caused by the dough's high viscosity, which was brought on by the APP's absorption of water.

Ranjitha et al. (2018) showed comparable results. The authors' analysis revealed that adding 15 and 20% mango peel powder to biscuits resulted in a decrease in diameter and thickness. This could be because the powder diluted the gluten in the soft-dough biscuits.

Table 3 : The physical parameters of biscuits contain apple peels powder

levels	Diameter (cm)	Thickness(cm)	spread ratio(%)
T0	12.3333±.33333a	5.0300±.03512ab	2.4333±.00882a
T1	13.0000±.28868a	5.2000±.05774a	2.5167±.00882b
T2	13.5000±.28868b	5.3000±.05774a	2.5500±.00577c
T3	12.8300±.16503a	4.8267±.16344b	2.6700±.00577d

T0=control ,T1=1%APP,T2=2%APP,T3=4%APP

The results are a triplicate analysis average with ± standard deviation. A column's mean values that differ by a superscript indicate a significant difference ($p<0.05$).

This could be the result of improving the fruit and vegetable powder, which dramatically reduced the biscuit's thickness. It is ascribed to the addition of apple peel powder, which is a rich source of fiber, which increased the fiber content. The same pattern has been noted by Sharif et al. (2009) in de-fatted rice bran-supplemented cookies that are enhanced with fiber and minerals. When the amount of rice bran incorporated increased, they saw a tendency for the width to reduce, from 44.15 to 36.53 mm.

Sensory analysis of biscuits with powdered apple peels

Table 4 and Figure 1. show the sensory analysis of biscuits made with varying amounts of apple peel powder in comparison to the control biscuit. The results showed that, up to a concentration of 4%, adding apple peel powder to prepared biscuits significantly improved their color, flavor, appearance, and textural profile. However, at higher concentrations, these same characteristics were drastically

reduced, including color, texture, flavor, and appearance. These findings concur with those of Sharoba *et al.* (2013).

Table 4 : sensory analysis of biscuits made with varying amounts of apple peel powder.

levels	flavor	color	appearance	texture	overall acceptability.
T0	8.1875±.22751a	8.3162±.21576a	7.6050±.35215a	7.8850±.38507a	7.9938±.14454a
T1	8.5825±.15051a	8.6337±.16804a	8.2513±.27721a	8.3913±.23803a	8.4600±.13148b
T2	7.9275±.13642a	8.1400±.05779a	7.9000±.19385a	6.3400±.42771b	7.5725±.12936a
T3	5.4850±.42198b	5.4300±.32232b	6.6275±.25222b	5.0550±.26807c	5.6450±.18548c

T0=control ,T1=1%APP,T2=2%APP,T3=4%APP

The results are a triplicate analysis average with ± standard deviation. A column's mean values that differ by a superscript indicate a significant difference ($p<0.05$).

results of the sensory calculation showed that the biscuit with 1% APP was the most desired option and received the highest rating for each of the sensory characteristics that were assessed, including flavor, color, appearance, texture, and overall acceptability. The 4% APP biscuit's color differed noticeably from the other samples. Additionally, compared to other samples, the 1% APP biscuit's flavor and texture were noticeably better, most likely as a result of the enhanced apple flavor.

0% 1% 2% 4%

The biscuits' texture score steadily increased as the amount of APP increased. The addition of APP gave the biscuits their distinctive apple flavor, which enhanced their flavor. biscuits kept a decent flavor even after adding 1%. Because of the formation of a distinct apple flavor, APP enhanced the biscuits' flavor. The biscuits' overall acceptability scores rose by 1% after the inclusion of APP, then fell. The sensory evaluations of the biscuits containing 4% APP were significantly lower. This alteration also takes place in the dough when APP is added. An excessively high hardness value, as that seen in the dough samples containing 4%APP, is

not suitable for biscuits processing in real industrial production.

4-Conclusions

An underutilized by-product that is rich in nutritional fiber and can be used to make biscuits with lots of fiber is apple peels. The spread ratio of the biscuits was higher with the addition of APP. Because of their better flavor and taste, biscuits containing 1% APP were determined to have the most acceptable level of integration. Nevertheless, it was discovered that excessive levels of integration had a negative impact on the biscuits' texture, color, and appearance, which decreased their appeal overall. The addition of APP caused the biscuits' fiber content to rise. As a result, apple peel powder can be added to biscuits to enhance their fiber content. One of the key elements influencing food shelf life is moisture absorption or loss. Controlling moisture exchange is essential to avoiding enzymatic or microbiological spoiling.

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Scientific Research

Application of High Encapsulation of Nutritional and Bioactive Compounds from Black Bean (*Cajanus* sp.) in Functional Ice Cream

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ABSTRACT

Functional ice cream enriched with encapsulated black bean extract (*Cajanus* sp.) represents a type of functional food. This concept aligns with the definition of functional foods as food products containing nutritional components, dietary supplements, or bioactive compounds that specifically enhance the function of certain body parts, improve overall health, boost the immune system, prevent specific diseases, or reduce the risk of illnesses. Functional foods are enriched with specific components or ingredients, such as minerals, vitamins, fatty acids, dietary fiber, bioactive compounds, or probiotics. The key ingredients used in this functional ice cream, based on the best research findings, include 55% low-fat milk powder, 15% encapsulated black bean extract, and 1% carboxymethyl cellulose. Supporting ingredients consist of 6% cornstarch and 26% powdered sugar. The ice cream contains 127,56 ppm of anthocyanins, with total phenolic, flavonoid, and antioxidant activity values of 32,07 mg GAE/g, 40,73 mg QE/g, and 11,08 IC50 (µg/mL), respectively. The unsaturated fatty acids identified in the product include linoleic acid, eicosatrienoic acid, octadecatrienoic acid, and eicosenoic acid. The functional ice cream, with the optimal formulation of 15% encapsulated black bean extract, 55% low-fat milk powder, and 1% CMC, offers a comprehensive nutritional profile. It contains 23.47% protein, 13.60% fat, 14.85% moisture, 3.28% ash, 15.90% total fiber, 28.94% carbohydrates, and 38.21% total dietary fiber. Additionally, the ice cream exhibits antioxidant activity with an IC50 value of 11.08 µg/mL. Phytochemical screening of the functional ice cream revealed the absence of alkaloids, while confirming the presence of terpenoids, flavonoids, polyphenols, and saponins, indicating the presence of bioactive compounds.

1-Introduction

Black peas (*Cajanus sp.*), locally referred to as "kacang lebui" on Lombok Island, are rich in bioactive compounds, including anthocyanins, polyphenols, flavonoids, phenolics, and terpenoids. Research has demonstrated that the phenolic content of *kacang lebui* ranges from 30.501 to 78.363 mg GAE/g, significantly surpassing the levels found in *Phaseolus lunatus* (0.11–9.72 mg GAE/g) and *Vigna angularis* (8.18 ± 0.12 mg GAE/g). Classified as moderate to high in phenolic content, *kacang lebui* falls within a range of 3,000 to >5,000 mg GAE/100 g, with anthocyanin levels between 107.120 and 153.350 ppm [1-3].

Given the potential of local ingredients as sources of bioactive compounds and the increasing demand for natural antioxidants in functional food products, the development of microencapsulation technology is essential. This advanced technique offers enhanced protection for the bioactive compounds in *kacang lebui*, outperforming conventional extraction methods. Moreover, it provides a foundation for product characterization, facilitating the industrial-scale development of functional foods enriched with these valuable compounds [4,5,18,19].

The inherent vulnerability of free bioactive compounds to environmental factors such as oxygen, heat, and light necessitates careful handling to ensure their effective utilization. Microencapsulation produces encapsulated particles that provide optimal protection for these critical compounds. This technology, a subset of encapsulation techniques, transforms materials into particles ranging in size from micrometers (1–1000 µm) to nanometers, enhancing their stability. The fortification of functional ice cream with encapsulated bioactive compounds derived from kacang lebui is an ideal application[16-17]. This approach is supported by existing literature, as the ice cream manufacturing process does not involve high temperatures, a critical factor in preserving the stability and efficacy of bioactive compounds. Therefore, this study aims to determine the optimal composition for producing ice cream that incorporates encapsulated kacang lebui extract, with the best nutritional and bioactive compound content [7-9].

2-Materials and Methods

Materials

The materials used in the preparation of encapsulated black bean (*Cajanus* sp.) extract include high-quality kacang lebui harvested at 3 months of age from a reliable source in Gunungsari Village, West Lombok. Other materials include n-hexane, 90% ethanol, 70% ethanol, Whatman no. 1 filter paper, filter paper, nitrogen gas (N2), *Rhizopus* sp. culture, PDA medium, maltodextrin coating agent, and additional components for forming encapsulated extract.

The main and supporting materials for this research include microencapsulated particles produced using the best methods and treatments from the previous research, low-fat milk powder, carboxymethyl cellulose (CMC), skim milk, granulated sugar, cornstarch, egg yolks, salt, 90% ethanol, 70% ethanol, Whatman no. 41 filter paper, screening chemicals for physicochemical and phytochemical analysis, profiling, antioxidant activity, and total anthocyanins, phenolics, and flavonoids.

The research equipment used in the third year includes an ice cream maker, freezer, Memmert oven, glassware, pH meter, thermometer, Barnstead SHKE2000 shaker, TLC set, Kiesel Gel GF254 TLC plates, UV rays (366-254nm), Buchi rotary evaporator, Konica Minolta CR10 colorimeter, UV-1700 PharmaSpec spectrophotometer, UV-Vis 1240 spectrophotometer, vortex, micropipette, and GC-MS. Additionally, equipment required for forming the microencapsulated particles includes an electric dryer, microwave, maceration-percolation apparatus, analytical balance, centrifuge, pH meter, autoclave, thermometer, incubator, microbiological analysis equipment, vortex, micropipette, sonicator, spray drying equipment, and glassware. This comprehensive list of materials and equipment is essential for the successful microencapsulation and ice cream production processes in this study.

Methods

The raw material in this study, in the form of encapsulates, incorporates the treatments and optimal stages from previous research [1,2].. The

encapsulates are obtained through a series of steps, including kacang lebui extraction, optimization of the pre-microencapsulation process, and the formation of encapsulates. The process optimization in this research employs Response Surface Methodology (RSM) with a Central Composite Design, involving three factors: low-fat milk powder concentration (S) at 35%, 40%, and 45%; encapsulate concentration (E) at 20%, 15%, and 10%; and CMC concentration (C) at 0.5%, 1%, and 1.5%. The chosen value for α, with k = 3, is calculated as 2^(k/4) = 2^(3/4) = 2^0.75 = 1.682, rounded to 1.68. The factor levels are then set based on the average concentration of each material at the central point, with factors a = 0, b = 0, and c = 0. The factor levels are determined by taking the central point of each factor from the average concentration of each material. The physicochemical characterization of whole kacang lebui and its powder includes the analysis of protein content, fat content, carbohydrate content, moisture content, ash content, and total fiber using the AOAC International method[11]. Total anthocyanin and dietary fiber (DF) content are measured according to the method of Asp et al. (1983)[12], while total phenolic and total flavonoid contents are based on the method from ULP (2015)[10].

Qualitative testing to confirm the presence of bioactive compounds in the extract is performed through phytochemical screening, which includes testing for alkaloids, terpenoids, steroids, flavonoids, and polyphenols. Flavonoid screening is conducted using several reagents: a 1N NaOH solution for a basic test, 1N HCl and 1N H2SO4 for acid tests, ferric chloride for a specific reaction, and thin-layer chromatography (TLC). Secondary metabolite and fatty acid profiling are carried out using GC-MS, following the method from ULP (2015) [10]. The main parameters in the third phase of the research are anthocyanin content and the determination of the highest anthocyanin concentration, which are obtained through the Response Surface Methodology (RSM) process, consisting of phase 0, phase 1, and phase 2 testing. The phase 1 diagnosis is evaluated through the lack of fit test. If the lack of fit shows a significant value, the linear model or interaction model cannot be used to model the response. A lack of fit is considered significant when the p-value is less than 0.05 (α = 5%). If significant, the curvature is then examined, which may indicate that the response can be modeled using quadratic or cubic equations if the p-value for curvature is less than 0.05 (α = 5%), suggesting the presence of a curve or an optimal point. The models used in Design Expert include linear, two-factor interaction (2FI), quadratic, and cubic models. Research parameters include the physicochemical quality of microencapsulates, antioxidant activity, as well as screening, profiling, and bioactive compound content. The main data on anthocyanin content from the treatments, including expanded treatments, will be processed with the Design-Expert DX 7.1.5 program for statistical analysis.

3-Results and Discussion

Selection of the Best Model

In this study, Response Surface Methodology (RSM) was used to optimize the formulation of ice cream with a combination of low-fat milk powder, encapsulation, and carboxymethyl cellulose (CMC) concentrations on anthocyanin content. Choosing the best model in RSM analysis is crucial to ensure that the selected model has both accuracy and good predictive capabilities. In this process, several models were tested, including linear, two-factor interaction (2FI), quadratic, and cubic models, to determine which best fits the research data.

Based on the analysis results, the quadratic model was proposed as the best model for this study. This is supported by a significant Sequential p-value of 0.0394, indicating that the addition of a quadratic component to the model significantly contributes to the variation in the data. Additionally, the Lack of Fit p-value of 0.8704 suggests that the quadratic model does not poorly fit the data, meaning that it is appropriate for the observed data. Furthermore, the Adjusted R^2 value for the quadratic model is 0.7873, and the Predicted R^2 value is 0.7236, indicating that this model has good predictive ability and explains approximately 78.73% of the variation in the measured data. While the cubic model had a slightly higher Adjusted R^2 value of 0.7953, it was not chosen due to its insignificant Sequential p-value of 0.3737. This suggests that adding the cubic component did not lead to a meaningful

improvement in the model. Therefore, the quadratic model was selected as the best model to describe the effects of the combination of low-fat milk powder, encapsulation, and CMC on the anthocyanin content in ice cream.

Table 1. Selection of the Best Model

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001	0,7020	0,5561	0,4976	
2FI	< 0.0001	0,8332	0,7458	0,6903	
Quadratic	**0,0394**	**0,8704**	**0,7873**	**0,7236**	**Suggested**
Cubic	0,3737	0,8773	0,7953	0,6364	Aliased

Assumption Evaluation

Evaluating the assumptions in Response Surface Methodology (RSM) is an essential step to verify the model's validity and reliability. The key assumptions to be met include the normality of residuals, homoscedasticity (the consistency of residual variances), and the appropriateness of data transformation. To assess these assumptions, tests were performed using the P-P Plot for normality, the residual vs. predicted plot, and the Box-Cox plot for determining the optimal power[14-15].

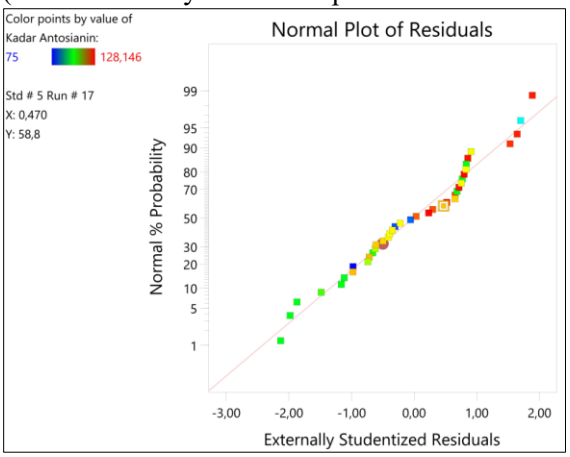

Figure 1. Normality Assumption Evaluation

The assumption check results indicate that the model meets the necessary criteria for the validity of the analysis. The P-P Plot for normality shows that the observation points align with the diagonal line, suggesting that the residuals are normally distributed. Residual normality is a crucial assumption, ensuring that the model's errors are random and unbiased, which means the analysis results can be considered valid.

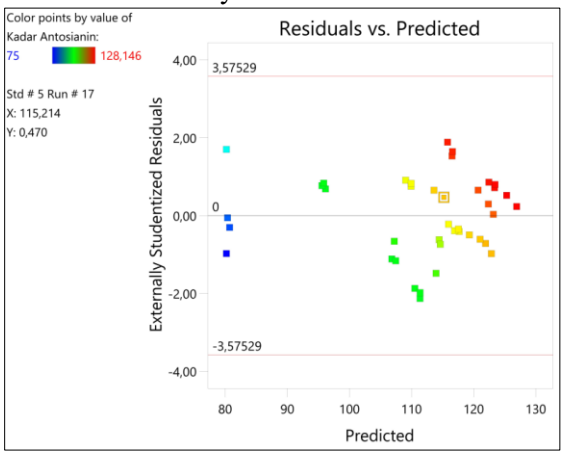

Figure 2. Homogenity Evaluation

Next, the residual vs. predicted plot shows a random scatter of points without any specific pattern, indicating that the assumption of homoscedasticity is met. This means that the residual variance is constant across the range of predicted values, suggesting that the model do es not exhibit heteroscedasticity, which could undermine the reliability of the predictions.

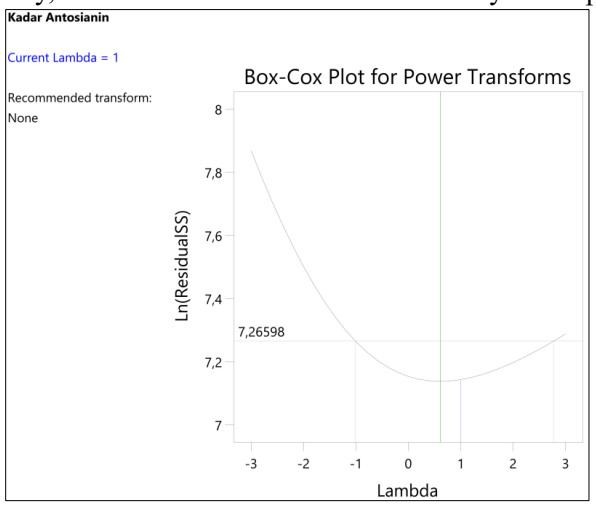

Figure 3. Evaluation of box cox transformation

The Box-Cox plot for power transformation, with a lambda value of 1, indicates that no further data transformation is necessary, as the data already satisfy the assumption of linearity. This lambda value confirms that the model is appropriate for the original data without requiring any changes to the data distribution. Overall, the assumption checks support the validity of the model used in this study, ensuring that the results and conclusions are reliable and trustworthy.

ANOVA Analysis

Analysis of Variance (ANOVA) was used to evaluate the significance of the quadratic model that describes the effect of low-fat milk powder, encapsulation, and Carboxymethyl Cellulose (CMC) on the anthocyanin content in ice cream. ANOVA helps identify factors that significantly affect the measured response as well as interactions between these factors. The ANOVA results also include a "Lack of Fit" test to assess the model's adequacy in fitting the observed data. The ANOVA results show that the quadratic model is overall significant in explaining the variation in anthocyanin content in the ice cream, with an F-value of 17.04 and a p-value of < 0.0001. This indicates that the model is effective in predicting the measured response. Among the individual factors, low-fat milk powder (A) and encapsulation (B) have a significant effect on anthocyanin content, with p-values of 0.0067 and 0.0328, respectively. This suggests that changes in the levels of low-fat milk powder and encapsulation significantly affect the anthocyanin content in the ice cream. In contrast, CMC (C) did not make a significant contribution to the response, as indicated by a p-value of 0.8537.

Table 2. ANOVA Results for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6472,42	9	719,16	17,04	< 0.0001	**significant**
A- low-fat powdered milk	357,76	1	357,76	8,48	0,0067	
B-Enkapsulat	211,37	1	211,37	5,01	0,0328	
C-CMC	1,46	1	1,46	0,0346	0,8537	
AB	291,94	1	291,94	6,92	0,0133	
AC	14,72	1	14,72	0,3489	0,5592	
BC	16,79	1	16,79	0,3979	0,5330	
A^2	162,31	1	162,31	3,85	0,0592	
B^2	396,38	1	396,38	9,39	0,0046	
C^2	1,60	1	1,60	0,0378	0,8471	
Residual	1265,84	30	42,19			

Lack of Fit	1167,84	29	40,27	0,4109	0,8704	**not significant**
Pure Error	98,00	1	98,00			
Cor Total	7738,25	39				

The interaction between low-fat milk powder content and encapsulation (AB) was found to be significant, with a p-value of 0.0133 ($p<0.05$), indicating that these two factors interact to affect the anthocyanin content. However, other interactions, such as between low-fat milk powder and CMC (AC), and between encapsulation and CMC (BC), did not show significance, with p-values of 0.5592 and 0.5330, respectively. The quadratic effect of encapsulation (B^2) was also significant on anthocyanin content, with a p-value of 0.0046, suggesting a significant non-linear effect of encapsulation. Meanwhile, the quadratic effect of low-fat milk powder content (A^2) approached significance (p-value 0.0592), while CMC (C^2) did not show a significant effect (p-value 0.8471). The Lack of Fit test yielded a p-value of 0.8704 ($p>0.05$), indicating that the model fits the data well and there are no patterns left unaccounted for. This reinforces the validity of the quadratic model in describing the influence of the combination of these factors on anthocyanin content in the ice cream.

Response Surface and Contour Analysis

The 3D response surface graph and the contour plot above are used to visualize the interaction between low-fat milk powder content (A) and encapsulation level (B) on anthocyanin content in ice cream, with CMC (C) held constant. The 3D response surface graph provides a three-dimensional representation, while the contour plot offers a more detailed two-dimensional view of how the combination of these two factors affects the anthocyanin content, measured in ppm. Both graphs illustrate the variation in anthocyanin content through color gradations, where blue represents the lowest anthocyanin levels and red indicates the highest. This investigation aims to identify the optimal combination of low-fat milk powder content and encapsulation level that can significantly increase anthocyanin content in the ice cream product.

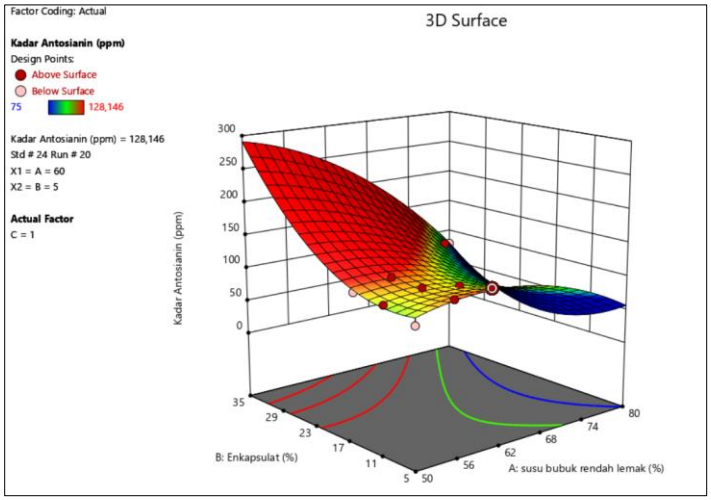

Figure 4. 3D Response Surface

Based on Figure 4, which presents the 3D graph, it is evident that there is a significant interactive relationship between the low-fat milk powder content (A) and encapsulation level (B) concerning the anthocyanin content in ice cream. The response surface shows variations in color, reflecting the anthocyanin levels, ranging from blue (low content) to red (high content). The red areas on the surface indicate the combinations of factors A and B that result in the highest anthocyanin levels, while the blue areas represent combinations that lead to the lowest anthocyanin content. The optimal combination for achieving

the highest anthocyanin content in the ice cream occurs at a low-fat milk powder content (A) in the range of 50-60%, an encapsulation level (B) around 25-30%, and a constant CMC (C) value of 1%. In this combination, the response surface shows a deep red color, indicating high anthocyanin content. This suggests that increasing the low-fat milk powder content along with the appropriate encapsulation level synergistically maximizes the anthocyanin content, while the effect of CMC remains constant at a non-significant value[13]. Thus, adjusting the composition to a higher low-fat milk powder content (A) and an optimal encapsulation level (B) is key to enhancing the anthocyanin content in the ice cream product.

The interaction between the two factors is non-linear, confirming the synergistic effect of low-fat milk powder content and encapsulation in influencing the anthocyanin levels. The optimal combination of these two variables is essential to achieving the highest anthocyanin content in the ice cream, as indicated by the surface pattern produced. This graph reinforces the statistical analysis results, which demonstrate the significance of the interaction between low-fat milk powder content and encapsulation level in the measured response[13].

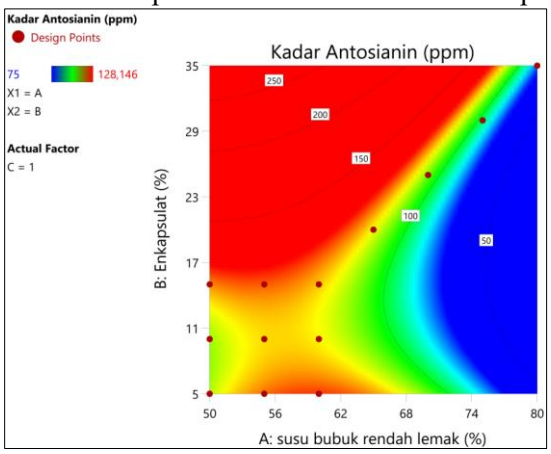

Figure 5. The interaction between low-fat milk powder content and encapsulation level with respect to anthocyanin content

The interaction between low-fat milk powder content and encapsulation level with respect to anthocyanin content is an important factor in determining the final anthocyanin concentration in the ice cream product. As the analysis indicates, these two factors exhibit a significant synergistic effect. When both the low-fat milk powder and encapsulation are optimized, there is a marked increase in the anthocyanin content, demonstrating that their combination plays a crucial role in enhancing the functional properties of the ice cream. From the 3D response surface and contour graphs, it is clear that higher low-fat milk powder content (within the range of 50-60%) combined with an optimal encapsulation level (around 25-30%) leads to the highest anthocyanin levels[13]. This interaction suggests that both factors work together in a non-linear fashion, where small changes in either factor can lead to significant changes in anthocyanin concentration. However, the effect of CMC remains relatively constant and does not significantly influence this interaction. This synergistic effect further supports the idea that the combination of these two variables (low-fat milk powder and encapsulation level) should be carefully adjusted to maximize the health benefits related to anthocyanin content, which is desirable for both its nutritional and antioxidant properties in the ice cream.

Based on Figure 5, the contour plot clearly shows a color gradient ranging from blue to green, yellow, and red, representing the anthocyanin content from low to high. The red areas indicate the highest levels of anthocyanin, while the blue areas represent the lowest levels. The plot indicates that higher low-fat milk powder content (A), particularly in the range of 50-65%, combined with encapsulation levels (B) above 25%, results in higher anthocyanin content, as shown by the red region in the plot. Conversely,

lower concentrations of low-fat milk powder and encapsulation levels lead to lower anthocyanin content, represented by the blue areas.
The interaction between these two variables demonstrates that increasing the low-fat milk powder content (A) along with higher encapsulation levels (B) significantly enhances the anthocyanin content in the ice cream. This plot further confirms the previous analysis, which showed that the optimal combination of these two variables effectively increases the anthocyanin levels. The transition areas between green and red indicate specific combinations that require further optimization to achieve the maximum anthocyanin content[13].

Formulation, Nutritional Composition, and Bioactive Compounds of Functional Ice Cream

In this section, the formulation of functional ice cream is discussed, including the selection of ingredients that contribute to its nutritional value and bioactive properties. The focus is on creating a product that not only provides essential nutrients but also offers health benefits through the incorporation of bioactive compounds, such as antioxidants, vitamins, and other functional ingredients derived from sources like local black bean extracts. The composition of the ice cream is carefully designed to optimize both the sensory qualities and the functional health benefits. Key components include low-fat milk powder, microencapsulated bioactive ingredients, and other nutritional additives, such as CMC, which enhances texture and stability. The aim is to balance the flavor, texture, and nutritional profile while ensuring the bioactive compounds remain effective in providing the desired health benefits. By considering the scientific principles of formulation and the properties of various ingredients, the functional ice cream is designed not only as a treat but also as a functional food that contributes to better health outcomes, particularly by boosting antioxidant levels and providing dietary fiber[13].
The ice cream enriched with encapsulates from black bean extract is made using an ice cream maker that is assembled and automated for the process. The resulting ice cream is considered a form of functional food. This is supported by the definition that functional foods are food products that contain nutritional components, food supplements, or bioactive compounds that can specifically enhance the function of certain body parts, improve overall health, strengthen the immune system, prevent specific diseases, and/or reduce the risk of illness. Such functional foods are products enriched or fortified with specific components or ingredients, such as minerals, vitamins, fatty acids, dietary fiber, bioactive compounds, or probiotics. They may also contain organic acids and other functional components.
The main ingredients used, based on the optimal results of the research, include 55% low-fat milk powder, 15% encapsulated black bean extract, and 1% carboxymethyl cellulose (CMC). Supporting ingredients include 6% cornstarch and 26% powdered sugar. All ingredients are mixed and processed in the ice cream maker for 45 minutes. The finished ice cream is then packaged in ice cream containers, labeled, and stored in a freezer to maintain the quality and consistency of the product. The ice cream produced from this process contains 127.56 ppm of anthocyanins. The total phenolic content, flavonoid content, and antioxidant activity are 32.07 mg GAE/g, 40.73 mg QE/g, and 11.08 (IC50, µg/ml), respectively. The unsaturated fatty acids found include linoleic acid, eicosatrienoic acid, octadecatrienoic acid, and eicosenoic acid[20,21].
The functional ice cream with the best treatment, containing 15% encapsulated black bean extract, 55% low-fat milk powder, and 1% CMC, has a complete nutritional composition with protein (23.47%), fat (13.60%), moisture (14.85%), ash (3.28%), total fiber (15.90%), carbohydrates (28.94%), and total dietary fiber (DF) of 38.21%. Furthermore, the ice cream also exhibits antioxidant activity with an IC50 of 11.08 µg/ml. Based on the results of the phytochemical screening of bioactive compounds in the functional ice cream, it was found that the ice cream sample does not contain alkaloids, but does contain terpenoids, flavonoids, polyphenols, and saponins[13,20,21].

4-Conclusion

The functional ice cream, with the optimal formulation of 15% encapsulated black bean

extract, 55% low-fat milk powder, and 1% CMC, offers a comprehensive nutritional profile. It contains 23.47% protein, 13.60% fat, 14.85% moisture, 3.28% ash, 15.90% total fiber, 28.94% carbohydrates, and 38.21% total dietary fiber. Additionally, the ice cream exhibits antioxidant activity with an IC50 value of 11.08 µg/mL. Phytochemical screening of the functional ice cream revealed the absence of alkaloids, while confirming the presence of terpenoids, flavonoids, polyphenols, and saponins, indicating the presence of bioactive compounds.

5-Acknowledgement

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6-Conflicts of interest

The authors declare no conflict of interest.

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The Role of Nutrition in Oral and Systemic Health: Bridging Food Science and Medicine

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ABSTRACT

The link between nutrition and health transcends mere systemic wellness, profoundly affecting oral health and reeling the complex interplay between food science and healthcare. Adequate nutrition preserves healthy teeth and gums, while nutritional deficiencies may cause cavities, gum disease, and enamel erosion. On the flip side, compromised oral health can also affect systemic ailments, including heart disease and diabetes, highlighting the reciprocal nature of this connection. Vital nutrients like calcium, vitamin D, and phosphorus are key for robust teeth and bones, whereas antioxidants and anti-inflammatory substances present in fruits and vegetables foster promote. In contrast, high sugar intake and unhealthy eating habits worsen dental health challenges, stressing the necessity of well-rounded nutrition. The fusion of food science and healthcare provides a comprehensive strategy for prevention and treatment, enhancing both oral and systemic health through dietary regimens customized to individual requirements. This cross disciplinary view emphasizes the importance of incorporating nutritional advice into healthcare to achieve optimal wellness.

1-Introduction

The research community has become increasingly fascinated by the implicit link between effects like artery disease, stroke, and overall mortality. Proposed mediators for this connection include infection, chronic inflammation, and genetic susceptibility to both oral and systemic diseases [1]. Commonly hypothesized arbiters of this relationship incorporate disease, incessant irritation, and hereditary inclination to both verbal and systemic illness. Nutrition has been proposed as a potential mediator [2].
The research community has grown increasingly interested in the link between oral health and systemic effects such as coronary artery disease, stroke, and overall mortality. Proposed mediators for this connection include infection, chronic inflammation, and genetic susceptibility to both oral and systemic diseases [1]. Nutrition has also been suggested as a potential mediator [2]. The inflammation caused by periodontal pathogens is thought to contribute to atherosclerosis, a condition characterized by the buildup of plaques in the arteries. Diabetes Mellitus There is a bidirectional relationship between diabetes and periodontal disease. Diabetes can increase the severity of gum disease, while periodontal disease can exacerbate diabetes management due to its impact on blood sugar control. Managing oral health becomes crucial for individuals with diabetes to mitigate complications. Respiratory Infections Oral health can significantly influence respiratory health. Bacteria from periodontal disease can be aspirated into the lungs, leading to conditions such as pneumonia [3].

The effects of different oral health issues on nutritional status may be closely tied to both nutrient intake and overall nutritional health [4] Numerous studies have shown a connection between nutrient consumption, nutritional wellbeing, and a range of systemic diseases. Recent research has clearly indicated a negative correlation between the intake of fruits and vegetables and the onset of cardiovascular issues. A higher intake of fruits and vegetables has additionally been linked to a lower likelihood of stroke. Several investigations have also hinted at a protective effect of fruits and vegetables against cancer. Nevertheless, studies have also indicated no clear connection with certain types of cancer [5]. Consumption of saturated fats has been associated with cardiovascular issues and might elevate the likelihood of breast and colorectal cancers. Antioxidants and dietary fiber have been evidenced in both epidemiological and interventional studies to lower the odds of cardiovascular diseases. However, the results have not been as definitive as those related to fruits and vegetables. For both fruits and vegetables, as well as individual vitamins and minerals, the reduction of health risks occurs through various mechanisms, including shielding against free radical damage, regulation of cytokine production, improvement of endothelial functionality, and modification of coagulation factors [6].
The intermediary function of nutrition in the link between oral health and systemic diseases has piqued our curiosity in exploring the connections between prevalent

oral issues and nutritional results. This article will succinctly assess and discuss the significance of nutrition and oral health and their relation to various ailments.
We will outline common criteria for nutritional evaluation employed in research and respond to inquiries about ho w particular oral health features relate to these nutritional results. We will encapsulate the methods, outcomes, and constraints of t he chosen studies.

2- Nutritional Components Essential for Oral Health

In 2016, the World Dental Federation redefined oral health, shifting from a limited focus on disease to a more expansive, multifaceted perspective. This new view encompasses the capability to communicate, grin, detect scents, savor, touch, chew, swallow, and express a wide array of emotions through facial gestures confidently and without discomfort or pain. Neglected oral care (e.g., tooth loss or poorly fitting dentures) may lead to discomfort and oral infections, significantly impacting one's quality of life [7]. These ramifications are often closely linked to decreased nutrient intake, behavioral effects on dietary choices, difficulties in communication, chewing issues, and a diminished appetite.
This section explores the essential nutritional components that support oral health and how they contribute to preventing oral diseases.
1. Calcium: building Strong Teeth Calcium is a vital mineral for maintaining strong teeth and bones. It plays a fundamental role in the development and maintenance of dental structures. Adequate calcium intake helps to support the enamel, the protective outer layer of the teeth, making them less susceptible to decay and erosion. Dairy products, leafy greens, and fortified foods are abundant in calcium, and sufficient intake of this mineral should be emphasized during childhood and adolescence when teeth are developing [8].
2. Vitamin D: enhancing Calcium Absorption Vitamin D works synergistically with calcium to promote optimal oral health. This vitamin enhances the absorption of calcium in the gut and helps in the remineralization of tooth enamel. A deficiency in vitamin D may lead to periodontal disease and tooth loss, as it compromises the immune system and affects bone density. Natural sources of vitamin D include sunlight exposure, fatty fish, and fortified foods. Thus, maintaining adequate vitamin D levels is crucial for preventing oral health issues [9].
3. Phosphorus: supporting Tooth Structure Phosphorus is another essential mineral that works collaboratively with calcium to create strong, healthy teeth. It is a critical component of hydroxyapatite, the mineral complex that comprises tooth enamel and dentin. Foods rich in phosphorus include meats, poultry, fish, eggs, nuts, and legumes. Including these foods in the diet can help maintain the structural integrity of teeth and support overall oral health [10].
4. Vitamin C: enhancing Gum Health Vitamin C is integral to oral health, particularly for maintaining healthy gums. This vitamin assists in collagen synthesis, which is vital for gum tissue integrity and healing. Insufficient vitamin C can lead to gum inflammation and periodontal disease. Citrus fruits, berries, bell peppers, and leafy greens are excellent sources of vitamin C. Regular consumption of these foods can significantly enhance gum health, reducing the risk of oral diseases [11].
5. Fiber: promoting Saliva Production Dietary fiber plays an indirect but significant role in oral health. High fiber foods stimulate saliva production, which serves as a natural defense mechanism against cavities and gum disease. Saliva neutralizes acids produced by bacteria in the mouth, aiding in the

remineralization of enamel. Fruits, vegetables, whole grains, and legumes are all rich sources of dietary fiber that should be incorporated into a daily diet [10,11].

6. Sugars: moderation is Key While carbohydrates, including sugars, provide energy, excessive sugar intake is detrimental to oral health. Sugars foster the growth of harmful bacteria in the mouth, leading to the production of acids that erode enamel and cause cavities. It is essential to limit refined sugars and explore healthier alternatives, such as natural sweeteners and whole fruits, which also provide beneficial nutrients [12].

7. Iron: iron is critical for the formation of hemoglobin, influencing the blood supply to oral tissues. Its deficiency can lead to anemia, which may manifest as a pale and swollen tongue, as well as impaired wound healing, increasing the risk of infections in the oral cavity [9,12].

A comprehensive understanding of nutritional components essential for oral health underscores the significance of diet in preventing dental issues. Incorporating sufficient calcium, vitamin D, phosphorus, vitamin C, and dietary fiber into daily meals, while moderating sugar intake, can promote optimal oral health. Developing a balanced diet focused on these nutrients is crucial for both maintaining healthy teeth and gums, ultimately contributing to overall well-being. Maintaining oral health through nutrition is both effective and essential in the prevention of complications, making it a key priority for individuals seeking to improve their dental hygiene. [7]. Establishing nutritious eating patterns alongside consistent consumption of vital vitamins and minerals holds considerable importance for both overall and dental wellness. Given the scarce understanding among dental professionals about the significance of trace elements in nutrition [10]. For example, maintaining good oral health requires a balanced diet rich in essential nutrients that support strong and healthy teeth and gums. Calcium and phosphorus are crucial for building and maintaining tooth enamel, and dairy products, leafy greens, nuts, and fish are excellent sources. Vitamin D plays a key role in calcium absorption and helps develop healthy teeth and bones, and can be obtained from exposure to sunlight, fatty fish, and fortified foods. Vitamin C is vital for gum health, as it helps prevent inflammation and strengthen connective tissues. Citrus fruits, strawberries, and bell peppers are good sources. Additionally, vitamin A supports saliva production, which is essential for washing away food particles and bacteria, while fluoride strengthens tooth enamel and prevents cavities. Limiting sugary and acidic foods also helps protect teeth from decay [8].

3-The Impact of Diet on Oral Diseases: Caries, Periodontal Disease, and Beyond

Drawing from more than a century of investigation, there is clear proof that dietary fermentable carbohydrates (sugars, starch) are an essential contributor, but by themselves, not a comprehensive cause for the onset and advancement of caries [11]. Variations in the cariogenic properties of different carbohydrates exist, even though only minor differences are observable in biofilm acid production. In this context, sucrose merits particular focus due to its rapid conversion into acid and its ability to be transformed into extracellular glucans, fructus, and intracellular reserve substances [12]. The cariogenic potential of starch varies significantly depending on the bioavailability of starches in processed foods. The concentration and bioavailability of carbohydrates in foods, in addition to the composition and stickiness of the diet, are further influential elements [13]. Behavioral aspects can play a role in the emergence or prevention of diseases. The frequency of carbohydrate consumption and physiological elements like oral clearance, biofilm makeup,

and salivary buffering capability have garnered significant focus over time. Evidence suggests that a diet where sugars account for less than 10% (50 g / day) of total caloric intake (E) is linked to reduced incidences of caries. Although the certainty of this evidence is low, there are signs that a noteworthy connection exists between sugar consumption and caries, even with free sugar intake below 5% E (25 g / day) [14]. The working group endorses the objective of eradicating sugars from current diets but acknowledges that even achieving daily consumption levels between 25–50 g / day will pose challenges due to the added free sugars in the form of mono and disaccharides in food and drinks [15]. Since "nutrition" acts both locally and systemically, lack of dietary micronutrients such as vitamin D, calcium, phosphates and vitamin K, has a negative impact upon tooth mineralization and tooth quality and size, and may also affect caries risk later in life through other mechanisms [16].

Research from both association studies and controlled clinical depletion experiments suggests that diet plays a significant role in periodontal disease. Deficiencies in micronutrients are found to have an inverse relationship with periodontal wellness. Multiple studies conducted across various populations have demonstrated a consistent inverse correlation between dietary vitamin C consumption, plasma vitamin C levels, and the prevalence of periodontitis, even after accounting for confounding variables [17]. Furthermore, it has been established that a deficiency in vitamin C can result in excessive bleeding of the gums [18]. Lower levels of serum magnesium/calcium, reduced antioxidant micronutrient levels, and decreased intake of docosahexaenoic acid have also been significantly linked to increased prevalence of periodontal diseases [19]. Although there is mixed evidence regarding the impact of vitamin D intake and serum concentrations on periodontal health (van der Velden et al. 2011), combining vitamin D supplementation with calcium has been shown to lessen tooth loss and enhance periodontal health [20]. At the macronutrient level, emerging data suggests that a diet high in carbohydrates poses a greater risk of inflammation and, consequently, gingival bleeding [21], I adopting a Paleolithic diet leads to a reduction in gingival bleeding [22]. Considering that the underlying mechanisms may differ between these two ailments, fermentable carbohydrates stand out as the most significant shared dietary risk factors for tooth decay and gum diseases [23]. In the case of tooth decay, this relates mainly to the fermentation process that occurs within the dental biofilm, leading to the production of harmful acids. For gum diseases, the predominant biological process appears to be glucose and advanced glycation end products inciting a state of heightened inflammation in white blood cells [24]. Additionally, research indicates that deficiencies in micronutrients can affect both conditions at various life stages. There is evidence to suggest that a lack of vitamin D may cause enamel hypoplasia or hypo mineralization, ultimately increasing the risk of tooth decay [25]. Furthermore, vitamin D deficiency has been linked with periodontitis in observational studies. A systematic review of randomized trials indicates that supplementation of vitamin B6 can diminish the incidence of tooth decay [26]. With regard to gum diseases, findings from a cohort study revealed that a deficiency of vitamin B12 was associated with the progression and damage of periodontal disease [27]. The vulnerability to tooth decay fluctuates significantly throughout an individual's life. Dietary habits evolve over time, especially concerning exposure to specific fermentable carbohydrates. An increase in tooth decay prevalence correlates with the frequency of sugar consumption and also varies according

to sugar consumption patterns. Tooth decay risk is particularly high among young children during the early years following the eruption of primary and permanent teeth. Early childhood tooth decay may stem from poor feeding practices (increased sugar exposure during weaning, bottle feeding, or prolonged nighttime breastfeeding) [28]. Increased consumption of sweets and soft drinks during adolescence heightens the risk of tooth decay. Although the evidence is limited, there may be a higher risk for adults based on various work environments (restaurants, food laboratories, and shift work). After retirement, dietary patterns may shift toward softer diets with increased sugar consumption. Starches are considered a risk factor for tooth decay in root surfaces, especially concerning for seniors [29].

Tooth decay risk can escalate in any age group due to physiological changes, including decreased nutrient absorption and diminished chewing function, which are often associated with higher medication use [30]. Nowadays, dietary guidelines are frequently provided to augment conventional medical treatments. As energy needs decline with age, dietary intake may also decrease, raising the risk of micronutrient deficiencies [31]. It is vital to ensure that diets, especially for frail and dependent older individuals, maintain optimal quality to aid in disease prevention. Currently, the influence of dietary risk factors on periodontal diseases throughout the lifespan remains uncertain [32].

4- The Role of Micronutrients in Maintaining Oral and Systemic Health

Numerous studies involving both animals and humans have effectively illustrated the influence of certain micronutrients on the inflammatory response of the host by reducing inflammatory markers and ultimately mitigating bone loss [33]. A comparable pattern was noted in one investigation included in this systematic review by Ehlers et al. (2011), where the authors observed that the C-reactive protein (CRP) levels, an indicator of inflammatory activity, exhibited a smaller increase among participants consuming nutritional supplements, alongside a slightly beneficial effect on gingival inflammation when contrasted with the control group, which comprised dental students facing significant examination pressure [34]. In another examination within this systematic review, it was found that the intensity of periodontal disease escalated with a reduced intake of vitamins A, B1, C, E, as well as iron, folate, and phosphorus [35]. Although the precise mechanisms linking nutrition to periodontal disease remain partially understood, Chapple et al. identified a negative correlation between overall antioxidant properties and periodontal disease, providing insight into the intricate connection between nutrition and inflammation that leads to periodontal disease [36]. These discoveries are bolstered by evidence that acknowledges the antioxidant capabilities of vitamins A, B1, C, and E [37].

The research conducted by Dodington et al. investigated the impact of nutritional intake on the recovery of periodontal health among both smokers and nonsmokers suffering from chronic generalized periodontitis after undergoing scaling and root planning. The researchers noted a substantial decrease in probing depth associated with increased consumption of fruits and vegetables, α tocopherol, vitamin C, β carotene, as well as EPA and DHA among nonsmokers, whereas such a correlation was absent in smokers. This relationship may be partly due to their higher intake of antioxidants from fruits and vegetables. Furthermore, the results indicated that whole food sources laden with antioxidants proved to be significantly more advantageous than supplements containing

isolated compounds for optimal periodontal recovery. Positive effects of EPA and DHA supplements were also recorded among participants in this investigation, which might be elucidated by the influence of a downstream metabolite of DHA, Resolving D1, known to reduce inflammatory mediators in an in vitro study on periodontal ligaments [38].

A study conducted by Adegboye et al. uncovered the beneficial impacts of elevated dietary calcium and dairy consumption (within recommended limits) on plaque levels among seniors aged 65 and older who also had higher vitamin D consumption (exceeding 6.8 μg/d). This correlation was evident even after controlling for various confounding variables, and participants with lower vitamin D intake (below 6.8 μg/d) did not exhibit this relationship between dietary calcium and plaque levels. This aligns with prior research suggesting an inverse correlation between increased consumption of calcium, dairy products, and vitamin D, and a decrease in dental caries and periodontitis [39]. Given that Adegboye et al. stratified the effect of calcium intake based on vitamin D levels, it appears that a greater intake of vitamin D can enhance the positive effects of elevated calcium consumption, likely by boosting calcium absorption. This is supported by findings from Laky et al., who noted that vitamin D deficiency was more prevalent among individuals with severe periodontal disease than among healthy adults [40]. In another study included in this systematic review, the second highest level of vitamin D intake (3.2–6.0 μg) was associated with a reduction in the severity of periodontal disease compared to the highest level of intake (≥ 6.0 μg), indicating a potential optimal range of 3.2–6.0 μg for vitamin D intake [41]. This underscores the necessity for further research into the ideal levels of vitamin D for promoting oral health and preventing periodontal disease [42].

4.1. The Interplay Between Micronutrients and Systemic Health

A. Immune Function: Micronutrients such as vitamins A, C, D, and E, along with minerals like zinc and selenium, have well documented roles in modulating the immune system. A robust immune system is essential for combating infections, including oral diseases. Thus, adequate intake of these micronutrients is beneficial for both oral and systemic immunity [43].

B. Inflammatory Response: Deficiencies in certain micronutrients may exacerbate inflammatory responses in various tissues, including the gums. For instance, adequate zinc status has been correlated with reduced inflammation and better periodontal health. Micronutrients help regulate inflammatory pathways, thus having systemic implications in conditions like cardiovascular diseases [44].

C. Bone Health: The combination of calcium, magnesium, phosphorus, and vitamins D and K is essential for maintaining bone health. The jawbone's health is crucial not only for oral integrity but also for systemic skeletal health. Osteoporosis, often linked with vitamin D deficiency, can directly impact oral health by leading to tooth loss [45].

4.2. Consequences of Micronutrient Deficiencies

A. Oral Manifestations: Micronutrient deficiencies can lead to a variety of oral health problems, such as:

1. Periodontal Disease: Insufficient vitamin C can increase the risk of gingival diseases.
2. Delayed Healing: Lack of vitamins A and C may prolong healing after dental surgery.
3. Altered Taste Sensation: Deficiencies may impact taste buds, affecting appetite and nutrition [44].

B. Systemic Health Issues: Oral health is intricately linked to systemic health. Poor

oral hygiene can contribute to systemic conditions such as:

1. cardiovascular diseases: Inflammation promoted by periodontal disease can increase the risk of heart disease.
2. Diabetes Management: There exists a bidirectional relationship between diabetes and periodontal disease. Poorly controlled diabetes can impair immune response and exacerbate gum problems [46]. Finally, according to the topics presented, essential in maintaining oral health and preventing systemic diseases. Their influence extends beyond the oral cavity, affecting overall health through immune modulation, inflammatory response regulation, and support of vital physiological functions. Ensuring adequate intake of various micronutrients through a balanced diet is essential for maintaining optimal oral and systemic health. It is crucial for individuals to be aware of the importance of micronutrients and to strive for a healthy and balanced diet that meets these needs.

5- Future Directions

Emerging pathways in the connection between nutrition and oral as well as systemic wellness are poised to emphasize individualized nutrition plans, the amalgamation of food science with tailored medicine, and enhancements in functional dietary options. With the advent of nutrigenomics, personalized dietary guidance rooted in genetic information is becoming increasingly attainable, enabling people to maximize both oral and general wellbeing. Furthermore, there is a rising enthusiasm for creating functional foods and dietary supplements tailored to specific health issues, such as probiotics for maintaining oral microbiome equilibrium or anti-inflammatory agents to combat chronic illnesses. Nevertheless, obstacles remain, including the necessity for rigorous clinical studies to validate health claims, addressing economic disparities in the availability of nutritious food options, and linking healthcare practitioners with nutrition specialists. Cooperation among food scientists, healthcare providers, and lawmakers will be vital to surmounting these hurdles and guaranteeing that nutrition-oriented strategies are effectively enacted for enhanced health results.

6- Conclusion

The intricates relationship between nutrition, oral, and systemic health underscores the need for a cohesive approach that bridges food science and medicine. By recognizing the significance of nutrition in maintaining oral health and its implications for systemic well-being, healthcare professionals can develop comprehensive strategies to promote better health outcomes. Through collaboration, education, and preventive care, it is possible to enhance the understanding and practice of nutrition as a cornerstone in achieving optimal health for individuals and communities alike.

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