Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Evaluating the effect of aqueous and ethanolic extracts of *Moringa oleifera* leaves on the microbial, chemical, and sensory properties of heat-treated non-carbonated doogh

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ARTICLE INFO

Article History:

Received: 2025/04/25

Review: 2025/09/30

Accepted: 2025/10/02

Keywords:

Moringa oleifera leaves,
Ethanolic extract,
Aqueous extract,
Heat-treated doogh, Shelf-life

DOI: **10.48311/fsct.2026.84036.0**

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ABSTRACT

This study was aimed to investigate the effect of adding aqueous and ethanolic extracts of *Moringa oleifera* separately at different concentrations (0.2, 0.4, and 0.8%) on the physicochemical (pH, acidity, syneresis, and viscosity), antioxidant (total phenolic content and DPPH radical scavenging activity), microbial (total microbial count, mold and yeast count, coliforms, *Staphylococcus aureus*, and *Escherichia coli*), and sensory (color, taste, smell, texture, and overall acceptance) characteristics of doogh during 42 days of refrigerated storage. The results showed that Moringa extracts, especially at higher concentrations, caused a significant increase in pH, viscosity, total phenolic content, and antioxidant properties of the doogh treatments compared to control sample, and decreased acidity, syneresis, total microbial count and mold and yeast count ($p<0.05$). Moreover, with increasing the storage time, pH, viscosity, and antioxidant properties decreased, while acidity, syneresis, and microbial load increased ($p<0.05$). Also, coliforms, *E. coli*, and *S. aureus* were negative in all the samples by day 42. Sensory characteristics, except for color, were affected by extract concentration and storage time; In such a way that increasing the concentration of the extracts led to the improvement of taste, smell, texture, and overall acceptance, but the color did not change significantly. In comparison of the two extracts studied, doogh treatments containing ethanolic extract revealed better results in improving physicochemical and microbial properties than aqueous extract, but no significant difference was observed in sensory properties ($p>0.05$). Finally, the use of *M. oleifera* extracts can be recommended as a natural preservative to extend the shelf life of doogh, and treatments containing 0.8% aqueous or ethanolic extract of Moringa are considered as superior treatments.

1- Introduction

Doogh is a traditional fermented dairy beverage made from a mixture of yogurt, water, and salt, sometimes enhanced with natural herbal extracts or essential oils. This drink is widely consumed in countries such as Iran and Turkey, and holds a special place in the diet because of its pleasant taste and digestive benefits [1]. Doogh is recognized as a healthy alternative to carbonated soft drinks because it has higher digestibility than milk, and it is rich in vitamins and minerals. Additionally, consuming doogh after meals can help inhibit the growth of pathogenic bacteria and strengthen the population of beneficial gut microbiota [2].

Nevertheless, due to its low pH and nutrient-rich composition, doogh provides a favorable environment for the growth of molds, yeasts, and bacteria. Microbial spoilage of this product can lead to undesirable changes in flavor, aroma, and shelf life, posing a serious challenge to the dairy industry. Chemical preservatives, like sorbic and benzoic acids, are commonly used to control microbial spoilage in various foods. However, these additives are not allowed in doogh, and due to safety concerns associated with them, it is important to search for natural alternatives [3].

Herbal extracts, due to their antimicrobial, anti-inflammatory, and antioxidant activities, have attracted attention as promising ingredients for use in food products [4]. Bioactive compounds present in plants, such as flavonoids and phenolics, not only improve the physicochemical and sensory properties of foods but can also help inhibit the growth of microorganisms [5, 6].

Moringa oleifera, commonly known as the "tree of life" or "miracle tree," is an herbal plant that has attracted significant attention due to its medicinal benefits and high nutritional value [7, 8]. The leaves of this plant are rich in vitamins, minerals, antioxidants, and bioactive compounds, and are used in the treatment of various diseases such as skin infections, anemia, diabetes, and bronchitis [9]. Research has shown that Moringa extracts, particularly those derived from its leaves, contain compounds such as n-hexadecanoic acid and cis-vaccenic acid, which exhibit notable antimicrobial and antioxidant effects [7]. In recent years, applying Moringa extracts as bioactive additives in food products such as yogurt, meat nuggets, and other food items has received considerable attention [10, 11]. These extracts can also improve the sensory attributes and shelf life of products in addition to enhancing nutritional quality [12].

Therefore, the present study was conducted to investigate the effects of aqueous and ethanolic extracts of *Moringa oleifera* leaves as natural preservatives in heat-treated, non-carbonated doogh. The effects of these extracts on the microbial, physicochemical, and sensory properties of doogh during refrigerated storage were assessed.

2- Materials and Methods

2.1. Materials

Dried *Moringa oleifera* leaves were purchased from Sabz Royan Company (Dezful, Iran). The ingredients used to produce the doogh included low-fat milk, skim milk powder, and milk protein concentrate powder (Pegah Co., Iran), a starter culture (Christian Hansen Co., Denmark), and salt (Golha Co., Iran). The chemicals required for the analyses, such as ethanol, gallic acid, Folin, Ciocalteu reagent, sodium hydroxide, and microbial medium cultures were obtained from Merck Co. (Germany).

2-2. Preparation of Ethanolic and Aqueous Extracts of *Moringa oleifera* Leaves

Dried *M. oleifera* leaves were ground into powder using a household grinder and sieved through a 60-mesh screen. For the aqueous extract, 10 g of Moringa leaf powder was soaked in 100 mL of distilled water at room temperature for 24 hours, then filtered through

filter paper. For the 80% ethanolic extract, 100g of the powdered leaves was immersed in 1000 mL of 80% ethanol, mixed for 10 minutes, and left at room temperature for 24 hours with continuous stirring using a glass stirring rod. The resulting solutions were filtered through Whatman No. 1 filter paper and concentrated at 55 °C using a rotary evaporator until the solvent was completely removed [13, 14].

2-3. Antimicrobial Activity of *Moringa oleifera* Extracts

Lyophilized strains of two bacteria (*Escherichia coli* and *Staphylococcus aureus*) and two fungi (*Aspergillus niger* and *Saccharomyces cerevisiae*) were obtained from the Iranian Research Organization for Science and Technology. Streptomycin was used as the positive antibiotic control. The minimum inhibitory concentration (MIC) was determined using a serial dilution antimicrobial susceptibility test. The negative control well contained no microorganisms, while the positive control well contained the test organism. Final concentrations in the wells ranged from 0.12 g/mL to 250 g/mL. To determine the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), inocula from the MIC and higher-concentration wells were plated. Bacterial cultures were transferred to sterile Mueller–Hinton agar and incubated at 37 °C for 48 hours. Fungal cultures were transferred to sterile Sabouraud dextrose agar: *S. cerevisiae* was incubated at 28 °C for 72 hours, and *A. niger* was incubated at the same temperature for 5–7 days in an incubator (IPP55 Memmert, Germany). Finally, thiazolyl blue tetrazolium bromide (TBTB) indicator was added to the positive and negative control wells, and color change was observed. The lowest concentration showing no visible growth was recorded as the MBC or MFC [15, 16].

2-4. Production of Doogh Samples

Doogh samples were produced on an industrial scale at the Domas Laban Dairy Co., Iran. The fat content of raw milk was standardized to 2.5%. To adjust total solids, 2% skim milk powder and 1.5% milk protein concentrate were added. After homogenization at 150–200 bar and 60–65 °C, the milk was heat-treated at 90 °C for 15 minutes. Once cooled to 42 °C, starter cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were inoculated according to the manufacturer's instructions, and fermentation continued until the pH reached 4.6. The resulting yogurt was thoroughly stirred and blended with purified water (1:1 ratio) and 0.8% salt to obtain a total solids content of 3.2%. The mixture was then preheated to 32 °C, homogenized at 110 bar and 60 °C, and pasteurized at 80 °C for 2 minutes. Finally, the samples were cooled to 8 °C [17]. The doogh was divided into seven groups. Aqueous and ethanolic Moringa extracts were separately added to six of the groups at concentrations of 0.2%, 0.4%, and 0.8%, while one group served as the control. All samples were packaged in 300 mL polypropylene bottles and stored at 4 °C for 42 days. Physicochemical, microbiological, and sensory evaluations were performed on days 1, 7, 14, 21, 28, 35, and 42.

2-5. Determination of Total Phenolic Content and Antioxidant Activity of Moringa Extracts and Doogh Samples

2-5-1. Total Phenolic Content

The total phenolic content of *M. oleifera* extracts and doogh samples was determined using the Folin–Ciocalteu method. Ten grams of each sample were mixed with 90 mL of a 50:50 methanol–water solution and agitated at room temperature for 2 hours. After filtration and centrifugation, the supernatant was collected, and total phenolics were expressed as milligrams of gallic acid equivalents per 100 g (mg GAE/100 g). Absorbance at 765 nm

was measured using a UV/Vis spectrophotometer (Jenway 6705, UK), and a calibration curve was prepared with gallic acid standards [13, 18].

2-5-2. Antioxidant Activity

Antioxidant activity was assessed using the DPPH radical-scavenging assay. One gram of each sample was diluted in 95 % ethanol and centrifuged at 440 × g for 20 minutes at 4 °C after thorough mixing. A 100 µM DPPH solution in methanol was prepared and mixed 1:1 with 100 µL of each diluted sample in a 96-well microplate. After 30 minutes of incubation, DPPH radical-scavenging activity was measured at 517 nm using the UV/Vis spectrophotometer (Jenway 6705, UK). The percentage reduction in absorbance relative to the control was calculated as the inhibitory concentration (IC_{50}) and reported as the percentage of free radical scavenging [13, 18].

2-6. Physicochemical Tests of Doogh Samples

The pH of the samples was measured using a digital pH meter (Metrohm 780, Switzerland). Titratable acidity was determined by titration with a standard sodium hydroxide solution using phenolphthalein as an indicator, and results were expressed in degrees Dornic [19].

Viscosity measurements were carried out at 23 ± 1 °C using a Brookfield DVE viscometer (USA) at shear rates ranging from 10 to 200 rpm (depending on torque), with results reported in centipoise [20].

Syneresis was evaluated using 50 mL Falcon tubes of identical shape and size. Equal volumes of each sample were poured into the tubes and stored in a refrigerator without agitation for 48 hours. After this period, the volume of the serum layer, the clear liquid separated from the top to the interface between phases, was measured in milliliters. The percentage of syneresis was calculated as the volume of separated serum divided by the initial doogh volume, multiplied by 100, and reported as the percentage of serum separation [21].

2-7. Microbiological Tests of Doogh Samples

For microbiological analysis, 10 mL of each doogh sample was diluted with 90 mL of sterile peptone water, and serial decimal dilutions were prepared. Total microbial counts were determined by surface-plating 0.1 mL of the appropriate dilutions on Plate Count Agar and incubating at 37 °C for 48 hours in an IPP55 Memmert incubator (Germany), with colonies expressed as CFU/mL [22].

Yeasts and molds were counted by plating 0.1 mL of the dilutions on Sabouraud dextrose agar containing chloramphenicol and incubating at 25 °C for 5–7 days before counting colonies [3]. Coliform bacteria were counted by surface-plating 0.1 mL of the dilutions on Violet Red Bile Glucose Agar and incubating at 30 °C for 24 hours [23].

Staphylococcus aureus was counted by plating 0.1 mL of the dilutions on Baird–Parker agar and incubating at 37 °C for 48 hours [3].

For the isolation and enumeration of *Escherichia coli*, 0.1 mL of the prepared dilutions using a sampler was transferred to Sorbitol MacConkey agar and plated by the surface culture method, then incubated at 37 °C for 48 hours [3].

2-8. Sensory Evaluation

Sensory attributes including color, taste, odor, texture, and overall acceptability were evaluated by 10 trained panelists using a five-point hedonic scale, where 1 = unacceptable, 2 = poor, 3 = acceptable, 4 = good, and 5 = excellent [3].

2-9. Statistical Analysis of Data

A completely randomized design was used in this study. All experiments were conducted in triplicate, and data were analyzed using SPSS 16. One-way analysis of variance (ANOVA) was applied to assess the significance of the studied variables, and Duncan's multiple range test at a 5% probability level was used to compare significant means. Microsoft Excel 2016 was used to draw the diagrams.

3- Results and Discussion

3-1. MIC, MBC, and MFC Results of *Moringa oleifera* Extracts

The MIC, MBC, and MFC of *M. oleifera* extracts against studied bacteria and fungi are presented in Table 1. The results indicate that both *M. oleifera* extracts exhibit antimicrobial activity against the studied bacterial and fungal species, with no significant difference between them ($p > 0.05$). However, it was observed that the antibacterial effect of the Moringa extract is stronger than its antifungal effect. Among the pathogenic bacteria tested, the extract was more effective against *E. coli* than *S. aureus*, and among the fungi, it was more effective against *S. cerevisiae* than *A.niger*.

This extract shows notable antibacterial effects against pathogens such as *E. coli* by inhibiting growth and interfering with bacterial cell function. These findings suggest its potential use and synergistic effects with antibiotics for combating resistant bacterial strains [24]. These effects may be attributed to the bioactive compounds and certain secondary metabolites present in *M. oleifera* leaves, which can disrupt bacterial cell membranes or inhibit essential enzymes [25]. In a study by Prasajak *et al.* (2021), the antimicrobial activities of *M. oleifera* leaf and pod extracts were evaluated, reporting that the pod extract exhibited the highest antibacterial activity against all tested bacterial strains, including *S. aureus*, *Bacillus cereus, E. coli*, and *Salmonella typhimurium*, with the lowest MIC (1.56 µg/mL) and MBC (3.13 µg/mL) [26]. Moreover, in a study by Mohammed *et al.* (2024) on the antifungal activity of *M. oleifera* extract, the MIC and MFC values against *A. niger* were 44.2 µg/mL and 56 µg/mL, respectively. This antifungal activity was attributed to bioactive compounds such as tannins, flavonoids, and phenolic compounds present in the plant's ethanolic extract [27]. Differences between these studies and the present research may be due to variations in the Moringa variety used, as well as differences in extraction methods and incubation conditions.

Table 1. Antimicrobial effects of ethanolic and aqueous extracts of *Moringa oleifera*

Microbial strain	Moringa ethanolic extract		Moringa aqueous extract	
	MIC (µg/mL)	MBC/MFC (µg/mL)	MIC (µg/mL)	MBC/MFC (µg/mL)
Escherichia coli	0.125	0.25	0.1	0.25
Staphylococcus aureus	0.25	0.5	0.25	0.5
Aspergillus niger	125	125	125	125
Saccharomyces cerevisiae	0.5	1	0.5	1

3-2. Total Phenolic Content and Antioxidant Activity of *Moringa oleifera* Extracts

The total phenolic content (TPC) of the ethanolic and aqueous extracts of *M. oleifera* was 80.39 ± 0.5 mg GAE/100g and 73.52 ± 0.7 mg GAE/100g, respectively, while the antioxidant activity (IC50) of the ethanolic

and aqueous extracts was 80.84 ± 1.2% and 77.51 ± 1.4%, respectively. These results indicate that the ethanolic extract contains a higher phenolic content and consequently exhibits greater DPPH radical scavenging activity compared to the aqueous extract ($p < 0.05$). This is attributed to the higher phenolic and flavonoid content in the Moringa ethanolic extract, which can effectively scavenge free radicals [13, 28].

The study by Moyo *et al.* (2012) demonstrated that the flavonoids, flavonols, phenols, and proanthocyanidins in *M. oleifera* extracts obtained using acetone were higher than those in water extracts of the plant [28]. In a study by El-Gammal *et al.* (2017), the aqueous extract of Moringa contained 340.82 mg GAE/g total phenolics and had an IC50 value of 75.82%, indicating that the addition of *M. oleifera* extract to yogurt could enhance its nutritional value [13]. Additionally, Prasajak *et al.* (2021) evaluated the phenolic content and free radical scavenging of *M. oleifera* leaf and pod extracts, reporting that the leaf aqueous extract had the highest TPC (67.18 mg GAE/g) compared to the pod aqueous extract (55.17 mg GAE/g). Moreover, the leaf extract exhibited the strongest DPPH radical scavenging activity with an IC50 of 85.49%, which is consistent with the present study [26].

3-3. Total Phenolic Content and Antioxidant Activity of Doogh Samples Containing *Moringa oleifera* Extracts

According to statistical analysis (Table 2), the effects of Moringa extracts, storage time, and the interactions of variations on TPC and antioxidant activity of doogh samples were significant ($p < 0.05$). Evaluation of antioxidant activity showed that increasing the concentration of Moringa extracts in doogh led to higher phenolic content and greater DPPH radical scavenging activity. Treatments E0.8 and A0.8 (containing 0.8% ethanolic and aqueous Moringa extracts, respectively) exhibited the highest TPC and antioxidant activity, while the control sample showed the lowest phenolic content and antioxidant property. Furthermore, with increasing storage time, TPC and antioxidant activity significantly decreased in all samples ($p < 0.05$). It should be noted that all treatments containing the ethanolic extract maintained significantly higher phenolic content and antioxidant activity than the aqueous extract at the same concentration throughout the storage period ($p < 0.05$). The DPPH radical scavenging property of plant extracts is likely due to the hydrogen-donating ability of hydroxyl groups in their phenolic compounds. The decrease in antioxidant activity during storage may result from the reduction of phenolic and anthocyanin compounds derived from the extract and the formation of complexes between phenolics and proteins in the sample [29, 30, 31]. Polyphenol content also gradually decreases over time due to bacterial metabolic activity and the enzyme polyphenol oxidase [32]. Specifically, the degradation of phenolic compounds can be attributed to their breakdown and the hydrolysis of polyphenols into aromatic acids such as phenylacetic, phenylpropionic, and benzoic acids by lactic acid bacteria [31]. In studies by Mohammadi and Esmaeilpour (2021) and Haseli *et al.* (2022), the antioxidant properties of doogh containing nettle extract and alcoholic Mufra extract were evaluated, respectively. Both studies reported that adding these plant extracts increased the antioxidant activity of doogh compared to the control group, and antioxidant activity decreased over storage time, consistent with the results of the present study [33, 34].

Table 2. Effects of *Moringa oleifera* extracts on phenolic content and DPPH radical scavenging activity of doogh samples (Mean±SD)

Test	Treatment	Storage Days						
		1	7	14	21	28	35	42
Phenolic content (mgGAE /100g)	C	27.59±0.04^{Ag}	22.37±0.01^{Bg}	20.45±0.01^{Cg}	17.59±0.03^{Dg}	15.93±0.05^{Eg}	12.55±0.04^{Fg}	7.72±0.11^{Gg}
	$E_{0.2}$	32.92±0.06^{Ae}	28.51±0.03^{Be}	24.35±0.04^{Ce}	20.24±0.04^{De}	17.42±0.04^{Ee}	14.49±0.03^{Fe}	10.26±0.04^{Ge}
	$E_{0.4}$	38.53±0.05^{Ac}	33.98±0.06^{Bc}	28.44±0.04^{Cc}	24.19±0.03^{Dc}	19.65±0.04^{Ec}	16.51±0.04^{Fc}	13.91±0.04^{Gc}
	$E_{0.8}$	42.71±0.04^{Aa}	37.86±0.04^{Ba}	33.59±0.04^{Ca}	27.68±0.05^{Da}	23.41±0.04^{Ea}	20.16±0.02^{Fa}	17.57±0.04^{Ga}
	$A_{0.2}$	32.42±0.06^{Af}	28.24±0.04^{Bf}	24.20±0.02^{Cf}	20.14±0.02^{Df}	17.26±0.04^{Ef}	14.34±0.03^{Ff}	10.11±0.04^{Gf}
	$A_{0.4}$	38.30±0.04^{Ad}	33.65±0.04^{Bd}	28.19±0.01^{Cd}	24.10±0.03^{Dd}	19.50±0.03^{Ed}	16.36±0.03^{Fd}	13.56±0.04^{Gd}
	$A_{0.8}$	42.36±0.03^{Ag}	37.49±0.04^{Bb}	33.28±0.04^{Cb}	27.40±0.03^{Db}	23.24±0.04^{Eb}	20.09±0.04^{Fb}	17.35±0.05^{Gb}
DPPH (IC50)	C	14.84±0.03^{Ga}	18.96±0.04^{Fa}	22.19±0.04^{Ea}	26.81±0.04^{Da}	32.04±0.08^{Ca}	35.15±0.05^{Ba}	39.09±0.10^{Aa}
	$E_{0.2}$	12.30±0.03^{Gc}	16.50±0.03^{Fc}	19.38±0.05^{Ec}	23.15±0.04^{Dc}	27.41±0.04^{Cc}	32.58±0.06^{Bc}	35.72±0.04^{Ac}
	$E_{0.4}$	8.38±0.04^{Ge}	14.81±0.04^{Fe}	17.00±0.02^{Ee}	20.40±0.04^{De}	24.18±0.04^{Ce}	28.28±0.05^{Be}	32.03±0.04^{Ae}
	$E_{0.8}$	5.84±0.03^{Gg}	11.31±0.02^{Fg}	14.70±0.02^{Eg}	17.20±0.06^{Dg}	21.39±0.04^{Cg}	24.83±0.05^{Bg}	27.15±0.04^{Ag}
	$A_{0.2}$	12.40±0.02^{Gb}	16.89±0.03^{Fb}	19.71±0.03^{Eb}	23.51±0.10^{Db}	27.79±0.06^{Cb}	32.92±0.04^{Bb}	35.97±0.04^{Ab}
	$A_{0.4}$	8.90±0.04^{Gd}	14.99±0.03^{Fd}	17.10±0.02^{Ed}	20.99±0.14^{Dd}	24.63±0.05^{Cd}	28.87±0.13^{Bd}	32.18±0.04^{Ad}
	$A_{0.8}$	6.00±0.02^{Gf}	11.89±0.04^{Ff}	15.00±0.06^{Ef}	17.90±0.02^{Df}	21.80±0.02^{Cf}	25.08±0.02^{Bf}	27.64±0.05^{Af}

A-G: Different capital letters in each row indicate significant differences between days of storage ($p<0.05$).
a-g: Different lowercase letters in each column indicate significant differences between doogh samples ($p<0.05$).
(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

3-4. Physicochemical Properties of Doogh Samples Containing *Moringa oleifera* Extracts

3-4-1. pH and Acidity

According to the results (Figures 1 and 2), pH and acidity differed significantly among the treatments and over storage days ($p < 0.05$). The samples containing *M. oleifera* extract showed the highest pH and the lowest acidity, whereas the control sample exhibited the lowest pH and the highest acidity. Measurements indicated that treatments E0.8 and E0.4 (containing 0.8 % and 0.4 % Moringa ethanolic extract, respectively) and A0.8 (containing 0.8 % Moringa aqueous extract) maintained the highest pH and the lowest acidity throughout storage, while the control sample had the lowest pH and highest acidity, differing significantly from the other treatments ($p < 0.05$). Between the two extract types, samples with ethanolic extract generally had significantly higher pH and lower acidity than those with aqueous extract at the same concentration. The higher pH of the treated samples may be related to the alkaline nature of the extract and its microbial-inhibitory effect [35, 36].

As storage time increased, pH decreased and acidity increased in all samples ($p < 0.05$), because longer storage promotes microbial activity and greater conversion of lactose to lactic acid, leading to pH reduction and acidity increase [17]. Overall, during fermentation and storage, the trend of increasing acidity and decreasing pH can continue until pH < 3.5. *Lactobacillus bulgaricus*, together with other acid-producing bacteria, can generate excess acid in doogh just as in yogurt [35, 36]. Similarly, higher acidity and lower pH over storage but at a slower rate than in controls have been reported when plant extracts such as fennel, *Echinophora cinerea,* mountain tea, dill, and garlic were incorporated into doogh [17, 36, 37], which are consistent with the present results.

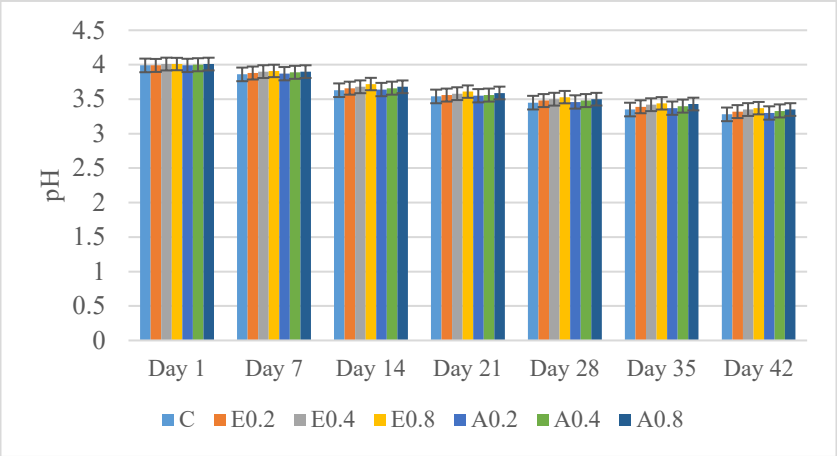

Fig 1. Effect of *Moringa oleifera* extracts on pH of doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

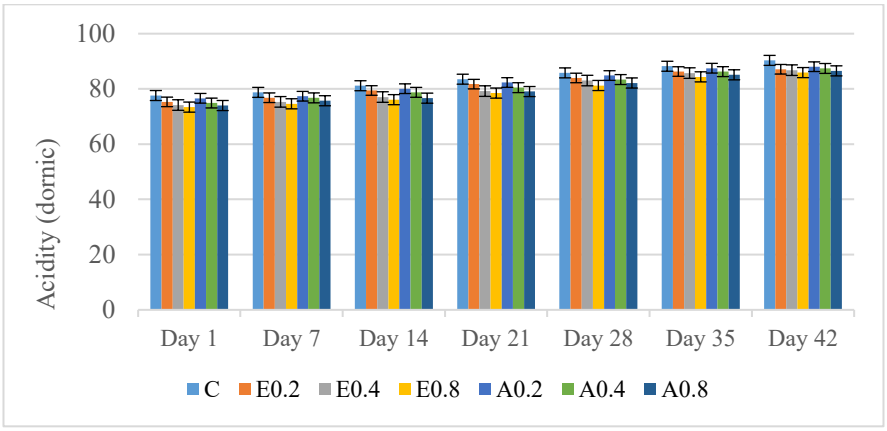

Fig 2. Effect of *Moringa oleifera* extracts on acidity of doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

3-4-2. Viscosity

Viscosity measurements (Figure 3) showed that increasing the concentration of Moringa extracts in doogh increased viscosity. The highest values were observed on day 1 in the treatments containing 0.8 % ethanolic extract (131.03 cp) and 0.8 % aqueous extract (131.24 cp), both significantly higher than the control and other treatments ($p < 0.05$). However, viscosity decreased in all samples as storage time progressed ($p < 0.05$). The phenolic compounds in plant extracts, due to their hydrophilic and hydrophobic properties, can form bonds between caseins and water, which increases resistance to external forces and results in higher viscosity [38]. Over time, as acidity rises and pH approaches the isoelectric point of milk proteins, proteins denature and break down, reducing water-binding capacity, increasing whey separation, and thus lowering viscosity. In other studies, the addition of plant ingredients such as Melissa extract and powder, and encapsulated essential oil of wild nasturtium and dill also increased the viscosity

of doogh, but the storage period caused a decrease in viscosity, which is consistent with the findings of the present study [39, 40].

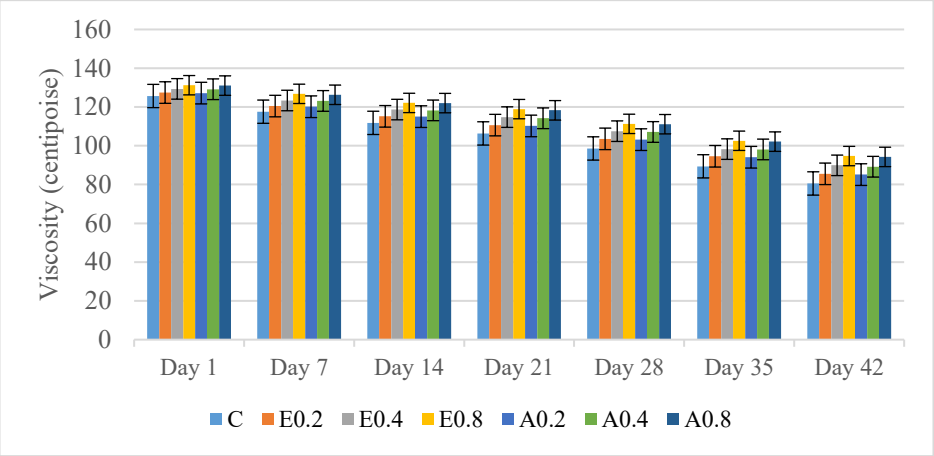

Fig 3. Effect of *Moringa oleifera* extracts on viscosity of doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

3-4-3. Results of syneresis

Based on the obtained results (Figure 4), the amount of syneresis in different samples and at different storage days showed statistically significant differences ($P<0.05$). Treatments containing higher concentrations of *M. oleifera* extracts (E0.8 and A0.8 treatments), especially the ethanolic extract, had lower syneresis, while the control sample showed the highest amount of syneresis. Furthermore, with increasing storage time, the amount of syneresis in all samples increased until day 42 of storage, although this trend was slower in the treatments containing Moringa extracts compared to the control sample. On the other hand, all treatments containing ethanolic extract had significantly lower syneresis compared to the aqueous extract treatments of Moringa at the same concentration ($p<0.05$). The presence of higher terpene and phenolic compounds in the ethanolic extract of Moringa, due to having hydrophilic and hydrophobic structures, increased the interaction between caseins and water and thereby reduced the syneresis of doogh. Another effective factor is the increase in product viscosity as a result of adding the extract to doogh, which itself leads to the entrapment of casein micelles [38].

One of the main problems of doogh is its syneresis during storage, which arises from low viscosity, pH, and their effect on proteins. The cause of instability and the increase in syneresis of doogh after production until the end of the storage period can be attributed to the low pH and the approaching of proteins to the isoelectric point, which as a result causes the proteins to begin aggregating and precipitating [41]. Furthermore, the reason for the difference in the stability of doogh treatments compared with the control sample may be attributed to the difference in the pH levels they created, since the pH levels of the doogh samples at different concentrations of extracts were different on each storage day. In studies conducted by Raftani Amiri *et al.* (2020) on the effect of nanoemulsion containing nettle extract on the stability of doogh, and by Ghosi Hoojaghan *et al.* (2022) on the characteristics of doogh enriched with tragacanth gum and fennel extract, it was reported that the addition of plant extracts increased stability and reduced syneresis in

doogh treatments, while the storage period increased the amount of syneresis in doogh, which is consistent with the findings of the present study [36, 42].

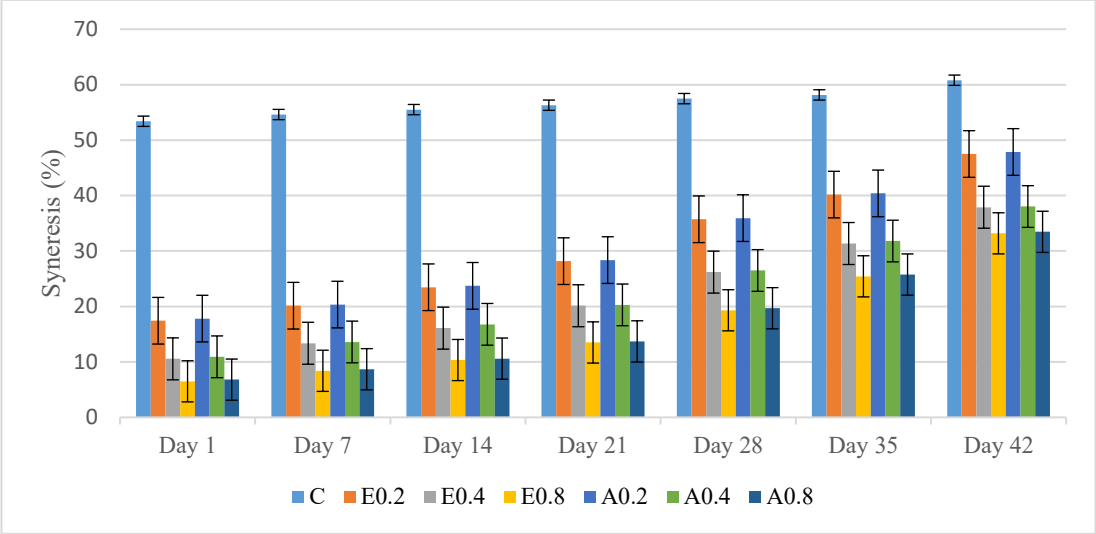

Fig 4. Effect of *Moringa oleifera* extracts on syneresis of doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

3-5. Microbial characteristics of doogh samples containing *Moringa oleifera* extract

3-5-1. Total microbial count results

Based on the obtained results (Table 3), it was found that the total microbial count of the doogh samples was significantly dependent on the type and concentration of ethanolic and aqueous extracts of *M. oleifera* as well as the storage period ($p<0.05$). Treatments containing higher concentrations of *M. oleifera* extracts, especially the ethanolic extract, had the lowest total microbial counts, while the control sample on day 42 showed the highest total microbial count (47.03 CFU/mL). As can be seen, in none of the treatments containing Moringa extracts on the first day, in treatments A0.4 and E0.4 up to the seventh day, and in treatments A0.8 and E0.8 up to day 21 of storage, microbial colony growth was observed and therefore no counting was performed. Moreover, treatments containing ethanolic extract had higher antimicrobial properties and lower microbial counts compared to the Moringa aqueous extract treatments at the same concentration, which is related to the higher phenolic content of ethanolic extract compared to aqueous extract. Similarly, in other studies on the proximate analysis of *M. oleifera* leaves and the antimicrobial activities of ethanolic and aqueous extracts of its leaves, it has also been reported that the ethanolic extract contains higher phenolic compounds than aqueous extract [43, 44].

In the present study, with increasing storage time, the total microbial count increased in all doogh samples, but the slope of increase was slower in the treatments containing Moringa extracts ($p<0.05$). Specifically, using higher concentrations (0.8%) of *M. oleifera* extracts during 35 and 42 days of storage led to a reduction of 1.34–1.39 log CFU/mL in total microbial count compared to the control sample. This is attributed to the antimicrobial properties of phenolic compounds, flavonoids,

and anthocyanins present in the Moringa extract. These compounds can react with cell wall proteins, cause permeability of the lipid membrane of the cell wall, alter and precipitate membrane proteins, denature and inhibit some microbial enzymes such as glycosyltransferase, and eventually lead to the disintegration of microbial cell membranes, resulting in leakage of cellular contents and cell death, or they may inhibit the growth and reproduction of the microbes [45, 46, 47].

3-5-2. Mold and yeast count results

Based on the obtained results (Table 3), it was found that the mold and yeast counts in the doogh samples were significantly dependent on the type and concentration of ethanolic and aqueous extracts of *M. oleifera* and the storage period ($p<0.05$). Specifically, in treatments containing higher concentrations (0.8%) of Moringa extracts, the mold and yeast counts were zero throughout the storage period, while the treatments containing lower concentrations (0.2% and 0.4%) of extracts also showed lower counts compared to the control sample. This is related to the minimum inhibitory concentration and minimum fungicidal concentration of the extracts against fungi. In fact, the absence of mold and yeast growth in the treatments containing the extract is due to the extension of the lag phase in the growth curve compared to the control. Research shows that *M. oleifera* extracts have effective antifungal properties and potentially prevent the growth of molds and yeasts. Key phytochemicals such as alkaloids, flavonoids, and phenolic compounds present in Moringa extracts contribute to this antimicrobial effect. The antifungal activity of Moringa extract is attributed to its ability to disrupt the fungal cell membrane, interfere with spore germination, and induce structural changes in fungi, leading to cell damage and growth inhibition [48]. However, in treatments containing lower concentrations of the extract, from day 21 onwards, with the decline in the extract's effectiveness, the mold and yeast counts increased, with the highest counts observed on day 42 of storage in the control sample. The increase in mold and yeast during storage can be attributed, on the one hand, to the synergistic effect of starter culture bacteria and their increased acid production over time, which creates favorable conditions for fungal growth, and on the other hand, to the reduced ability of plant extracts to control mold and yeast growth with the passage of time. The release of phenolic compounds into cells increases due to the hydrophobic nature of the active compounds, but as storage time progresses, the level of phenolic compounds decreases, and consequently, antimicrobial activity diminishes, leading to increased microbial counts over time [49]. Zarali *et al.* (2015), in a study on the effect of *Echinophora cinerea* and mountain tea extracts, and Dinpajhooh *et al.* (2019), in a study on the effect of dill and garlic extracts on the microbial characteristics of heat-treated non-carbonated doogh, reported that adding plant extracts significantly reduced mold and yeast counts, while storage increased their counts, which is consistent with the results of the present study [37, 17].

Table 3. Effects of *Moringa oleifera* extracts on microbial counts of doogh samples (Mean±SD)

Test	Treatment	Storage Days						
		1	7	14	21	28	35	42
Total plate count (CFU/mL)	C	0.0	4.29 ± 0.04^{Fa}	10.50 ± 0.04^{Ea}	17.39 ± 0.04^{Da}	26.62 ± 0.04^{Ca}	31.91 ± 0.03^{Ba}	47.03 ± 0.09^{Aa}
	$E_{0.2}$	0.0	0.64 ± 0.01^{Fc}	2.60 ± 0.03^{Ec}	10.35 ± 0.05^{Dc}	18.39 ± 0.03^{Cc}	22.16 ± 0.05^{Bc}	33.43 ± 0.03^{Ac}
	$E_{0.4}$	0.0	0.0^{Fd}	1.19 ± 0.06^{Ee}	5.30 ± 0.04^{De}	7.14 ± 0.04^{Ce}	11.33 ± 0.03^{Be}	17.23 ± 0.03^{Ae}
	$E_{0.8}$	0.0	0.0^{Dd}	0.0^{Df}	0.0^{Df}	0.32 ± 0.01^{Cg}	0.73 ± 0.01^{Bg}	2.05 ± 0.10^{Ag}

	$A_{0.2}$	0.0	0.72 ± 0.01^{Fb}	2.98 ± 0.02^{Eb}	10.44 ± 0.04^{Db}	18.61 ± 0.02^{Cb}	22.27 ± 0.03^{Bb}	33.72 ± 0.04^{Ab}
	$A_{0.4}$	0.0	0.0^{Fd}	1.43 ± 0.03^{Ed}	5.40 ± 0.02^{Dd}	7.46 ± 0.02^{Cd}	11.49 ± 0.03^{Bd}	17.61 ± 0.03^{Ad}
	$A_{0.8}$	0.0	0.0^{Dd}	0.0^{Df}	0.0^{Df}	0.36 ± 0.01^{Cf}	0.77 ± 0.02^{Bf}	2.17 ± 0.03^{Af}
Mold & yeast count (CFU/mL)	**C**	0.0	0.0	0.0	0.32 ± 0.04^{Da}	2.59 ± 0.04^{Ca}	5.05 ± 0.09^{Ba}	7.93 ± 0.05^{Aa}
	$E_{0.2}$	0.0	0.0	0.0	0.0	1.03 ± 0.04^{Cc}	2.09 ± 0.05^{Bc}	3.72 ± 0.04^{Ac}
	$E_{0.4}$	0.0	0.0	0.0	0.0	0.0	0.93 ± 0.02^{Be}	1.78 ± 0.04^{Ae}
	$E_{0.8}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	$A_{0.2}$	0.0	0.0	0.0	0.0	1.08 ± 0.01^{Cb}	2.14 ± 0.04^{Bb}	3.81 ± 0.02^{Ab}
	$A_{0.4}$	0.0	0.0	0.0	0.0	0.0	0.98 ± 0.01^{Bd}	1.89 ± 0.03^{Ad}
	$A_{0.8}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0

A-G: Different capital letters in each row indicate significant differences between days of storage ($p<0.05$).
a-g: Different lowercase letters in each column indicate significant differences between doogh samples ($p<0.05$).
(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

3-5-3. Results of Enumeration and Detection of Coliforms, *E. coli*, and *S. aureus*

The results of the enumeration of coliforms, *E. coli*, and *S. aureus* in the control sample and all doogh treatments containing *M. oleifera* extract were negative during the storage period. Therefore, according to the National Standard No. 2406 (2024), which sets the maximum level of coliforms in doogh at 10 CFU/mL and requires *E. coli* and *S. aureus* to be negative, all samples complied with the standard and had high hygienic quality [50]. The results of the present study, along with other studies, have shown that the ethanolic extract of *M. oleifera* exhibits significant antibacterial effects. The presence of bioactive compounds such as alkaloids, flavonoids, tannins, and saponins in Moringa contributes to its antibacterial properties. These compounds disrupt bacterial cell walls and interfere with cellular processes, thereby inhibiting bacterial growth [51].

3-6. Sensory Evaluation Results of Doogh Samples Containing *Moringa oleifera* Extract

Based on the ANOVA statistical results, the effects of *M. oleifera* extracts and storage time on all sensory properties (except for color) in doogh samples were significant ($p<0.05$), whereas the interaction effects between variables on sensory attributes were not significant ($p>0.05$). As shown in Figure 5, the sensory score for color in all doogh samples was very satisfactory throughout the 42 days of storage, with scores higher than 4. In contrast, other sensory attributes of doogh samples on day 42 of storage were evaluated as less than acceptable, with scores below 3, and only the E0.8 and A0.8 treatments (containing 0.8% ethanolic and aqueous extracts of Moringa) maintained scores above the acceptable level (score >3) for taste, odor, texture, and overall acceptance up to day 35 of storage (Figure 6). In other words, increasing storage time resulted in a significant decrease in sensory scores (except for color), while increasing the concentration of aqueous and ethanolic *M. oleifera* extracts increased the sensory scores compared to the control sample ($p<0.05$). Moreover, during the storage period, the treatments containing the ethanolic extract did not show statistically significant differences in sensory scores compared to the aqueous extract treatments at the same concentration ($p>0.05$).

Over time, and with the increase in acidity, the adverse effect of sourness on sensory properties was particularly observed in the control sample. This can be attributed to the

direct relationship between bacterial activity and souring of the product, as well as off-flavor development due to yeast activity, which results from the production of new metabolites in doogh, leading to sourness, bitterness, and reduced acetaldehyde [37]. The decrease in texture scores during storage may be related to the breakdown of proteins in doogh over time, reduced water-binding capacity, and, with increasing acidity and reaching the isoelectric point of milk proteins, higher syneresis and lower viscosity [37]. These results are consistent with similar studies reporting the positive effects of plant extracts on improving the sensory characteristics of dairy products [33, 40]. However, in the study by Saad and Elkhtab (2019), evaluating the effect of adding 80% ethanolic extract of Moringa leaves on yogurt quality, it was reported that although the addition of 0.8% *M. oleifera* leaf extract extended the shelf life of yogurt up to 28 days, the addition of 0.4% had the best effect on the organoleptic properties of yogurt up to 21 days of refrigerated storage [14].

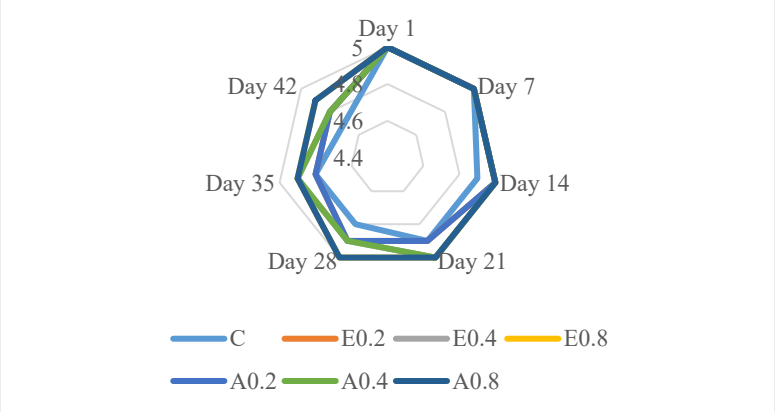

Fig 5. Effect of *Moringa oleifera* on color of Doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

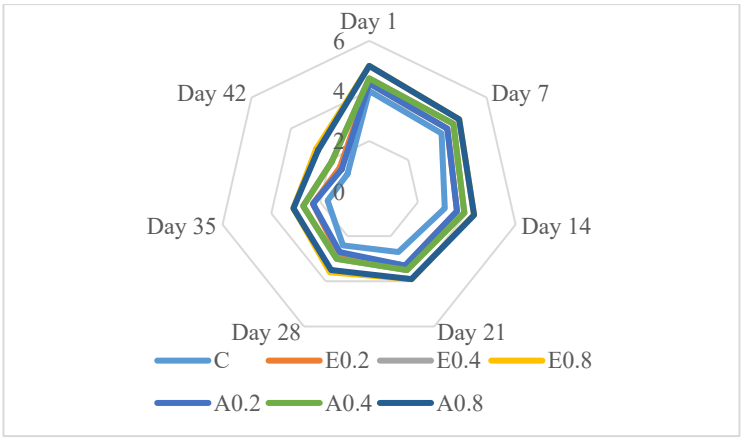

Fig 6. Effect of adding *Moringa oleifera* on odor, taste, texture, and overall acceptance of doogh samples

(C: control, $E_{0.2}$: 0.2% ethanolic extract, $E_{0.4}$: 0.4% ethanolic extract, $E_{0.8}$: 0.8% ethanolic extract, $A_{0.2}$: 0.2% aqueous extract, $A_{0.4}$: 0.4% aqueous extract, $A_{0.8}$: 0.8% aqueous extract)

4-Conclusion

The results of the present study showed that doogh treatments containing *Moringa oleifera* extract, especially at higher concentrations, exhibited higher pH, viscosity, total phenolic content, and antioxidant activity (DPPH) compared to the control, while showing lower acidity, syneresis, total microbial counts, and mold and yeast counts. Moreover, enumeration of coliforms, *E. coli*, and *S.* were negative in all doogh samples up to day 42. Sensory evaluation results also indicated that all sensory attributes of doogh, except for color, were significantly influenced by the concentration of ethanolic and aqueous extracts of *M. oleifera* and storage time. Specifically, prolonged storage significantly reduced the sensory scores of taste, odor, texture, and overall acceptance of doogh samples, whereas higher concentrations of Moringa ethanolic and aqueous extracts improved these sensory attributes in the treated doogh samples.

In conclusion, the addition of *M. oleifera* extract improves the physicochemical, antioxidant, microbiological, and sensory properties of doogh, and this extract can be considered as a natural and health-promoting preservative in doogh. The treatment containing 0.8% Moringa ethanolic extract, followed by the treatment containing 0.8% Moringa aqueous extract, achieved the highest sensory scores as well as the best physicochemical and microbiological results, and is therefore recommended as the superior treatment.

Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

Conflict Of Interest

The authors have no conflicts interest to report.

Funding Statemant

The researchers did not receive any specific grant from funding agencies the public, commercial or not-for-profit sectors.

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مجله علوم و صنایع غذایی ایران

سایت مجله: www.fsct.modares.ac.ir

مقاله علمی-پژوهشی

بررسی اثر عصاره‌های آبی و اتانولی برگ *مورینگا اولیفرا* بر ویژگی‌های میکروبی، شیمیایی و حسی دوغ گرمادیده بدون گاز

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۴/۰۲/۰۵

تاریخ داوری: ۱۴۰۴/۰۷/۰۸

تاریخ پذیرش: ۱۴۰۴/۰۷/۱۰

کلمات کلیدی:

برگ مورینگا اولیفرا،

عصاره آبی،

عصاره اتانولی،

دوغ گرمادیده،

مدت ماندگاری

DOI: 10.48311/fsct.2026.84036.0

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چکیده

این مطالعه با هدف بررسی تاثیر افزودن دو عصاره اتانولی و آبی مورینگا اولیفرا به طور جداگانه در غلظت های مختلف (۰/۲، ۰/٤ و ۰/۸ درصد) بر ویژگی‌های فیزیکوشیمیایی (pH، اسیدیته، میزان دو فاز شدن و ویسکوزیته)، آنتی‌اکسیدانی (محتوای فنل کل و فعالیت مهار رادیکال DPPH)، میکروبی (شمارش کلی میکروبی، کپک و مخمر، استافیلوکوکوس اورئوس، اشریشیا کلی و کلی فرم) و حسی (رنگ، طعم، بو، بافت و پذیرش کلی) دوغ در طی مدت ٤۲ روز نگهداری در یخچال مورد ارزیابی قرار گرفت. نتایج نشان داد عصاره مورینگا، به‌ویژه در غلظت‌های بالاتر، موجب افزایش معنی‌دار pH، ویسکوزیته، محتوای فنل کل و فعالیت آنتی‌اکسیدانی در تیمارهای دوغ نسبت به شاهد شدند و میزان اسیدیته، دو فاز شدن، شمارش کلی میکروبی و شمارش کپک و مخمر را کاهش دادند ($p<0.05$). همچنین با افزایش زمان نگهداری، میزان pH، ویسکوزیته و خاصیت آنتی‌اکسیدانی کاهش یافت، در حالی که اسیدیته، دو فاز شدن و بار میکروبی افزایش پیدا کرد ($p<0.05$). شمارش کلی‌فرم، اشریشیا کلی و استافیلوکوکوس اورئوس تا روز ٤۲ در تمامی نمونه‌ها منفی بود. ویژگی‌های حسی به‌جز رنگ، تحت تأثیر غلظت عصاره و زمان نگهداری قرار گرفتند؛ به‌طوری که افزایش غلظت عصاره منجر به بهبود طعم، بو، بافت و پذیرش کلی شد، اما رنگ تغییر معنی‌داری نداشت. در مقایسه دو عصاره مورد بررسی، تیمارهای دوغ حاوی عصاره اتانولی عملکرد بهتری در بهبود ویژگی‌های فیزیکوشیمیایی و میکروبی نسبت به عصاره آبی داشتند، اما تفاوت معنی‌داری در ویژگی‌های حسی مشاهده نشد ($p>0.05$). در نهایت می‌توان استفاده از عصاره‌های مورینگا اولیفرا را به‌عنوان یک نگهدارنده طبیعی برای بهبود ماندگاری دوغ توصیه نمود و تیمارهای حاوی ۰/۸ درصد عصاره آبی یا اتانولی مورینگا به‌عنوان تیمارهای برتر معرفی می‌شوند.