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OLIVE LEAF EXTRACT INCORPORATED CHITOSAN FILMS FOR ACTIVE FOOD PACKAGING

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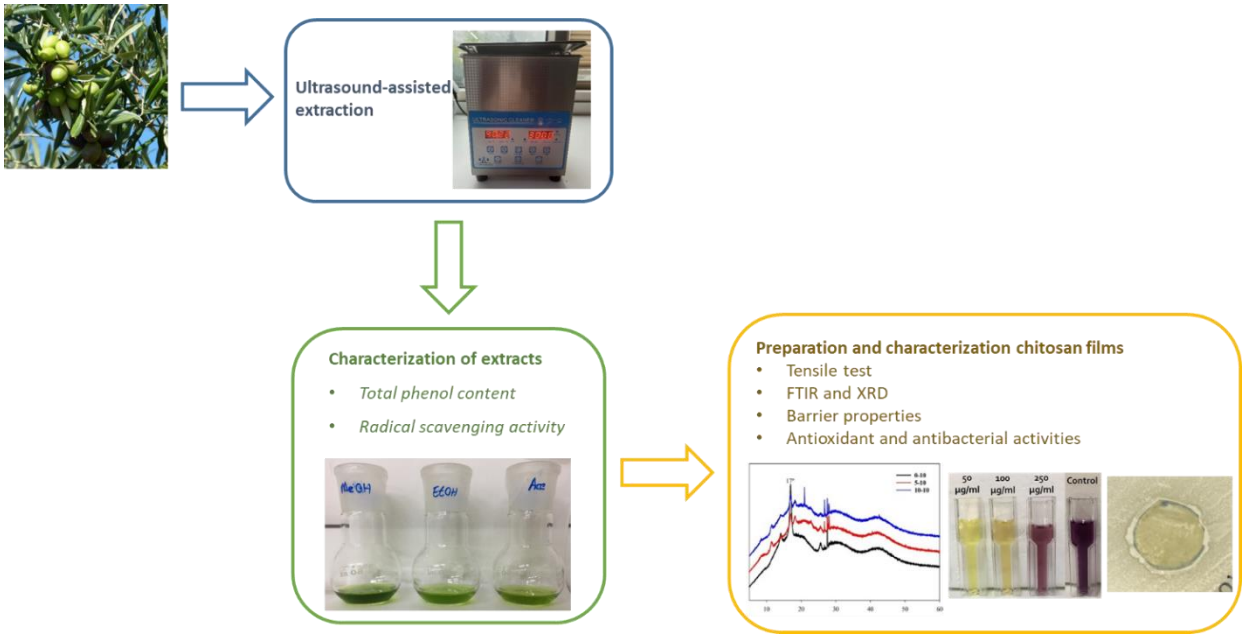
Highlights

- Olive leaf extract (OLE) added chitosan films were produced.
- Barrier properties were improved by OLE addition.
- Tensile strength and elongation of OLE-added chitosan films were 32 MPa and 9.3%, respectively.
- Prepared films possessed antioxidant and antibacterial activities.
- OLE containing chitosan films were proved to be an alternative active packaging.

Graphical Abstract



Flowchart for the preparation and characterization of olive leaf extract incorporated chitosan films

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ABSTRACT: Packaging materials serve as a barrier to protect the food from the environment and new approaches with improved properties, such as active packaging, is gaining more attention nowadays. In this study, chitosan films containing olive leaf extract (OLE) as an additive were prepared and characterized in terms of mechanical, structural and biological properties. The addition of OLE improved not only the tensile strength (32 MPa) and elongation (9.3%) of chitosan films but also their barrier properties such as water vapor transmission rate of 657.52 g/m²day and moisture retention capability of 90.41%. Furthermore, chitosan films gained antibacterial properties with the addition of OLE and possessed a dose and time-dependent antioxidant activity compared to their extract-free equivalents. As a consequence, the present study suggests that chitosan films incorporated with OLE are a promising alternative as an active food packaging with enhanced mechanical, barrier, antioxidant and antibacterial properties.

Keywords: *Active packaging, Antioxidant activity, Chitosan film, Mechanical properties, Olive leaf extract*

1. INTRODUCTION

Packaging is defined as a barrier that separates the product from the environment, protects it from any damage that may occur during transportation and storage, and maintain its quality until consumption [1, 2]. The main goal of traditional packaging is to conserve quality as long as possible while reducing the interaction between the product and the packaging material. However, changes in consumer interests led to the search for packaging materials with enhanced properties and new packaging approaches have emerged, one of them being active packaging. Unlike traditional packaging, direct interaction between the product and the packaging material is consciously utilized in active packaging to contribute to the extension of the shelf life rather than merely serving as a barrier. The starting point for the design of an active packing is usually improvement of the most significant features for the product quality such as scavenging of oxygen, carbon dioxide or free radicals, removal of odors and flavors, reduction of spoilage, and prevention of color change [1, 3, 4].

Antioxidant additives such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) have been used for the packing of oxygen-sensitive foods. However, the utilization of synthetic antioxidants is questioned since they have potential risks for human health and strict control is required for their utilization. As they are nontoxic, natural antioxidants have been used to overcome these concerns during the preparation of active packaging as natural-based and eco-friendly alternatives [5, 6]. Most common natural additives are essential oils, fruit, and plant extracts especially rich in phenolic compounds and they have been used in the forms of crude extracts as well as isolated components. Among them, phenolic compounds are very popular since they not only improve the antioxidant and antimicrobial properties of the packaging material but also contribute to its physical and mechanical features by acting as crosslinking agents [7].

Olive (*Olea europaea*) is an evergreen tree belonging to the Oleaceae family that is native to the Mediterranean coast and cultivated for its fruit and oil [8, 9]. Large quantities of by-products such as olive

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pomace, olive mill waste water, stones, seeds and leaves are released during olive oil production [10]. Olive leaf and its extract have been used in folk medicine for centuries due to their health promoting benefits. The leaves of the olive tree have been shown to possess a higher radical scavenging activity compared to the different parts of the plant [11].

Chitosan is a deacetylated derivative of chitin, the second most abundant polysaccharide after cellulose, and is used for various applications as being nontoxic, biocompatible, and biodegradable. In addition, its film forming ability, good mechanical features and barrier effect to gases such as CO_2 and O_2 make chitosan an excellent material for food packaging [12–14].

The aim of this study was to prepare and characterize chitosan films with OLE as an alternative active food packaging. For this purpose, chitosan films with and without OLE were prepared and compared in terms of mechanical, structural, antioxidant, and antibacterial properties.

2. MATERIAL AND METHODS

2.1. Chemicals and Plant Material

Folin-Ciocalteu agent and acetone are from Merck (Germany). Chitosan (85% deacetylated) was purchased from Alfa Aesar (Germany). Sodium carbonate was from AFG Bioscience (USA). Ethanol and methanol were obtained from Honeywell (Germany). Gallic acid was from Isolab (Germany) and 2,2-diphenyl-1-picrylhyrazyl hydrate (DPPH) from TCI (Japan). Olive leaves were purchased from a local market as dried and ground form.

2.2. Preparation and Characterization of Plant Extracts

2.2.1. Ultrasonic-assisted extraction of olive leaf

0.5 g of ground leaves were mixed with 10 ml of extraction solvent (methanol, ethanol or acetone) and immersed in an ultrasonic bath with a constant temperature of 40°C. Continuous ultrasound was applied for 40 minutes. At the end of the extraction duration, leaves were removed by filtration and filtrate was centrifugated at 4000 rpm for 5 minutes. After centrifugation, the supernatant was evaporated under vacuum condition at 40°C. All extracts were stored at -20°C until experiments.

2.2.2. Total phenol content of OLE

Extracts were characterized by Folin-Ciocalteu method in terms of total phenol content (TPC). 750 µl of Folin-Ciocalteu agent which was diluted in water for 10 folds, was added to 60 µl aliquot of extract. The mixture was vortexed and left to stand at room temperature for 5 minutes. Then, 600 µl saturated Na_2CO_3 solution was added, vortexed, and kept in the dark at room temperature for 2 hours. The absorbance of the mixture was determined at 760 nm by a spectrophotometer [15]. Gallic acid was used as a reference standard and the total phenol content of the extracts was stated as mg gallic acid equivalent (GAE)/g extract. The analyses were carried out in duplicate.

2.2.3. Radical scavenging activities and EC_{50} values of OLE

The extracts were dissolved in 4 ml of methanol at three different concentrations as 50, 100, and 250 µg/ml. Then, 0.5 ml of DPPH solution (1 mM in methanol) was added and vortexed for 10 s. The mixture was kept at room temperature for 30 minutes. Absorbance measurement was conducted by a spectrophotometer at 517 nm and radical scavenging activity (RSA) was calculated by the following formula:

$$RSA\% = \frac{A_{DPPH} - A_{Extract}}{A_{DPPH}} \times 100 \quad (1)$$

where A_{DPPH} is the absorbance of DPPH solution without extract [16]. After determination of RSA% for different extract concentrations, EC_{50} values corresponding to 50% radical scavenging activity were calculated for each sample.

2.3. Preparation and Characterization of Chitosan Films

2.3.1. Preparation of chitosan films

The film casting method was used to prepare the chitosan films. The chemical contents of the films are given in Table 1. For film production, chitosan was dissolved in acetic acid and distilled water mixture by mixing at 50°C for 48 hours. Then, a certain amount of glycerol was added to the solution and mixed for 1 hour at room temperature. While various amounts of methanol specified in Table 1 were included in the solution in pure chitosan samples, in doped samples OLE in methanol at 10% v/v concentrations were added to the solution and mixing was continued for one more hour at room temperature. The obtained solutions were poured into 9 cm diameter Petri dishes, left to dry for 48 hours at 40°C, and kept for 24 hours at room temperature before being removed from Petri dishes. The produced films were stored in a desiccator until tests were performed. The prepared films were named according to the amount of extract and methanol in their contents.

Table 1. Chemical compositions of the prepared films

Sample	Chemical Contents					
	Chitosan (g)	Acetic acid (ml)	MeOH (ml)	Distilled water (ml)	Glycerol (g)	Olive Leaf Extract (mg)
0-5	0.50	0.25	1.25	23.50	0.15	0
0-10	0.50	0.25	2.50	22.25	0.15	0
0-15	0.50	0.25	3.75	21.00	0.15	0
5-10	0.50	0.25	2.50	22.25	0.15	25
10-10	0.50	0.25	2.50	22.25	0.15	50

2.3.2. Thickness and light barrier properties of prepared films

The thickness measurement of chitosan films was carried out at three random positions for each sample by using Leica-M125 stereomicroscope (Leica, Germany) under 100x magnification. The transmittance spectrum of each film between 200-800 nm was recorded by Shimadzu-UV3600 spectrophotometer (Shimadzu, Japan) and film transparency was calculated described by Terzioğlu et al. [17].

2.3.3. Fourier transform infrared spectroscopy and X-ray diffraction analysis of prepared films

The prepared films were characterized with Fourier-transform infrared (FTIR-Thermo Nicolet, iS50 with an attenuated total reflectance (ATR) accessory) and X-ray diffraction (XRD-Bruker, D8 Advance) analyses. The FTIR spectra of the samples were recorded in the wavenumber range of 400 to 4,000 cm^{-1} with 16 scans at 4 cm^{-1} resolutions. XRD analysis of chitosan films was performed at experimental conditions of 3-70° scanning range and 0.5 $step^{-1}$ step size.

2.3.4. Mechanical properties of prepared films

The mechanical performances of the prepared films were determined with a tensile test using a Shimadzu AGS X-Static Mechanical Tester following the modified ASTM Standard Test Method D 882-

12. (6.5 x 1 cm) samples were prepared and tested with an initial grip separation of 5 cm and 5 mm/s crosshead speed. Five replicates were tested for each film sample.

2.3.5. Water vapor transmission rate of prepared films

The water vapor transmission rate (WVTR) of the prepared films was determined according to the method used in a previous study [17] with slight modifications. 10 mL of distilled water was filled in a 1.13 cm diameter bottle and the bottle mouth was covered with prepared films with the help of parafilm. The film covered bottle samples were kept in a 40°C vacuum oven and weighted after 24 h, 48 h, 72 h, and 96 h. WVTR (g/m²day) of the films were performed for 3 replicates of each film sample and calculated by using the following formula

$$WVTR = \frac{W_i - W_t}{A \times t} \times 10^6 \quad (2)$$

where, W_i is the weight of the bottle before being placed in the oven, W_t is the weight of the bottle after being removed from the oven at a certain time (t) (day), and A is the bottle mount area (mm²), respectively.

2.3.6. Moisture retention capability of prepared films

The moisture retention capability of the prepared films was determined according to a previous study [17]. 1 x 1 cm² square shape three samples of both films were placed into an 60°C oven for 6 h. The moisture retention capability of the films was calculated by using the following formula;

$$Moisture\ Retention\ Capability = \frac{W}{W_0} \times 100 \quad (3)$$

where, W_0 is the weight of the films being placed in the oven and W is the weight of films after being removed from the oven, respectively.

2.3.7. Radical scavenging activities of prepared films

Chitosan films with and without OLE were cut into small pieces, and 20 mg of film samples were transferred to test tubes containing 4 ml of methanol. Then, 0.5 ml of 1 mM methanolic DPPH solution was added to each tube and absorbance was measured at 517 nm at time intervals. The RSA% of samples was calculated by using Eq. 1.

2.3.8. Antibacterial properties of prepared films

Inhibition zones of the chitosan films presence and absence of OLE were determined by disc diffusion method against *Staphylococcus aureus* (ATCC 25923). Bacterial suspension adjusted to the 0.5 McFarland Standard were dispersed homogeneously on nutrient agar and chitosan films with a diameter of 10 mm discs were placed onto the medium. After incubation at 37°C for 24 h, petri dishes were examined for zone formation and zone diameters were measured. Clear inhibition zones around the disks indicated antibacterial activity [18].

2.3.9. Statistical analysis

The paired Student's *t*-test was used and the normality of the data was analyzed by Kolmogorov-Smirnov test. In all tests, significant differences were considered when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of Plant Extracts

Extraction of plant material was carried out with three different solvents and TPC of extracts was determined by Folin-Ciocalteu method. As shown in Table 2, the highest total phenolic content was obtained by methanol extraction and calculated as 106 mg GAE/g. Brahmi et al. [19], tested different solvents for the extraction of phenolic compounds from olive leaves and reported that the extraction solvent considerably affected the phenolic content of the obtained extracts and the highest amount of TPC was extracted by methanol. Kontogianni and Gerothanassis [20] extracted olive leaves with methanol by a one-week maceration process and the extracts were fractionated by solvents with different polarities. Total phenol content and EC_{50} value of the chloroform fraction were determined as 106 mg/g and 69.1 μg/ml, respectively. These results are in agreement with the results obtained in our study.

EC_{50} value, which is defined as the efficient concentration to reduce the initial amount of DPPH radical was calculated for extracts obtained by methanol, acetone and ethanol. The lowest EC_{50} was obtained for methanolic extract while there was no significant difference between the RSA and EC_{50} values of extracts obtained by acetone and ethanol. These results also indicated that there is a strong relationship between the TPC and RSA as previously reported [21, 22].

Table 2. Total phenol content, radical scavenging activity and EC_{50} value of OLE obtained by different solvents

Extraction solvent	TPC (mg GAE/g)	RSA (%)	EC_{50} (μg/ml)
Methanol	106.29±0.63	73.74±0.88	64.62±0.97
Ethanol	87.73±3.93	56.52±0.74	98.68±5.54
Acetone	90.84±2.04	57.19±0.74	94.49±1.30

3.2. Characterization of Chitosan Films

3.2.1. Thickness and light barrier properties of prepared films

The thickness of chitosan films with and without OLE was determined to vary between 0.05 mm and 0.065 mm. As seen in Figure 1, prepared films with and without OLE showed a homogenous structure and were clear enough to be utilized as see-through packaging. The incorporation of OLE in film structure resulted in a slight color change probably due to the chlorophyll content of OLE. Chitosan films containing 25 mg and 50 mg of OLE showed similar transparency as 25.49 mm^{-1} and 26.35 mm^{-1}, respectively.

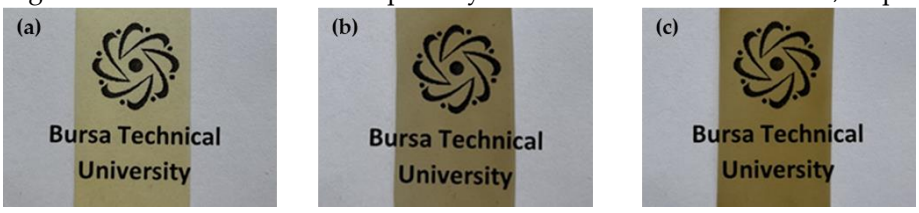

Figure 1. Visual appearance of chitosan films absence and presence of OLE: 0-10 (a), 5-10 (b) and 10-10 (c).

3.2.2. FTIR and XRD analysis of prepared films

Figure 2 demonstrated the FTIR spectra of the produced films according to the changes depending on the methanol ratio (Figure 2a) and OLE addition (Figure 2b) in 10% methanol-added films. In Figure 2a,

the asymmetric stretching of NH_2 and OH caused a broad and strong band in the range of 3200 cm^{-1} and 3450 cm^{-1}. Amide I, Amide II, and Amide III related bands were shown at 1638 cm^{-1}, 1561 cm^{-1}, and 1327 cm^{-1}, respectively. C-O-C bridge asymmetric stretching and skeletal vibrations involving the C-O stretching were characteristic of saccharide structure and seen at 1158 cm^{-1} and 1030 cm^{-1}, respectively [23–25].

There were a number of characteristic bands in the FTIR spectrum of the OLE. The 3310 cm^{-1} and 1690 cm^{-1} bands corresponded to the oleuropein and other active compounds being presented in the OLE. The 1607 cm^{-1} band represented the fingerprint region of functional groups such as C-O and O-H in the OLE structure. The 1515 cm^{-1} and 1386 cm^{-1} bands belonged to the Amide II and scissoring vibrations of the methylene in the proteins, respectively [26]. However, the FTIR spectra of the OLE added films were very similar to the 0-10 chitosan film and no evidence was found to show the interaction of chitosan and the OLE in the FTIR spectra of the films, as seen in Figure 2b. Similar results were obtained in the literature for OLE added chitosan microspheres [25].

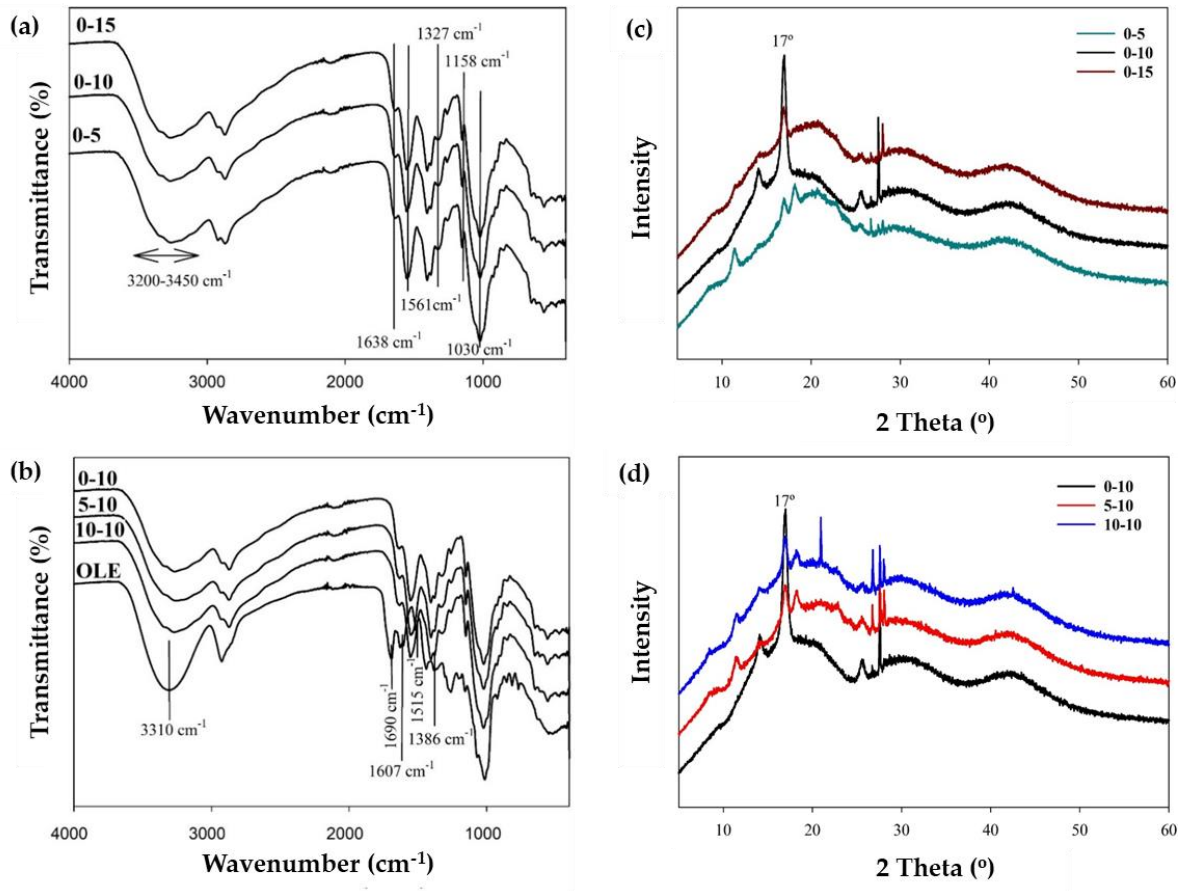

Figure 2. FTIR spectra (a), (b) and XRD pattern (c), (d) of chitosan films for different methanol and extract concentrations, respectively

XRD analysis was applied to the prepared films to demonstrate the effects of methanol addition and the interaction of the polyphenolic groups of OLE with chitosan. The XRD patterns of the 0-5, 0-10, and 0-15 were given in Figure 2c. As seen in Figure 2c and known in the literature, the solvent evaporation rate has an effect on the crystallization behavior of polymers, and the addition of methanol changed the crystallization behavior of chitosan [27]. Increasing the methanol ratio in the film solution up to 10% increased the crystallization rate due to the rapid evaporation of the solvent. However, increasing the solvent ratio further increased the solvent removal time, and the crystallization rate started to decrease again. Since 0-10 film showed the highest crystallization behavior, the OLE additive was included in the recipe at 10% methanol. The XRD patterns of the OLE added films (0-10, 5-10, and 10-10) were given in

Figure 2d. As seen in the figure, the addition of the OLE decreased the main peak intensity at 17°. However, with the addition of OLE, new crystallizations have been seen in the film structure. The effect of the change in the crystallization of the chitosan films was also seen in the mechanical test results.

3.2.3. Mechanical properties of prepared films

A tensile test was applied to determine the impact of methanol concentration and OLE addition on the mechanical properties of prepared films. Chitosan films prepared with 5% methanol concentration had the lowest tensile strength of 25 MPa and an increase in methanol concentration resulted in improved tensile strength. However, the difference between the tensile strengths of the chitosan films prepared with 10% and 15% methanol concentrations was insignificant (Figure 3a). To reduce the organic solvent consumption, 10% of the methanol concentration was chosen for subsequent experiments, which was also the concentration resulting in the highest crystallinity. Although the addition of OLE resulted in a slight decrease in the tensile strength from 32.8 MPa to 29.9 MPa compared to chitosan films without extract, this alteration was not statistically significant (Figure 3b).

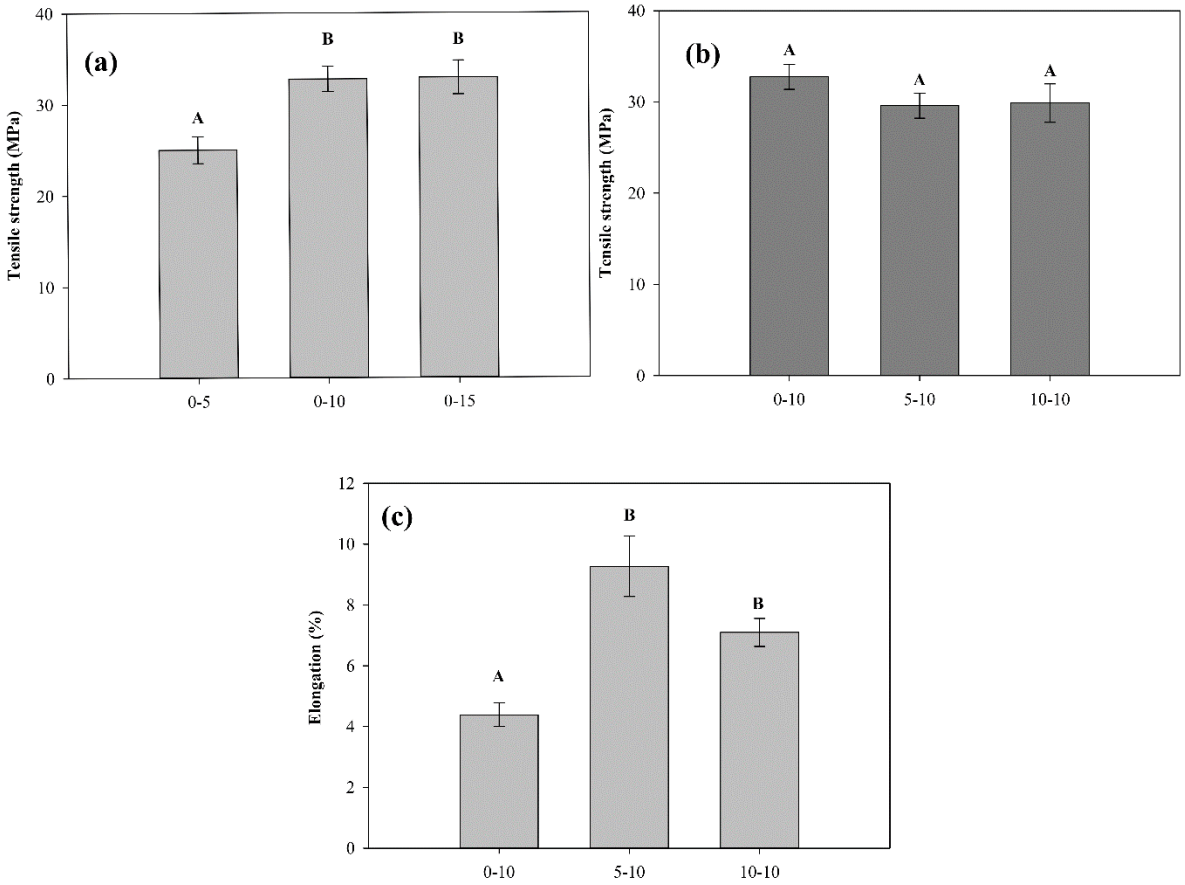

Figure 3. Mechanical properties of chitosan films: tensile strength at different methanol (a) and extract (b) concentration and percent elongation for different extract amount (c)

Chitosan films are known to be brittle and rigid in nature and commonly need plasticizers to improve their mechanical properties. However, the majority of commercial plasticizers are synthetic and may cause the migration of chemicals from packaging to food which may lead to consumer health risks and a change in the quality of food [28, 29]. In this study, prepared films were characterized in terms of percent elongation, which is a measure of stretching ability. The results showed that varying the methanol concentration from 5 to 15% did not lead to a significant difference in percent elongation while the addition

of OLE enhanced the flexibility and thus processability of prepared films. The additives are typically stated to affect the percent elongation and tensile strength of chitosan films inversely [12, 30]. Musella et al. [31], used OLE as an additive to produce chitosan films, however, the extract has not been uniformly distributed through the film structure resulting in the formation of visible insoluble particles. It has been reported that this non-homogeneity caused inconsistencies between the repetitions and affected the results of mechanical tests for chitosan films. On the other hand, the results of our study revealed that OLE can be used successfully to improve both the strength and flexibility of chitosan films (Figure 3c). Such a positive influence of OLE on tensile strength and percent elongation can be attributed to the good distribution of OLE in the chitosan film matrix with the help of methanol addition which was also revealed by the results of FTIR analysis.

3.2.4. Water vapor transmission rate and moisture retention capability of prepared films

It is known in the literature that the barrier and mechanical properties of the packaging films were affected by the water absorption and diffusion properties of the film. Therefore, WVTR and moisture retention capability are crucial properties for food packaging applications [23, 32, 33]. As seen in Figure 4, while the water vapor transition rate of the 10-10 sample was the lowest at 657.52 g/m^{2}day, the moisture retention capability was the highest at 90.41%. These results demonstrated that the addition of OLE to the film structure has increased the potential of chitosan films to be utilized in food packaging applications.

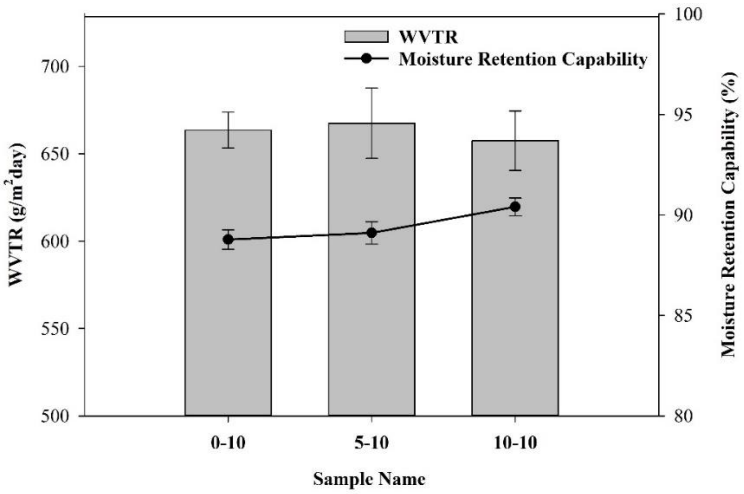

Figure 4. Water vapor transmission rate and moisture retention capability of chitosan films

3.2.5. Radical scavenging activities of prepared films

Radical scavenging activity of prepared films was also elicited and results are presented in Table 3. Chitosan films without OLE have not possessed radical scavenging activity and the presence of the extract resulted in a significant increase of radical scavenging ability of the films. In addition, prepared films showed dose-dependent radical scavenging activity, which increased from 14% to over 35% by doubling the OLE content of chitosan films.

Table 3. Radical scavenging activity of prepared films at 300 min

Film	RSA%
0-5	NA
0-10	NA
0-15	NA
5-10	14.62
10-10	36.64

As seen in Figure 5, the RSA of OLE added chitosan films showed a time-dependent behavior. These results revealed the migration of active ingredients from prepared films and also the necessity of adding active components to improve the antioxidant properties of chitosan films.

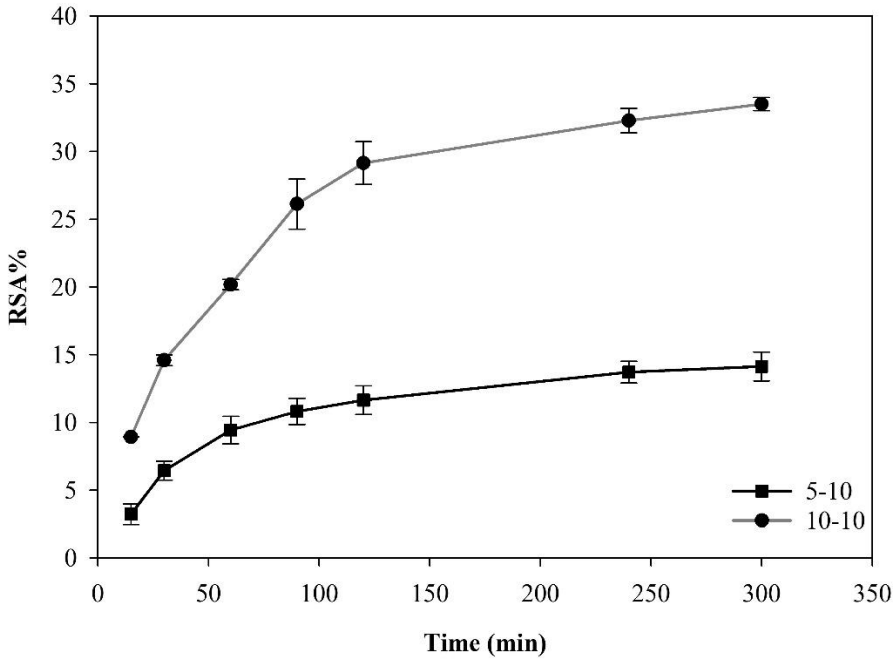

Figure 5. Radical scavenging activity of prepared films at different time intervals

3.2.6. Antibacterial properties of prepared films

Antibacterial activity is a desirable feature of active food packaging that inhibits spoilage and contributes to shelf life extension [34]. In this study, disk diffusion method was applied to chitosan films with and without extract. The results showed that chitosan films without extract have not possessed antibacterial activity. Although chitosan is known to have antimicrobial properties, the limited diffusion in agar medium could be the reason for the absence of an inhibition zone [35]. Chitosan films containing 25 mg and 50 mg OLE showed inhibition zones with diameters of 11.9 mm and 11.4 mm, respectively (Figure 6). These findings clearly revealed the effect and potential of OLE containing chitosan films to extend the shelf life of food.

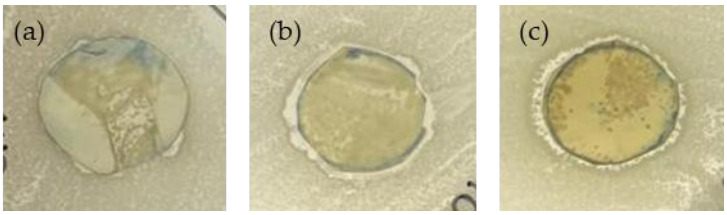

Figure 6. Inhibitory effect of chitosan films on the growth of *Staphylococcus aureus* absence (a) and presence (b), (c) of extract

4. CONCLUSIONS

In this study, chitosan films were prepared for active food packaging and characterized in terms of mechanical, structural and biological properties. OLE was used as an active ingredient and the most suitable solvent for the ultrasonic-assisted extraction was determined as methanol yielding 106.3 mg GAE/g extract total phenol content and 64.6 μg/ml EC_{50} value for radical scavenging activity. By addition of OLE, chitosan films gained antioxidant and antibacterial activities compared to their extract-free equivalents. Furthermore, OLE added films possessed a time-dependent radical scavenging activity indicating the migration of active components from the film which is an important feature to be used as active packaging. XRD analysis indicated that the crystallization behavior of chitosan films was also affected by the addition of methanol and OLE to the film formulation while their barrier properties were also improved. The effect of methanol content and extract amount on the mechanical and structural properties of chitosan films was also examined. OLE was successfully distributed in chitosan films as elicited by FTIR spectra and the presence of methanol and OLE greatly influenced the mechanical properties resulting in more resistant and stretchable films compared to the control group. This study suggests that chitosan films incorporated with OLE are a promising alternative as active food packaging with enhanced mechanical, antioxidant and antibacterial features.

Declaration of Ethical Standards

The authors declare that all ethical guidelines including authorship, citation, data reporting, and publishing original research are followed.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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