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Investigation of the Properties of Whey Protein-Sodium Alginate Edible Films Containing Extracts of Chia (*Graminifolius Tragopogon*) and Peppermint (*Mentha piperita*) Using a Mixture Design

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ABSTRACT

Food films made from biodegradable materials play a significant role in addressing consumer concerns about environmental pollution and food contamination. One of these modern packaging technologies is active packaging based on biopolymers, which offers a sustainable and environmentally friendly way to improve food shelf life. Edible coatings and films, layers They are edible compounds and biopolymers that are placed on the surface of food or among their compounds, and their use is considered one of the important methods to control physicochemical, microbial and physiological changes in food. Alginate is a linear polysaccharide extracted from brown algae, which consists of L α (1-3) glucuronic acid and β D (1-4) mannuronic acid units. This biopolymer is characterized by its unique colloidal properties, including: thickness, stability, film formation, gel production and emulsion stability, has a high potential for film and coating formation. Considering that polysaccharide films such as sodium alginate have better tensile strength compared to protein films, while protein films such as whey protein have a greater elongation than polysaccharide films, it is possible to combine sodium alginate with whey protein. It strengthened the properties of this film. Whey protein and sodium alginate solutions were prepared separately according to Table 1. First, whey protein solution (w/v) was prepared in distilled water at 80°C and stirred for 30 minutes. Glycerol (30% w/w film base) was added to the resulting solution and homogenized at 2400 rpm for 10 minutes (IKA T25 digital Ultra turrax, Germany). Then, a sodium alginate solution (w/v) was prepared in distilled water, 0.5 g of glycerol was added, heated to 80°C, and homogenized for 5 minutes at 2400 rpm. The two prepared solutions (whey protein-sodium alginate) were mixed and adjusted to pH=5-5.5. 0.1% Tween 80 was added to the resulting solution and homogenized for 4 minutes. In the next step, the resulting solution was cooled and the extracts of *Graminifolius Tragopogon*, *Mentha piperita* were added to the solution according to Table 1 and mixed for 2 minutes. The samples were poured into a plastic petri dish with a diameter of 15 cm and kept under a hood under vacuum for one hour and transferred to an oven at 37 degrees Celsius. The samples were dried for 20 hours and kept in a desiccator for testing. The highest opacity was associated with a 1% concentration of Peppermint extract, while the lowest opacity at the same concentration was noted for, *Graminifolius Tragopogon* extract. Increasing the concentration of Peppermint up to 0.75% resulted in an increase in permeability by 9 grams per meter per second Pascal. The highest tensile strength and Young's modulus were recorded for whey protein at a concentration of 0.75% and sodium alginate at 0.25%, with values of 17 and 85 MPa, respectively. The antioxidant property of the edible film was significantly validated using DPPH radicals ($p < 0.05$), and increased with the concentration of Peppermint up to 1%. Evaluation of the antimicrobial activity of the films, using the disk diffusion method, indicated that the largest inhibition zone was obtained at a concentration of 0.5% whey protein-alginate-Peppermint-*Graminifolius Tragopogon* extracts against *Staphylococcus aureus*, with an average diameter of 24 mm. Subsequently, at concentrations of 0.75% and 1%, the average inhibition zone diameter for *Escherichia coli* was 20 mm, attributed to Peppermint and whey protein, respectively. Overall, the results demonstrated that the addition of the Peppermint- *Graminifolius Tragopogon* extract led to the production of films that not only inhibited the growth and proliferation of bacteria but also exhibited strength, making them suitable for use in perishable food items

1- INTRODUCTION

In recent years, the main reason for the changes in food packaging technology has been the increasing consumer demand for minimally processed foods [1]. Edible films and coatings must possess essential features for food packaging, including moisture barrier effects, improved color and appearance, desirable mechanical properties, and non-toxicity. These characteristics depend on the type of food used, the formation process, and their applications [2]. One of these modern packaging technologies is active packaging based on biopolymers, which provides a sustainable and environmentally friendly way to improve food shelf life [3], and is considered one of the main methods for controlling physiological, microbial, and physicochemical changes in food products [4]. To produce biodegradable films, polysaccharides, proteins, fats, or a mixture of these are used [5]. Alginate, a linear polysaccharide extracted from brown seaweed, is composed of units of α -L (3-1) guluronic acid and β -D (4-1) mannuronic acid. This biopolymer, due to its unique colloidal properties, including thickness, stability, film formation, gel production, and emulsion stability, has high potential for film and coating formation [6]. Numerous domestic and foreign studies emphasize the positive effects of storing this material [7, 8, 9, 10, 11, 12, 13, and 14]. In this context, considering that polysaccharide films such as sodium alginate have better tensile strength compared to protein films, while protein-based films like whey protein have greater elongation at break, it is possible to enhance these properties by combining sodium alginate with whey protein [15]. Several polysaccharides and their derivatives, including starch, agarose, alginate, carrageenan, chitosan, pectin, gellan, pullulan, and gums (gum arabic, guar gum, xanthan gum, etc.), are commonly incorporated into protein films [16]. The mechanical and barrier properties of whey protein films tend to attract more attention

because these films generally perform better than polysaccharide-based films [17]. Whey protein concentrate (WPC) is one of the products derived from whey. Due to its properties such as digestibility, high solubility, viscosity formation, gelation ability, emulsification, foaming, and foam formation, it holds high value and has numerous applications in various food industries, including dairy, baking, pastry products, meat products, and others [18]. Whey protein isolate (WPI), as a packaging material in the form of films, as well as in multilayer films and coatings, has been extensively studied [19]. Whey proteins, in addition to their nutritional value, possess several notable physical properties, such as water solubility and emulsification capability, in edible films [17]. The availability of these proteins creates significant interest in packaging industries, enabling them to be used as films/coatings on food surfaces to protect foods against chemical or microbiological spoilage, thereby increasing shelf life and maintaining high product quality. Evidence regarding the antimicrobial activity of bioactive agents present in whey protein-based films is limited and mainly evaluated in laboratory conditions using agar disk diffusion methods with aromatic oils such as thyme and rosemary [20, 21]. To date, few studies have focused on the effect of polyphenol-rich plant extracts on the properties of films made from different protein sources. Whey protein-sodium alginate is often considered as carriers of plant-based antioxidants for creating active packaging (AP). However, the obtained materials have yet to be systematically compared to demonstrate their potential advantages and disadvantages. Polyphenols can interact with various proteins through weak reversible bonds (such as hydrogen bonds, hydrophobic interactions, and Van der Waals forces) and irreversible interactions (such as covalent bonds formed during oxidation and nucleophilic addition processes) in different ways [22]. *Peppermint* (*Mentha piperita*) is a plant belonging to the Lamiaceae family and is recognized as a

healthy plant. The entire plant possesses beneficial properties and is widely used in the cosmetic, pharmaceutical, and food industries. The active chemical compounds in *peppermint* include monoterpenes, caffeic acids, flavonoids, tannins, and active oils such as menthol. Other compounds found in peppermint include polymerized polyphenols, carotenoids, tocopherols, betaine, and choline [23]. Ashrafi and colleagues (2019) demonstrated that natural extracts of plants (rosemary and peppermint) are useful additives to edible films (pectin, chitosan, and starch polymers), which enhance their functional properties. They exhibited the highest inhibitory activity against all pathogenic strains (*Bacillus subtilis*, *Escherichia coli*, and *Listeria monocytogenes*) [24]. In a study, Musavian and colleagues (2017) used *peppermint* essential oil in a cassava starch film, and they reported that adding the oil reduced the film's water affinity and increased its resistance to moisture and vapor [25]. The plant *Tragopogon graminifolius*, known as *Tragopogon*, belongs to the Asteraceae family. The identified compounds in this plant are heterocyclic, including flavonoids, terpenes, saponins, benzyl and hydro-isocoumarins, phenolic compounds, and sterols, many of which are present in this genus. Shahraki et al. conducted a study to examine the antioxidant activity and antimicrobial properties of methanolic extracts of wild barberry (*Berberis integerrima*) and *Tragopogon graminifolius*. The findings showed that both extracts exhibited different antibacterial effects. *Bacillus subtilis* was the most sensitive bacterium, while *Salmonella Typhimurium* was the most resistant. Additionally, the wild barberry extract demonstrated greater inhibitory effects on the microorganisms studied. The wild barberry extract also showed a significant advantage over *Tragopogon* in terms of phenolic content, antioxidant capacity, and antimicrobial activity [26]. Chitosan, gelatin, edible proteins, sodium caseinate, sodium alginate, whey protein, soy

protein, and others are among the natural coatings used. When combined with plant essential oils, their antibacterial and antioxidant properties are enhanced, which can reduce spoilage rates in various products during storage. These edible films are water-soluble and help preserve product quality attributes such as aroma, taste, flavor, color, and nutritional value (including vitamins and essential amino acids). The aim of this research is to investigate the effects of *Tragopogon (Sheng)-peppermint* extracts on the physicochemical, mechanical, antioxidant, and antimicrobial properties of whey protein-sodium alginate edible films. Atarés and colleagues (2016) reported in their study on whey protein-sodium alginate films that this combination improves the tensile strength and flexibility of the film. Their research showed that an optimal ratio of these two components could significantly enhance the film's mechanical properties [27]. Shahraki and colleagues (2020) investigated the antioxidant and antimicrobial properties of *Tragopogon graminifolius* extract and found that the methanolic extract contained phenolic and flavonoid compounds, exhibiting moderate antioxidant activity. The extract demonstrated stronger antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis*) compared to Gram-negative bacteria (*Salmonella Typhimurium*). Additionally, the antioxidant activity of *Tragopogon* extract was weaker in comparison to wild barberry extract [28].

2-Materials and Methods

2.1. Materials

Whey protein, sodium alginate, and the chemicals used in this study were purchased from Sigma-Aldrich (USA). The plant *Tragopogon graminifolius* was collected from the mountains of Ilam Province, and *Mentha piperita* (*peppermint*) was obtained from the Medicinal Plants Research Center at the Faculty of Agriculture, Ilam University.

2.2. Aqueous Extract Preparation

For extraction via cold maceration, *Tragopogon graminifolius* and *Mentha piperita* were separately dried and powdered. The plant powders were then mixed with the solvent water-ethanol (70:30) at a ratio of 1:10 and macerated for 48 hours at 25°C in the dark. After filtering through Whatman No. 1 filter paper, the extracts were concentrated using a rotary evaporator at 40°C and then lyophilized into powder.

To prepare the edible films, whey protein (5% w/v) and sodium alginate (2% w/v) were dissolved in distilled water, and glycerol (1.5% w/v) was added as a plasticizer. The extracts were incorporated into the formulation based on a mixture design with various concentrations (0.5, 0.75, 1, and 3.25%). The homogeneous mixture was poured into petri dishes and dried in an oven at 45°C for 24 hours [29].

2.3. Preparation of the Composite Film

The whey protein and sodium alginate solutions were prepared separately according to Table 1. The whey protein solution (w/v) was prepared in distilled water at 80°C with continuous stirring for 30 minutes. Glycerol (30% w/w of the film base) was added to this solution, and it was homogenized at 2400 rpm for 10 minutes using an IKA T25 digital Ultra Turrax (Germany).

Similarly, the sodium alginate solution (w/v) was prepared in distilled water, with 0.5 g of glycerol added, heated to 80°C, and homogenized for 5 minutes at 2400 rpm. The two solutions (whey protein and sodium alginate) were mixed and adjusted to pH 5.5–6.0. Ten percent Tween 80 was then added and homogenized for 4 minutes. The cooled mixture was then combined with extracts of *Tragopogon graminifolius* and *Mentha piperita* according to Table 1 and mixed for 2 minutes. Finally, the samples were poured into 15-centimeter diameter plastic Petri dishes, placed under vacuum inside a hood for one

hour, transferred to an oven at 37°C, and dried for 20 hours. The dried films were stored in desiccators for subsequent testing [30].

2.3.1. Measurement of Film Thickness

The thickness of the films was measured to evaluate their mechanical properties, water vapor permeability, and optical properties. The thickness of the produced films was determined using a micrometer (caliper) with an accuracy of approximately 0.0001 mm at 10 different points on each sample. The average thickness was then calculated and used in the assessment of tensile strength, water vapor permeability, and opacity [31].

2.3.2. Solubility

Film samples (2 cm × 2 cm) were dried in an oven at 105°C overnight and weighed (initial dry weight, W1). The samples were then immersed in 25 mL of distilled water and kept at 25°C for 24 hours. After recovery, the films were blotted and dried again in an oven at 105°C to determine their final dry weight (W2). Solubility in water was calculated using the following equation, where W1 is the initial dry weight and W2 is the final dry weight:[32]

$$\text{Solubility (\%)} = (W1 - W2) / W1 \times 100$$

2.3.3. Permeability to Water Vapor (Vapor Transmission Rate)

Water vapor permeability was measured using gravimetric methods (ASTM-E96-95, 1995). A 14 mL container was filled with 10 mL of distilled water and sealed with the examined films, each having an area of 1.5 cm². The sealed bottles were weighed initially and then placed in a desiccator containing silica gel with a specified relative humidity and temperature. The bottles were weighed every 12 hours over a period of 3 days. The weight changes over time were plotted, and the slope of the graph

was calculated. Water vapor permeability (WVP) was then determined using the following formula:[33]

$$\text{WVP (gm}^{-1}\text{pa}^{-1}\text{s}^{-1}) = \frac{W \times X}{A \times t \times \Delta p}$$

W=the weight difference of the bottle,

X=the thickness of the gelatin film,

A=the surface area of the gelatin film (m²),

t=time in seconds,

Δp = the vapor pressure difference between the two sides of the film — the container filled with pure water and the chamber containing silica gel.

2.3.4. Optical Properties Measurement

Optical properties of the films were assessed using a full spectrum spectrophotometer. Samples measuring 9×40 mm were cut and placed inside spectrophotometer cuvettes. A blank sample was used as a control. The light transmittance at various wavelengths and absorbance at 500 nm were measured. Film haze was calculated using the following formula:[34]

$$\text{Film thickness} \times \text{absorbance at 500 nanometers} = \text{film opacity (haze)}$$

2.3.5. Mechanical Properties Measurement

Mechanical properties, including tensile strength (MPa), elongation at break (%), and Young's modulus (MPa), were determined according to ASTM-D882 standards using a texture analyzer (TA-Plus, England) [34]. Initially, film strips of 10×1 cm were cut, and thickness was measured at 10 different points. Films were conditioned in a desiccator containing magnesium nitrate solution at 55% humidity and 25°C for 3 days. The samples were then mounted between the grips of the texture analyzer, with an initial distance of 50 mm between the grips and a crosshead speed of 50 mm/min. Mechanical properties—tensile strength, elongation at break (calculated as

(change in length / original length) × 100), and Young's modulus—were calculated based on a standard tensile test, indicating the product's stiffness within the linear elastic range.

2.3.6. Antioxidant Properties

The antioxidant activity of the gelatin films was evaluated by measuring the color change of the DPPH radical from purple to yellow hydrazines. For this purpose, 30 mg of each film was dissolved in equal parts of distilled water and methanol (3:3 ratio). After complete dissolution, the solutions were subjected to centrifugation at 4000 rpm for 15 minutes using a Universal centrifuge (Germany). The samples were then stored at 4°C for 12 hours (overnight rest) and centrifuged again at 4000 rpm for 15 minutes.

Next, 1 mL of the upper layer of each centrifuged solution was mixed with 4 mL of 0.1 mM methanolic DPPH solution and kept in the dark at room temperature for 30 minutes. The absorbance was then measured at 517 nm using a spectrophotometer (AS). As a control, 1 mL of a mixture of water and methanol (3 mL distilled water + 3 mL methanol) was combined with 4 mL of 0.1 mM methanolic DPPH solution and incubated similarly for 30 minutes at room temperature in an incubator (Mettler, Germany). The absorbance of this sample was also measured at 517 nm using a spectrophotometer (Shimadzu, Japan).

Finally, the DPPH radical scavenging activity was calculated using the following equation:[34]

$$\text{DPPH scavenging activity} = \frac{(A_b - A_s)}{A_b} \times 100$$

2.3.7. Antimicrobial Properties of the Composite Films

The disk diffusion agar method was used to assess the antimicrobial activity of the films against model bacteria, including *Escherichia*

coli, *Staphylococcus aureus*, and *Pseudomonas*. For this purpose, 100 μL (10^{-6} CFU/g) of each bacterial suspension was spread onto Muller-Hinton agar plates. Subsequently, 6-mm diameter disks were cut from each film and placed onto the surface of the inoculated agar. The plates were then incubated at $35 \pm 2^\circ\text{C}$ for 24 hours. The zones of inhibition (measured in millimeters) around each disk were recorded to evaluate the antimicrobial activity [35].

2.4. Statistical Analysis

In this study, a mixed experimental design (Simplex Lattice) was used to investigate the main effects and interaction effects of two essential oils. The experimental design, variance analysis (ANOVA), and graph plotting were performed using Design Expert 13 software according to Table 1.

Table 1 - Experiments designed using the response surface method and calculated responses in investigating the effect of independent variables on the characteristics of produced films

	Mix 1-1	Mix 1-2	Mix 2-1	Mix 2-2
Run	A:Whey	B:Sodium Alginate	C: Graminifolius Tragopogon	D: piperita Mentha
1	0	1	0	1
2	1	0	0.25	0.75
3	0.5	0.5	1	0
4	1	0	1	0
5	0.5	0.5	1	0
6	1	0	0	1
7	0	1	0.5	0.5
8	0.5	0.5	0	1
9	0.5	0.5	0	1
10	1	0	0.75	0.25
11	0.5	0.5	0.5	0.5
12	1	0	0.5	0.5
13	0.25	0.75	0.25	0.75
14	0.5	0.5	0.5	0.5
15	0.25	0.75	0.75	0.25
16	0.75	0.25	0.25	0.75
17	0	1	0.5	0.5
18	0	1	1	0
19	0.5	0.5	0.5	0.5

3-Results and Discussion

3.1. Mechanical Tests (Elongation at Break, Tensile Strength, Young's Modulus):

The results of the interaction effects of different concentrations of *Tragopogon graminifolius* and *peppermint* extracts with the whey protein-sodium alginate composite films are shown in Figure 1. The statistical analysis indicated significant differences in the tests for elongation at break, tensile strength, and Young's modulus across the treatments ($p < 0.05$).

(A) Elongation at break (elongation capacity) showed no significant effect at maximum concentrations of *Tragopogon* and *peppermint* extracts. However, as the whey protein concentration increased, elongation at break rose and reached its maximum at 75% concentration. Conversely, increasing sodium alginate concentration decreased elongation at break, with the combined film (75% whey protein and 25% sodium alginate) reaching the highest value of 17 MPa, likely due to improved structural bonding and alignment with whey protein molecules.

(B) Tensile strength showed no significant change with increasing concentrations of the extracts. The highest value (~32 MPa) was observed at an intermediate ratio of 0.5-0.75 of *peppermint* and *Tragopogon* extracts, increasing with higher sodium alginate concentrations up to 1%, while decreasing whey protein.

(C) Young's modulus increased with higher whey protein concentrations, reaching a maximum of 85 MPa at 75% whey protein and 25% sodium alginate. However, in edible films, increasing sodium alginate concentration

resulted in a decrease in Young's modulus, with the lowest at 1%, approximately 65 MPa.

Polysaccharides generally interact with proteins to enhance the mechanical and barrier properties of films and coatings. Electrostatic interactions between proteins and polysaccharides (such as alginate, carboxymethyl cellulose, and pectin) improve the cohesion of the polyprotein network through cross-linking [17]. Two factors strongly influence the mechanical properties of WP-based films: first, the development of a three-dimensional protein network during film formation creates a dense, cross-linked matrix leading to stronger and tougher materials with higher modulus and tensile strength; second, this material tends to have less flexibility due to lower elongation capacity, which aligns with our experimental findings [36].

Multiple factors, including particle shape, molecular structure, particle size distribution, and interparticle interactions, affect mechanical properties. Pinrouves et al. (2017) studied starch-based films and attributed the reduction in tensile strength and Young's modulus to the influence of essential oils on the heterogenous structure of the film and weak interactions between glycerol and starch [37]. The incorporation of plasticizers reduces the extent of molecular interactions among protein chains, affecting mechanical performance not only by ratio but also by the type of plasticizer used [38].

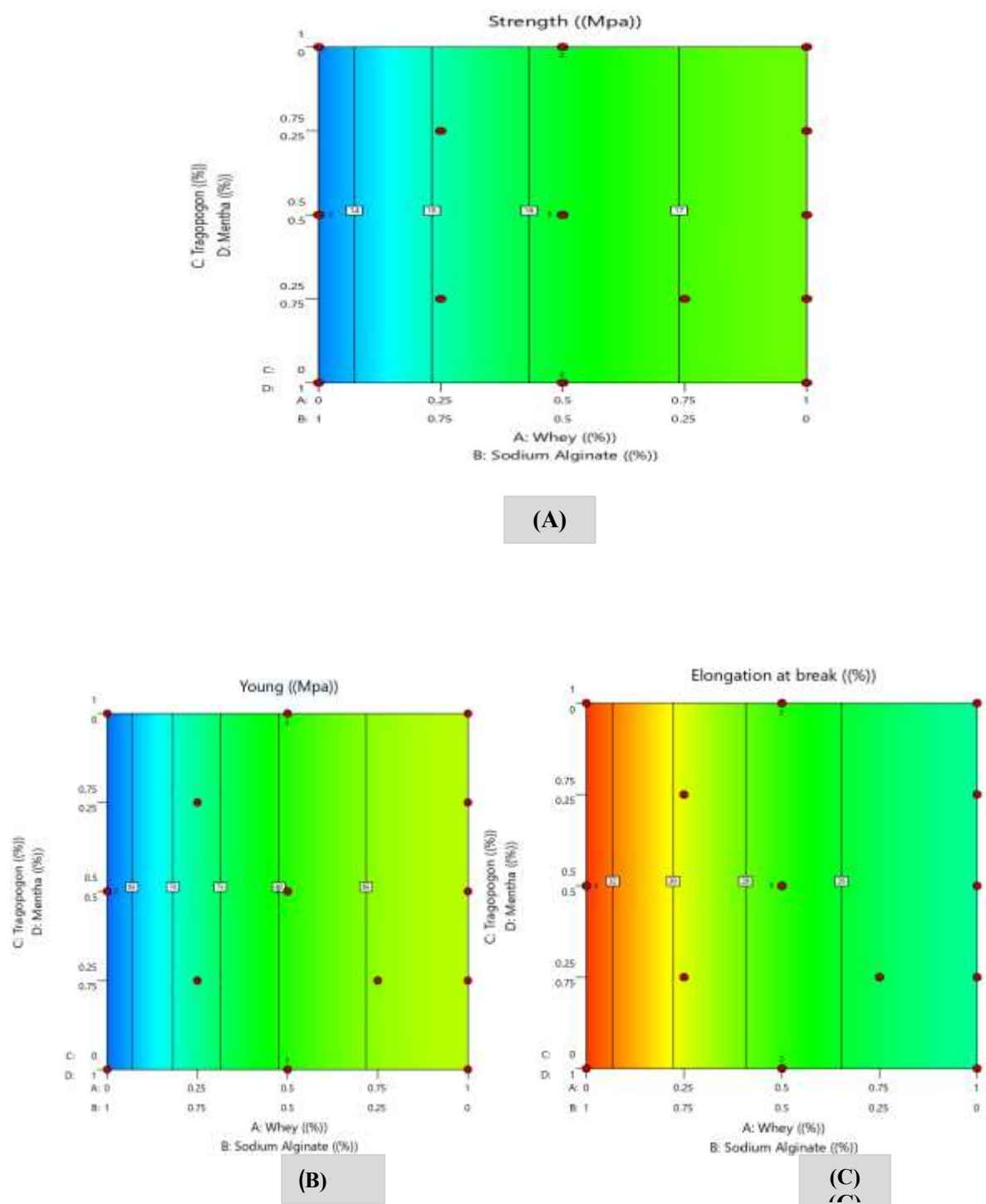


Fig 1. Interactive effects of *Graminifolius Tragopogon*, *Mentha piperita* extracts on: A) tensile strength B) elongation at break C) Young's modulus of whey protein-alginate edible films

3.2. Physical Properties of the Produced Films

3.3. Opacity and Solubility

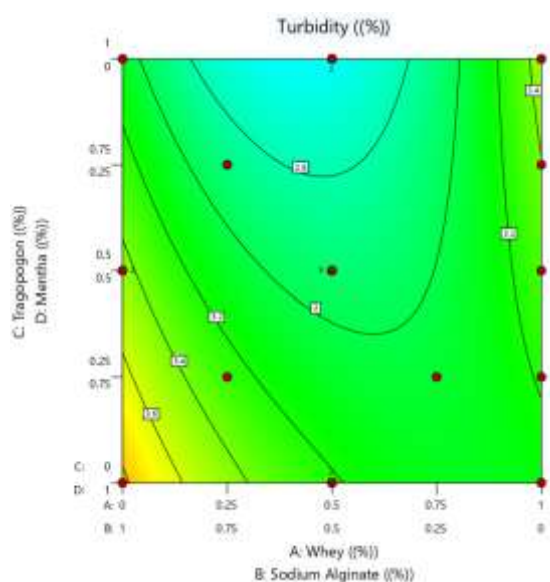
The results of opacity and solubility are shown in Figures 2(A) and (B). Opacity is a measure used to evaluate the transparency of films; lower opacity indicates higher transparency [39]. Comparison of the opacity of the produced

films revealed no significant differences between the *Tragopogon graminifolius* and *peppermint* extracts and the whey protein-sodium alginate films ($p > 0.05$). Increasing the concentration of *peppermint* extract to 1% resulted in the highest opacity, attributed to its heterogeneous structure with pores in the film, caused by ununiform particle distribution and increased thickness leading to light absorption by phenolic compounds in the extract. Conversely, increasing the concentration of *Tragopogon* extract to 1% decreased opacity.

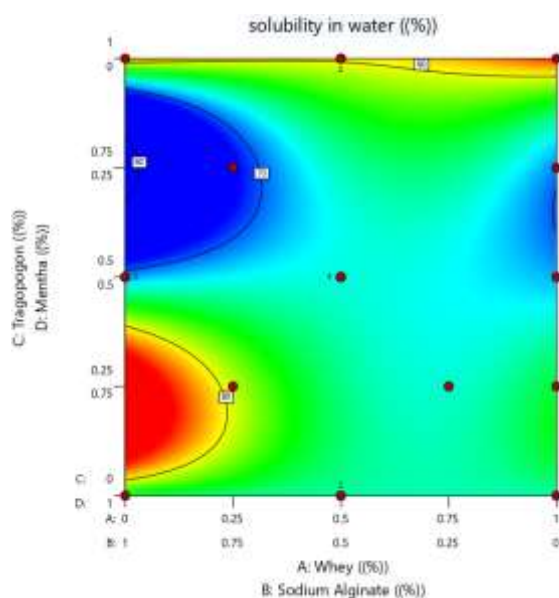
In this context, Alizadeh Sani et al. (2022) reported that adding saffron and red berry extracts significantly reduced the transparency of gelatin/Carrageenan-based films. The addition of extracts, due to the migration of some extract components to the film surface during drying, increased light scattering and decreased opacity [32]. These findings are consistent with results showing increased transparency in hybrid films and films containing essential oils, as reported by us and others. For example, in 2016, the use of soybean

oil emulsions and cinnamon essential oil in chitosan films, and the effects of fatty acids and plant extracts on gelatin-based composite films, were reported to have similar effects [40, 41]. The type of edible film (whey protein-sodium alginate) did not significantly affect opacity. Hosseini and colleagues studied the effect of different concentrations of thyme, oregano, and sage essential oils on gelatin-based edible film characteristics; they found that the opacity of the films was not significantly affected. The highest opacity was observed in films with the lowest concentration of oregano oil [42].

Figure 2(B) shows the interaction effects of different concentrations of edible films on solubility, which was not significantly affected across all extract concentrations used ($p > 0.05$). Overall, the highest solubility (~80%) was observed at 25% *Tragopogon* extract and 75% *peppermint* extract. As *Tragopogon* extract concentration increased, solubility decreased, reaching its minimum at 75%.



(A)



(B)

Fig 2 . Interactive effects of *Graminifolius Tragopogon*, *Mentha piperita* extracts on : A) turbidity and B) solubility of whey protein-alginate edible films

3.4. Thickness and Water Vapor Permeability

The film thickness, influenced by the formulation matrix, is always affected by the addition of various components such as emulsifiers, plasticizers, dry matter content, and additives like essential oils [5]. This behavior can be attributed to the amount of different substances incorporated, which cause significant changes in the optical properties of the films. The results of film thickness are shown in Figure 2(A). As observed, the interaction effects of film thickness at different essential oil concentrations are statistically significant ($p < 0.05$). Increasing the whey protein concentration led to a reduction in thickness. The maximum thickness was observed at a concentration of 0.5-0.5% whey protein and sodium alginate, reaching 0.35 mm. Increasing the percentages of *Tragopogon* and *peppermint* extracts had no significant effect on the thickness of the produced films.

The influence of polymer type and film composition on the resulting thickness can also be seen in the results of other studies. Fernandez et al. (2004) used various plasticizers such as glycerol, erythritol, and sorbitol, and the resulting composite films showed different thicknesses, with the film containing glycerol having the greatest thickness. Therefore, the observed variation in thickness is affected by the nature and composition of the film and the interaction between different components, which create different structures [43].

Permeability results with water vapor showed that high concentrations of *Tragopogon* extract decreased permeability. Conversely, increasing *peppermint* extract to 75% significantly increased permeability to 9 ($p < 0.05$). This behavior is likely due to the pores and cracks formed by the evaporation of the essential oil incorporated into the formulation. These findings are consistent with Hosseini et al. (2023), who reported that increasing thyme essential oil increased water vapor permeability of gelatin-based films [42]. Water vapor transmission occurs primarily through the film's hydrophilic section, and permeability depends on the ratio of hydrophilic to lipophilic domains within the film [37, 44]. The interaction between essential oils and other film constituents reduces the hydrophobicity of the matrix, leading to increased water vapor permeability at higher *peppermint* concentrations.

Figure 3(B) shows that the increase in permeability in the presence of high *peppermint* oil concentration reaches its maximum, which can be attributed to the cohesive matrix or polymer network in the presence of the oil. Ahmad et al. (2018) observed that higher surface area mixing of citrus oil increased permeability, consistent with our observations. Additionally, higher concentrations of lemon oil resulted in decreased permeability of gelatin films, aligning with previous findings, which they attributed to differences in the nature of the essential oils used [45].

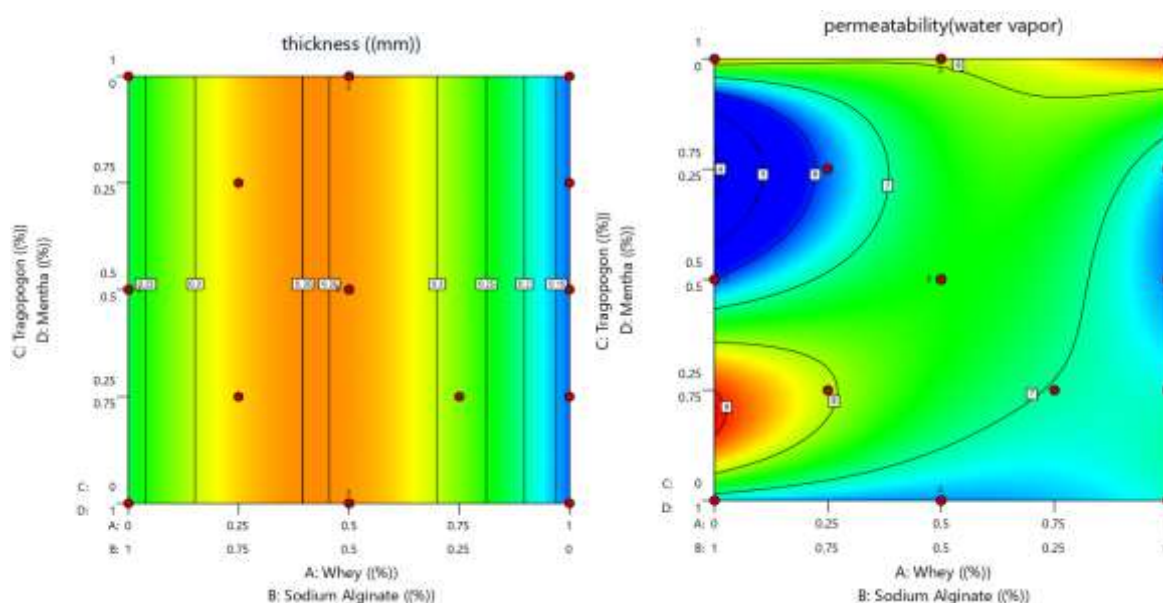


fig3. The interactive effects of *Graminifolius Tragopogon*, *Mentha piperita* extracts: A) thickness and B) water vapor permeability of whey protein-alginate edible films

3.5. Antioxidant Activity

Regardless of its health benefits, antioxidant activity prevents the oxidation of the product during storage. The total phenolic compounds are an indicator of the plant's antioxidant capacity. An increase in antioxidant activity may be directly related to the phenolic compound content in the extract. Antioxidants are used in active packaging films to inhibit oxidation reactions and extend the shelf life of food products [26]. Figure 4 presents the results of the antioxidant activity of the whey protein-sodium alginate composite film, with significant effects observed for the interaction of different concentrations of *Tragopogon graminifolius* and *peppermint* extracts ($p < 0.05$).

The identified compounds in *Tragopogon graminifolius* include flavonoids, terpenes, saponins, benzyl derivatives, hydroisocoumarins, phenolic compounds, and sterols [26]. The *Tragopogon* and *peppermint* extracts exhibited different effects on the antioxidant capacity of the produced films, with

peppermint extract showing markedly stronger effects, likely due to its higher phenolic content. The main constituents of *peppermint* essential oil include α -pinene, β -pinene, limonene, isomenton, terpinene, and menthol [46].

In this context, Momeni et al. (2024) attributed the weak antioxidant activity of *Salvilia* species' essential oils, in reducing DPPH radicals, to their low phenolic compound content [35]. Variations in antioxidant potency among different extracts can be related to the plant species, cultivar diversity, environmental conditions, climate, soil type, harvest time, and numerous other factors. Increasing the concentration of the whey protein-sodium alginate film did not significantly affect the antioxidant activity, indicating the absence of radical scavenging functional groups in the structure of the whey protein-sodium alginate matrix. Akhtar et al. (2019) reported that the nature of the film and differences in the antioxidant capacity of added compounds influence the overall antioxidant activity of the films [24].

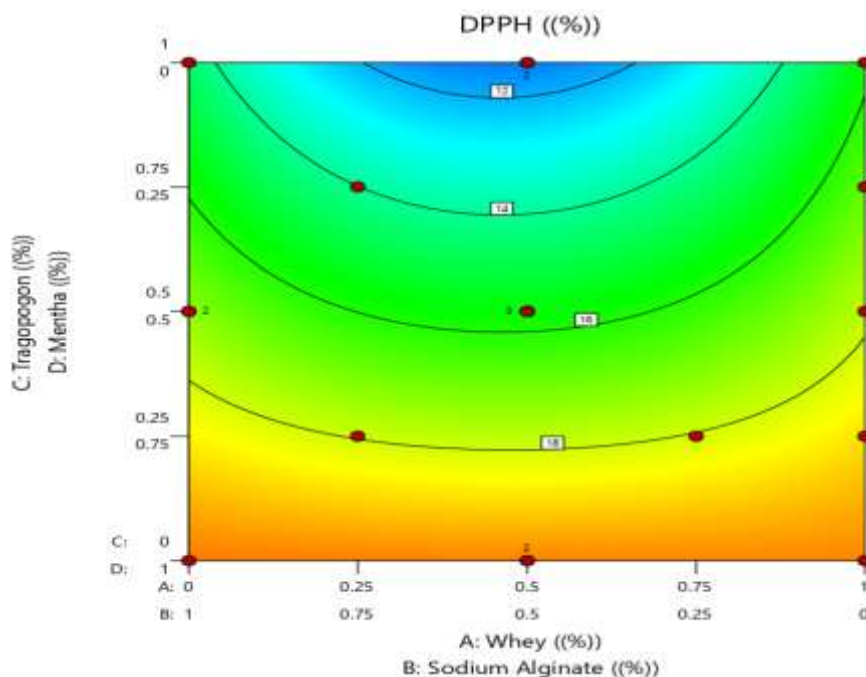


Fig4. Antioxidant properties of Whey Protein - Sodium Alginate films with different concentrations of *Graminifolius Tragopogon*, *Mentha piperita* extracts

3.6. Antimicrobial Activity

The ability to inhibit or prevent spoilage or the growth of pathogenic microorganisms on food items is another important feature of active packaging materials [26]. Figures 5(A), (B), and (C) depict the significant interaction effects of different concentrations of *Tragopogon graminifolius* and peppermint extracts on the growth inhibition zone around edible whey protein-sodium alginate films against *Pseudomonas*, *Escherichia coli*, and *Staphylococcus aureus*, with statistically significant differences observed in *E. coli* and *Pseudomonas* ($p < 0.05$).

In Figure 5(A), at concentrations of 0.5-0.5%, the highest inhibition zone (~19.5 mm) against *Pseudomonas* was observed with *Tragopogon* and *peppermint* extracts. Increasing the concentrations of these extracts led to a decrease in microbial inhibition. However, increasing the whey protein and *peppermint* concentrations did not significantly affect bacterial growth inhibition.

In Figure 5(B), increasing the concentration of *Tragopogon* extract resulted in decreased antimicrobial activity, whereas increasing *peppermint* extract concentration enhanced microbial growth inhibition, reaching the highest inhibition zone of 20 mm at 75% concentration. Talaei et al. (2008) found that *Tragopogon* extract at concentrations below 1900 micrograms per milliliter does not have inhibitory properties against *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, or *Staphylococcus aureus*. As the concentration of the whey protein-sodium alginate edible film increased, the inhibition of *E. coli* growth also increased, reaching its maximum at 1% concentration.

Overall, the antimicrobial effectiveness of the edible film depends on the nature of the extract and the type of microorganism [47]. However, increasing the concentration of the sodium alginate film reduced the microbial inhibition. When combined with the sodium alginate and whey protein edible films, the extracts (*tragopogon* and *peppermint*) demonstrated activity against both Gram-positive and Gram-

negative bacteria, likely due to possible chemical interactions between hydroxyl groups in the film and phenolic compounds, which may block active sites of the extracted compounds [48], thereby reducing or halting microbial growth. Studies have indicated that minor components (at low concentrations) in the film formulations can play a significant role in the antimicrobial activity of the films [49, 50].

Figure 5(C) shows the interaction effect of different concentrations of *Tragopogon graminifolius* and *peppermint* extracts on edible whey protein-sodium alginate films against *Staphylococcus aureus*, which was not statistically significant ($p > 0.05$). However, at concentrations of 0.5-0.5%, whey protein and sodium alginate, the maximum inhibitory effect on the growth of *Staphylococcus aureus* was observed, with a zone of inhibition measuring 24 mm. The 1% whey protein concentration showed the lowest inhibition zone, measuring 18 mm. Plant essential oils and their active compounds are recognized for their antimicrobial activity against a broad spectrum of microorganisms, including both Gram-positive and Gram-negative bacteria. However, some studies indicate resistance in Gram-positive bacteria and activity primarily against Gram-negative bacteria.

Momeni et al. (2023) investigated the properties of gelatin-based edible films enriched with sage essential oil at concentrations of 0%, 1/4%, 1/2%, and 3/4% [35]. The antimicrobial activity of the films was evaluated using the disc

diffusion method. The largest inhibition zones were observed at a sage essential oil concentration of 3/4%, with activity primarily against *Staphylococcus aureus*, followed by *Pseudomonas aeruginosa* and *Escherichia coli*. Lopez et al. (2023) studied the antimicrobial and antioxidant properties of alginate edible coatings combined with plant extracts. The results showed that, except for the coating with sage extract, all other coatings entirely inhibited or reduced the growth of all tested bacteria, including *Pseudomonas aeruginosa*, *E. coli*, *S. aureus*, and *Listeria monocytogenes* [51]. However, only films combined with licorice and eucalyptus could inhibit the growth of Gram-positive bacteria, while none of the films could inhibit Gram-negative bacteria.

Rihana Akhtar and colleagues (2019) examined biocomposite films containing rosemary and mint essential oils, concluding that the antimicrobial activity against both Gram-positive and Gram-negative bacteria resulted from the polyphenolic components present in the films [24]. Therefore, it can be concluded that the combination films based on whey protein-sodium alginate can effectively inhibit the growth of both Gram-positive and Gram-negative bacteria, making them potentially useful for food preservation, increasing shelf life, and maintaining product quality and safety.

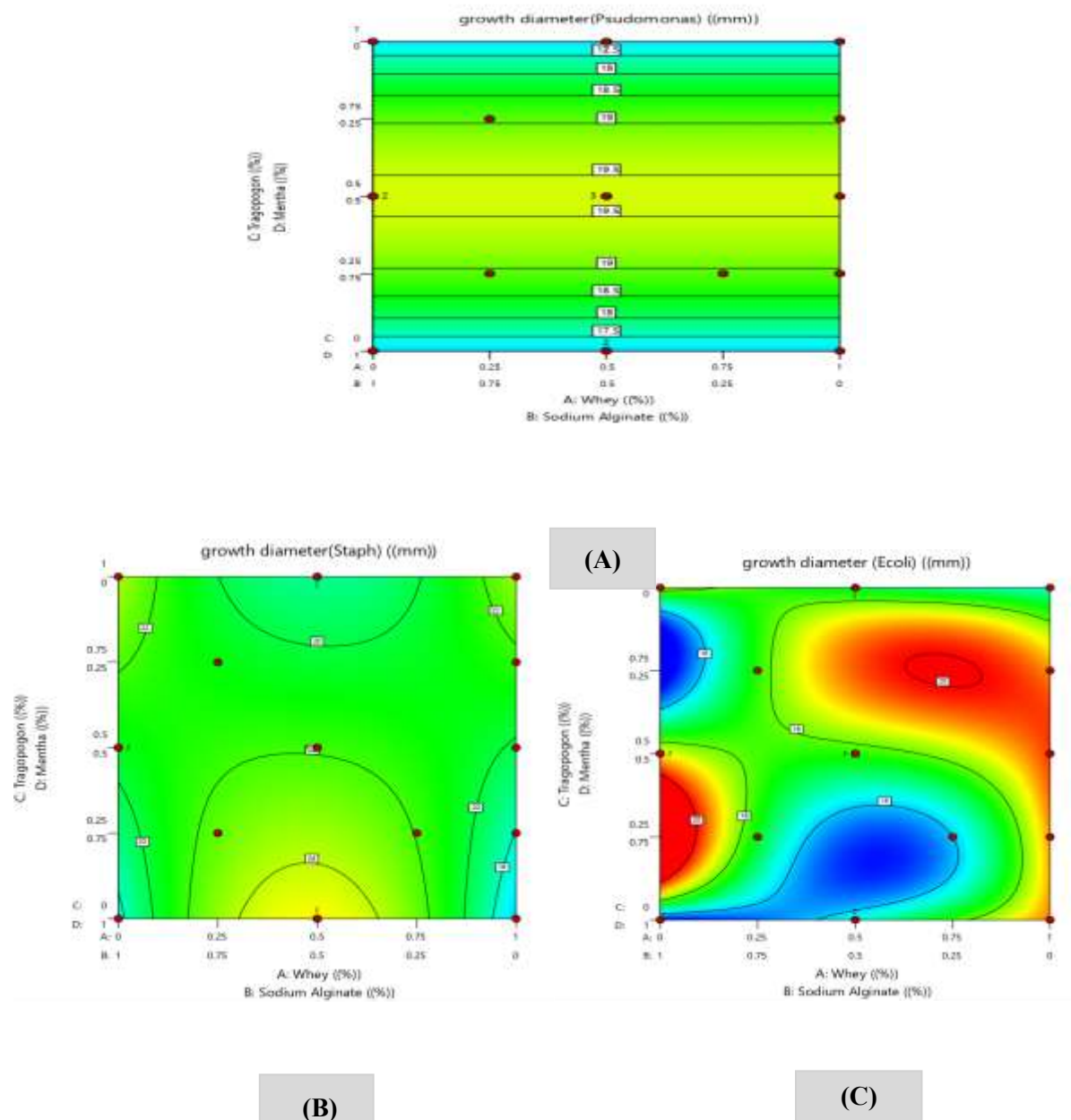


Fig5. The interactive antimicrobial effects *Graminifolius Tragopogon*, *Mentha piperita* extracts A) *Pseudomonas*, B) *Escherichia coli*, C) *Staphylococcus aureus* in

Whey Protein - Sodium Alginate Edible Films

4-Conclusion

In recent years, numerous studies have been conducted on edible films based on natural materials enhanced with various plant extracts and essential oils. In this study, the main focus was on examining the interaction effects of the film base, particularly on mechanical properties, as well as the combined effects of *Tragopogon graminifolius* and *peppermint* extracts on antimicrobial activity and

antioxidant capacity. The results showed that the treatments significantly affected permeability, thickness, whey protein-sodium alginate films ($p < 0.05$). Increasing the concentration of *peppermint* oil up to 0.75% led to an increase in permeability, while the lowest solubility and thickness were observed in the control group. The highest opacity was noted in the 1% *peppermint* extract group, and the lowest opacity in the 1% *Tragopogon* extract group. The proportion of edible film compositions and the extracts of *Tragopogon*

and *peppermint* had no significant effect on solubility; however, the proportion of the film formulations did not influence moisture content. Increasing *Tragopogon* extract concentration up to 1% reduced permeability, whereas increasing *peppermint* extract concentration up to 1% increased permeability. The increase in elongation at break and Young's modulus was statistically significant ($p < 0.05$). The maximum thickness was observed at the ratio of 0.5-0.75 whey protein-sodium alginate with *Tragopogon* and *peppermint* extracts. The extract type significantly affected antioxidant activity and antimicrobial properties; specifically, antioxidant activity increased with the rising concentration of *peppermint* extract, and microbial growth inhibition reached up to 75%. Overall, it can be concluded that the active films containing whey protein (0.75%), sodium alginate (0.25%), and a combined concentration (0.5-0.75%) of *peppermint* and *Tragopogon* extracts exhibit the highest performance and desirable characteristics. These films can potentially be used as effective coatings to delay lipid oxidation and improve mechanical and physicochemical properties, making them suitable for food preservation applications.

Author Contributions

All activities were performed by the authors.

Data availability

All data supporting the findings of this study are available within the article.

Conflict of Interest

The author confirms that there are no financial conflicts of interest or competing interests in this study.

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بررسی ویژگی‌های فیلم خوراکی پروتئین آب پنیر- آلژینات سدیم حاوی عصاره های شنگ (*Graminifolius*)

Tragopogon و نعناع فلفلی (*Mentha piperita*) با استفاده از طرح mixture

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فیلم‌های خوراکی به‌عنوان مواد جدید تجزیه‌پذیر در بسته‌بندی مواد غذایی، نقش مهمی در رفع نگرانی‌های مصرف‌کنندگان در مورد آلودگی محیط زیست و آلودگی مواد غذایی دارند. در این مطالعه، دو تیمار عصاره‌های شنگ و نعناع فلفلی بر پایه آب پنیر-آلژینات سدیم با غلظت‌های مختلف (۰/۵، ۰/۷۵، ۱/۵ و ۳/۲۵ درصد) بر روی ویژگی‌های فیزیکی (حلالیت، نفوذپذیری به بخار، ضخامت و کدورت)، مکانیکی (ازدیاد طول تا نقطه شکست، مقاومت به کشش و مدول یانگ)، آنتی‌اکسیدانی و ضد میکروبی تهیه شدند. طراحی آزمایشات، آنالیز واریانس و همچنین رسم نمودارها با استفاده از نرم افزار Design Expert 13 انجام گرفت. تمامی آزمایشات در سه تکرار (n=3) با نمونه‌گیری کاملاً تصادفی انجام شد. بالاترین میزان کدورت مربوط به غلظت (۱٪) عصاره نعناع و کمترین کدورت در همین غلظت مربوط به عصاره شنگ بود. با افزایش غلظت نعناع تا (۰/۷۵٪) نفوذپذیری افزایش یافت و به مقدار ۹ گرم بر مترثانیه پاسکال افزایش یافت. بالاترین میزان کشش پذیری و مدول یانگ مربوط به پروتئین آب پنیر با غلظت (۰/۷۵٪) و سدیم آلژینات با غلظت (۰/۲۵٪) به ترتیب ۱۷ و ۸۵ مگا پاسکال بود. خاصیت آنتی‌اکسیدانی فیلم خوراکی با استفاده از رادیکال‌های DPPH معنی‌دار بود ($p < 0.05$) و با افزایش غلظت شنگ تا ۱٪ افزایش یافت. نتایج ارزیابی فعالیت ضد میکروبی فیلم با کمک روش دیسک‌های انتشاری نشان داد که بیشترین قطر هاله بازدارندگی در غلظت (۰/۵-۰/۵٪) پروتئین آب پنیر-سدیم آلژینات و نعناع فلفلی - شنگ مربوط به *استافیلوکوکوس اورئوس* با متوسط قطر ۲۴mm بود. سپس در غلظت (۰/۷۵٪) و (۱٪) به ترتیب مربوط به عصاره نعناع و پروتئین آب پنیر مربوط به *شرشیاکلی* با متوسط قطر هاله ۲۰mm بود. نتایج کلی نشان داد که افزودن اسانس نعناع فلفلی - شنگ باعث تولید فیلم‌هایی گردید که علاوه بر مهار رشد و تکثیر باکتری‌ها، دارای استحکام همچنین قابلیت استفاده در مواد غذایی فساد پذیر را دارد.