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Original article

Chitosan Nanofibers Enriched with Oleuropein via Electrospinning: Potential as Active Packaging Materials

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Abstract

The development of sustainable and functional food packaging materials is crucial to addressing global challenges such as environmental pollution, food waste, and the demand for eco-friendly solutions. This study explores the fabrication of oleuropein-incorporated chitosan nanofibers using the electrospinning technique. Oleuropein, a phenolic compound known for its potent antioxidant and antimicrobial properties, was integrated into chitosan-based nanofibers to enhance their functional characteristics. The structural, antioxidant, and antimicrobial properties of the nanofiber films were thoroughly evaluated. Results revealed that increasing oleuropein concentration significantly influenced the viscosity of the polymer solutions and nanofiber morphology while having negligible effects on electrical conductivity. The films exhibited enhanced total phenolic content (TPC) and antioxidant activity with higher oleuropein concentrations, with the OLE_3.75 sample achieving the highest TPC (31.66±3.29 mg GAE/g film) and strong DPPH scavenging activity (87.15±1.14%). Antimicrobial tests demonstrated selective inhibition of Staphylococcus aureus (Gram-positive bacteria), with inhibition zones of 18.50±0.15 mm and 17.50±0.25 mm for OLE_2.5 and OLE_3.75 films, respectively, while showing no activity against Escherichia coli (Gram-negative bacteria). These findings underscore the potential of oleuropein-loaded PEO/chitosan nanofiber films as innovative active packaging materials with robust antioxidant and selective antimicrobial properties. This research contributes to the advancement of multifunctional, biodegradable packaging solutions that align with sustainability and food safety goals, offering promising applications in food preservation and biomedical fields.

Keywords: Chitosan Nanofibers, Active Packaging, Oleuropein, Electrospinning.

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INTRODUCTION

Global challenges such as environmental pollution, resource depletion, and food waste have intensified the search for sustainable and functional alternatives to conventional materials in various industries. In the realm of food packaging, traditional petroleum-based plastics, while effective as barriers, pose significant environmental risks due to their non-biodegradability and contribution to landfill overflow (Geyer et al., 2017). These concerns have catalyzed the development of innovative biopolymer-based materials that not only reduce environmental impact but also address growing consumer demands for safer, eco-friendly, and functional packaging solutions. The growing demand for sustainable and functional materials in food packaging has driven significant advancements in the development of biopolymer-based solutions. Among these, active packaging has emerged as a transformative technology, offering not only a physical barrier but also functionalities like antimicrobial and antioxidant activity to extend food shelf life and maintain quality (Lourenço et al., 2019). This approach has gained considerable traction due to consumer preferences for safer, eco-friendly alternatives to synthetic additives. Active packaging systems commonly incorporate natural bioactive agents, such as phenolic compounds, to enhance material performance (Rambabu et al., 2019); (Kurek et al., 2018). This not only ensures a uniform distribution of active agents but also enhances their controlled release during storage (Bourbon et al., 2011); (Trigueiro et al., 2024). Chitosan, a biodegradable and biocompatible polymer, is frequently employed in such applications due to its inherent antimicrobial properties and ability to form stable films (Siripatrawan & Harte, 2010); (Peng et al., 2013).

Recent advancements in electrospinning technology have further expanded the potential of active packaging materials. This technique enables the fabrication of nanofibers with high surface area-to-volume ratios, which can encapsulate bioactive compounds and control their release effectively (Wang et al., 2017); (Fabra et al., 2016). Oleuropein, a phenolic compound derived from olive leaves, is a prime candidate for such applications due to its strong antioxidant and antimicrobial properties. Studies have demonstrated its efficacy in inhibiting foodborne pathogens like Staphylococcus aureus and its potential to improve the oxidative stability of packaging materials (Sudjana et al., 2009); (Serra et al., 2008).Furthermore, electrospun fibers exhibit superior functional characteristics, including enhanced tensile strength and reduced water vapor permeability, making them suitable for advanced active packaging applications (Kadam et al., 2021); (Bumbudsanpharoke et al., 2022). This study aims to develop oleuropein-incorporated chitosan nanofibers using the electrospinning technique, with a specific focus on leveraging the advantages of emulsion film technology. By optimizing oleuropein concentration and electrospinning parameters, the research seeks to evaluate the morphological, antioxidant, and antimicrobial properties of the resulting materials. These efforts contribute to the broader goal of creating eco-friendly, multifunctional packaging solutions capable of addressing both food safety and sustainability challenges.

MATERIALS and METHODS

Materials

Chemicals, including Tween 80, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, and sodium carbonate, were also sourced from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany). Chitosan (medium molecular weight, 75–85% deacetylated) and Polyethylne oxide (PEO) were purchased from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany), while oleuropein powder was provided by Kale Naturel Ltd. Şti. For antimicrobial assays, Mueller–Hinton agar, nutrient broth, peptone water, and plate count agar were obtained from Condalab (Madrid, Spain).

Solution preparation

A homogeneous PEO stock solution (4% w/v) was prepared by dissolving 4 grams of PEO in 100 mL of distilled water using a magnetic stirrer. A separate chitosan (CS) solution (1% w/v) was obtained by dissolving 1 gram of chitosan in 100 mL of 80% (v/v) acetic acid. Afterward, the PEO and CS solutions were combined at a 4:1 weight ratio (PEO:CS), followed by the addition of the surfactant Tween 80 at a concentration of 2% (w/v). Oleuropein (5% w/v) was dissolved in an ethanol/water mixture with an 80/20 (v/v) ratio. To eliminate any undissolved particles, the resulting suspension was centrifuged (Nüve, NF 800, Turkey) at 3500 rpm for 10 minutes. Finally, varying volumes of the oleuropein solution (12.5%, 25%, and 37.5% v/v) were incorporated into the polymeric blend.

Electrospinning Process

The electrospinning process was carried out using an electrospinning apparatus (ECAY Technology, Çanakkale, Turkey). For each formulation, the prepared solutions were transferred into a 10 mL syringe equipped with a metallic needle. The syringe was mounted horizontally on a syringe pump and connected to the positively charged electrode, which was powered by a direct current (DC) high-voltage generator. The metallic collector, acting as the negatively charged electrode, was covered with aluminum foil to facilitate nanofiber deposition. The flow rate of the polymer solutions was precisely set to 1 mL/h, while the distance between the syringe needle tip and the collector surface was kept constant at 30 cm. The applied voltage was consistently maintained at 12 kV throughout the process.

The electrospinning experiments were conducted in a controlled environment with a temperature of 25°C and a relative humidity ranging between 30–40%. The process was sustained for a total duration of 48 hours to ensure consistent nanofiber production. Based on the oleuropein content incorporated into the polymeric solutions, the resultant nanofibers were categorized and designated as OLE_1.25, OLE_2.5, and OLE_3.75, corresponding to increasing concentrations of oleuropein. The nanofibers produced without the addition of oleuropein were used as a control group and labeled as OLE_0.

Characterization of Nanofibers

Viscosity and Electrical Conductivities

The viscosity of the polymer solutions was measured using a Brookfield viscometer (Model DV-II + Pro, USA). The solutions were carefully transferred into the viscometer's stainless- steel container, and the measurements were performed under controlled conditions at a temperature of 25°C. A S34 spindle was employed for the analysis, operating at a rotational speed of 50 revolutions per minute (RPM) to ensure consistent viscosity readings. Additionally, the electrical conductivity of the solutions was determined using a Hanna EC conductivity meter (Woonsocket, RI, USA). The measurements were conducted at room temperature and repeated three times to ensure accuracy and reproducibility.

Morphological Analysis of Electrospun Nanofibers

The morphological characteristics of the electrospun nanofibers were examined using Field Emission Scanning Electron Microscopy (FE-SEM, JSM-7100F; JEOL, Japan) at a magnification of 10,000×. Prior to imaging, the nanofiber samples were securely mounted onto metal stubs and coated with a thin layer (approximately 10 nm) of gold-palladium to enhance their conductivity and imaging quality.

Total Phenolic Content of Electrospun Nanofibers

The total phenolic content (TPC) of the electrospun nanofibers was quantified using a modified Folin–Ciocalteu colorimetric method. The absorbance of the reaction mixture was recorded at 760 nm using a UV-visible spectrophotometer (UV-1800, Shimadzu, Columbia, USA). The TPC was calculated using Equation (2.2):

$$TPC\ (mg\ GAE/g\ film) = \frac{C \times V \times D}{WS}$$

where C is the concentration (mg/L) determined from the calibration curve, V represents the solution volume (L), D is the dilution factor, and W_S is the weight of the nanofiber (g).

Antioxidant Activity of Electrospun Nanofibers

The antioxidant activity of the electrospun nanofibers was determined with 0.1 g of the nanofiber was dissolved in 10 mL of an ethanol/water solution (80:20 v/v) to extract active compounds. After filtration to remove any insoluble residues, 100 µL of the resulting extract was added to 3.9 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution. The absorbance of the solution was measured at 517 nm using a UV-visible spectrophotometer (UV- 1800, Shimadzu, Columbia, USA). The percentage of DPPH radical scavenging activity (%AA) was calculated using Equation (2.3):

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{film}}{A_{control}} \times 100$$

where A_control represents the absorbance of the control sample (DPPH solution without nanofiber extract), and A_film corresponds to the absorbance of the sample extract.

Antimicrobial Activity of Electrospun Nanofibers

The antimicrobial activity of the electrospun nanofibers was evaluated using the agar diffusion method, following the protocol described by Liu et al. Cultures of Gram-negative *Escherichia coli* (ATCC 11229) and Gram-positive *Staphylococcus aureus* (ATCC 43300) were prepared by inoculating bacterial strains into 10 mL of LB broth. The cultures were incubated at 37°C for 24 hours to allow bacterial growth. The bacterial suspensions were adjusted to a 0.5 McFarland standard using a turbidity meter (DEN-1 B, Biosan, Latvia).

Circular discs of nanofiber films (1 cm in diameter) were cut and placed on Mueller-Hinton agar plates that had been evenly coated with 0.1 mL of the bacterial suspensions. The plates were incubated at 37°C for 24 hours under aerobic conditions. Following incubation, the diameters of the clear zones of inhibition around the films were measured using a digital caliper. The inhibition zones were used as an indicator of the antimicrobial efficacy of the nanofiber samples. Each test was performed in duplicate to ensure reproducibility of the results.

RESULTS and DISCUSSION

Viscosity and Electrical Conductivities Results

The viscosity of the polymer solutions is a key parameter influencing the electrospinning process and the resulting fiber morphology. As presented in the table, the viscosity of the solutions decreases significantly with increasing oleuropein concentration. For instance, the viscosity drops from 1196.0±2.83 cp in the OLE_0 sample to 595.9±2.82 cp in the OLE_3.75 sample. This trend can be attributed to the presence of ethanol in the oleuropein-containing solutions, which likely disrupts the interactions between polymer chains, leading to a reduction in viscosity. A similar phenomenon has been observed in other studies where ethanol, as a solvent, weakened intermolecular forces within the polymer matrix, thereby reducing viscosity. Maintaining an optimal viscosity is crucial for electrospinning, as excessively high viscosity impedes jet formation, while very low viscosity may prevent continuous fiber formation.

The electrical conductivity of the solutions plays a critical role in determining the elongation of the polymer jet and, subsequently, the morphology of the electrospun fibers. According to the results, the OLE_0 solution exhibits the highest conductivity value at 1201.3±89.6 µS/cm, while the conductivity decreases to 771.0±52.8 µS/cm in the OLE_1.25 solution. However, further increases in oleuropein concentration (OLE_2.5 and OLE_3.75) show no significant variation, with conductivity values stabilizing around 820 µS/cm. This reduction in conductivity may be related to the ethanol present in the oleuropein solution, as ethanol has inherently low conductivity compared to water. Despite the decrease in viscosity, the slight variations in conductivity suggest that the ionic mobility and charge distribution within the polymer matrix remain relatively stable across the different formulations.In summary,

increasing oleuropein concentration in the solutions reduces their viscosity while slightly influencing electrical conductivity. These findings highlight the importance of optimizing both parameters to ensure successful electrospinning and consistent nanofiber morphology. While the presence of ethanol plays a key role in reducing viscosity, its effect on conductivity appears to be minimal, allowing for stable jet elongation during fiber formation.

Table 1. Viscosity and electrical conductivities of solutions

Solution	Viscosity (cp)	Electrical conductivity (µs/cm)
OLE_0	1196.0±2.83[a]	1201.3±89.6[a]
OLE_1.25	786.8±9.89[b]	771.0±52.8[b]
OLE_2.5	696.9±1.42[c]	823.6±100.5[b]
OLE_3.75	595.9±2.82[d]	820.5±31.8[b]

Morphological Analysis of Electrospun Nanofibers

Figure 1 illustrates the morphological characteristics of the electrospun fibers along with their,. As shown in Figure 1, the samples OLE_0, OLE_1.25, and OLE_2.5 exhibited a uniform, bead-free morphology. However, the OLE_3.75 sample displayed, irregularities, and non-homogeneous structures. This suggests that the reduced viscosity at higher oleuropein concentrations may have compromised the solution's ability to form stable, continuous fibers. Additionally, independent of the oleuropein content, surface irregularities such as tubercle-like formations, fiber overlapping, and sticking were observed across the samples. These irregularities could be attributed to the incomplete evaporation of the solvent during the electrospinning process. It is well-established that viscosity plays a crucial role in producing bead-free fibers, as it reflects the extent of polymer chain entanglements in the solution (Aydogdu, Sumnu, & Sahin, 2018). For the formation of smooth, beadless fibers, sufficient entanglement of polymer molecules is necessary. When viscosity decreases further, as seen in the case of OLE_3.75, the polymer jet can no longer withstand the surface tension and electrical forces, leading to the inability to maintain a stable Taylor cone and surface irregularities.

Another possible explanation is the differing volatilization rates of the components in the multi-solvent system used in the preparation of the solutions.In conclusion, while the polymer solution viscosity primarily governed fiber morphology, solvent evaporation dynamics and the multi-component nature of the solvent system also played a role in influencing surface irregularities and structural variations.

In a recent study of Aydogdu Emir et al. 2024, a similar outcome was reported. As the concentration of elenolic acid, a compound similar to oleuropein, increased, the viscosity of the solution decreased. This reduction in viscosity led to a decrease in polymer chain entanglement, resulting in the formation of nanofibers with beads. This highlights the critical balance required in polymer concentration and viscosity to achieve bead-free nanofibers, emphasizing the importance of sufficient molecular interactions for stable fiber formation.

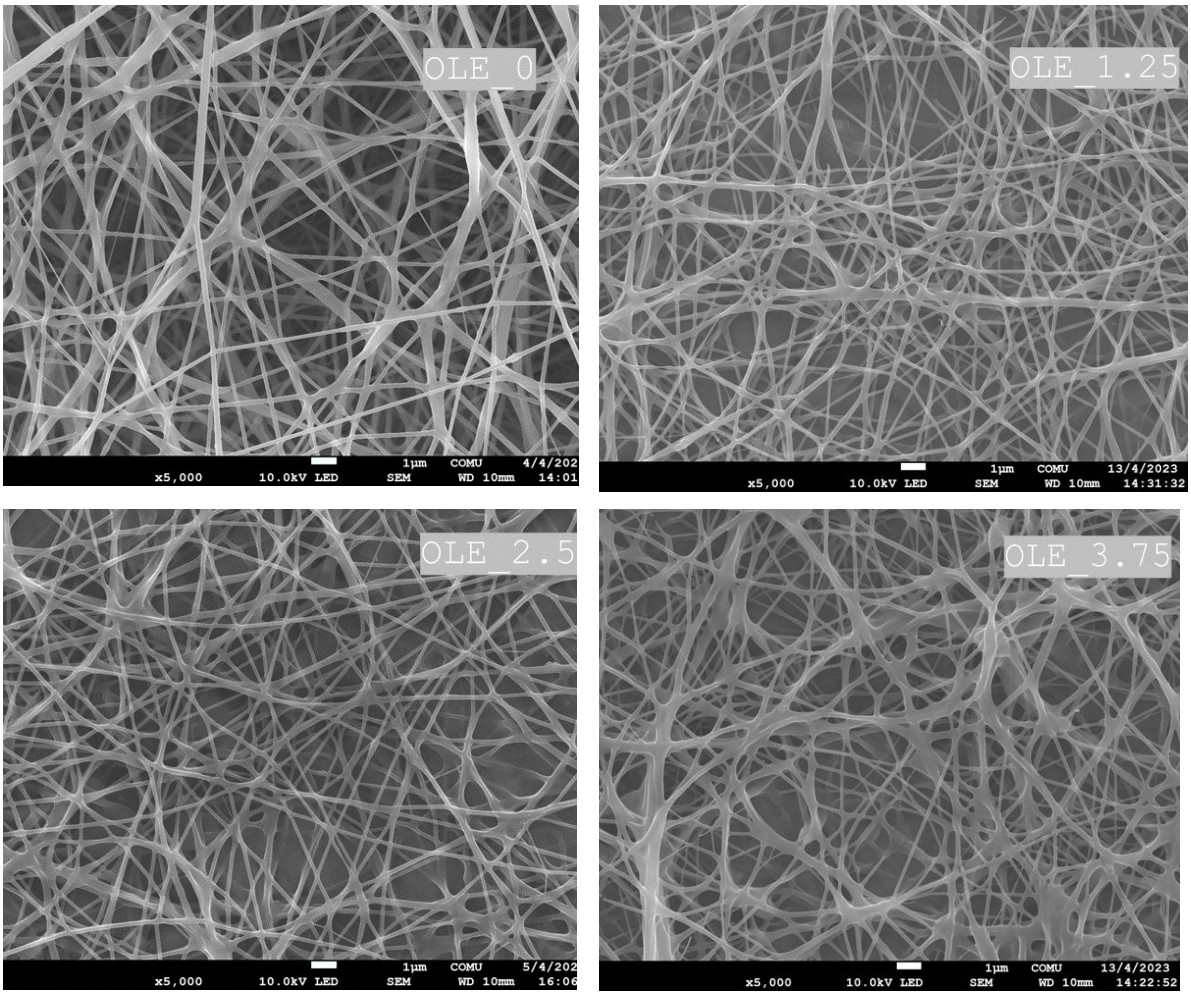

Figure 1. SEM images of the fibrous films.

Total Phenolic Content (TPC) and Antioxidant Activity Results

Electrospinning is a highly efficient technique for encapsulating active compounds, providing a cost-effective means of maintaining bioactivity due to its ability to produce fibers with a high surface-to-volume ratio. As shown in Table 2, the OLE_0 sample, which does not contain oleuropein, exhibited no total phenolic content (TPC) or antioxidant activity. In contrast, the incorporation of oleuropein into the polymer matrix significantly enhanced both parameters. The OLE_1.25 sample exhibited a TPC value of 7.52±0.66 mg GAE/g film, corresponding to an antioxidant activity of 35.15±1.82% inhibition. Increasing the oleuropein concentration further improved these values, with the OLE_2.5 film reaching a TPC of 22.56±1.52 mg GAE/g film and a markedly higher DPPH scavenging activity of 87.56±1.02%. Similarly, the OLE_3.75 sample demonstrated the highest TPC (31.66±3.29 mg GAE/g film) and maintained a strong antioxidant activity (87.15±1.14%).

The correlation between increasing oleuropein content and enhanced antioxidant activity highlights the effectiveness of the PEO-chitosan matrix in preserving and releasing phenolic compounds. The antioxidant activity can be attributed to the redox properties of phenolic hydroxyl groups in oleuropein,

which play a key role in scavenging free radicals. In conclusion, the oleuropein-loaded films demonstrated promising antioxidant potential, particularly at higher concentrations. These findings suggest that such nanofiber-based systems are highly suitable for applications where oxidative stability is critical, such as in food packaging and preservation systems.

Table 2. Total phenolic content (TPC), antioxidant activity of the nanofibers.

Film	TPC (mg Gallic Acid Equivalent (GAE)/g film)	DPPH activity (% inhibition)
OLE_0	-	-
OLE_1.25	7.52±0.66^{c}	35.15±1.82^{b}
OLE_2.5	22.56±1.52^{b}	87.56±1.02^{a}
OLE_3.75	31.66±3.29^{a}	87.15±1.14^{a}

Antimicrobial Activity of Oleuropein-Loaded Films

The antimicrobial activity of the oleuropein-incorporated films was evaluated using the disc diffusion method, and the inhibition zone diameters are presented in Table 3.

Table 3. Inhibition zone values of Oleuropein-Loaded Films

	Diameter of inhibition zone (mm)	
Films	***S. aureus***	***E. coli***
OLE_1.25	15.00±0.05	0.00±0.00
OLE_2.5	18.50±0.15	0.00±0.00
OLE_3.75	17.50±0.25	0.00±0.00

As shown, the OLE_1.25 sample exhibited an inhibition zone of 15.00±0.05 mm against *S. aureus*, while no inhibitory effect was observed against *E. coli*. Increasing the oleuropein

concentration enhanced the antimicrobial effect against *S. aureus*, with the OLE_2.5 and OLE_3.75 samples showing inhibition zones of 18.50±0.15 mm and 17.50±0.25 mm, respectively. However, none of the films demonstrated activity against *E. coli* regardless of the concentration of oleuropein (Figure 2). The variation in the antimicrobial agents' effectiveness against Gram-positive (G+) and Gram-negative (G-) bacteria can be explained by differences in their cell wall structures. According to Lv et al. (2011), the cell wall of G(-) bacteria is composed of lipopolysaccharides, which act as a barrier, preventing antimicrobial agents from reaching the cytoplasmic membrane. In contrast, G(+) bacteria lack this additional membrane, allowing antimicrobial agents to penetrate their cells more easily, leading to stronger inhibitory effects.

In the study of Aydogdu Emir et al. 2023, it was also reported findings consistent with this outcome. Despite increasing the concentration of sumac in the active film formulation, no significant antimicrobial effect was observed against *E. coli*. This aligns with the understanding that Gram-negative bacteria, like *E.*

coli, possess a lipopolysaccharide-rich outer membrane, which acts as a barrier to antimicrobial agents. As a result, the active compounds in sumac were unable to penetrate this protective structure effectively, thereby failing to exhibit inhibitory activity against *E. coli*. This result highlights the inherent resistance of Gram-negative bacteria to certain plant-based antimicrobials, despite their effectiveness against Gram-positive counterparts.

Chitosan, known for its polycationic structure, typically interacts with the negatively charged cell walls of microorganisms, facilitating antimicrobial activity. However, its inhibitory effect depends on various factors such as molecular weight, pH, and concentration. In this study, the absence of inhibition against *E. coli* suggests that the chitosan concentration (1% w/v) may not have been sufficient to penetrate the outer membrane of Gram-negative bacteria, which acts as a barrier. In contrast, Gram-positive *S. aureus* is more susceptible due to its simpler cell wall structure, allowing better interaction with chitosan and oleuropein. The enhanced antimicrobial activity with increasing oleuropein concentration indicates a potential synergistic effect between oleuropein and chitosan. Oleuropein's antimicrobial properties are primarily attributed to its phenolic hydroxyl groups, which disrupt the bacterial cell membrane, leading to cellular leakage and metabolic disturbances. Additionally, surface irregularities and beaded fiber morphology may influence the release and interaction of active agents. In conclusion, the films demonstrated selective antimicrobial activity, effectively inhibiting *S. aureus* while showing no effect against *E. coli*. This highlights the role of oleuropein concentration in enhancing antimicrobial efficacy, particularly against Gram-positive bacteria.

Figure 2. Inhibition zones of the Oleuropein-Loaded Films on agar plates.

Conclusion

In this study, oleuropein-incorporated PEO/chitosan nanofiber films were successfully produced via electrospinning, and their structural, antioxidant, and antimicrobial properties were evaluated. The results

demonstrated that increasing oleuropein concentration significantly influenced solution viscosity and nanofiber morphology, while having minimal effect on electrical conductivity.The total phenolic content (TPC) and antioxidant activity of the films increased proportionally with oleuropein concentration. The OLE_3.75 sample exhibited the highest TPC (31.66±3.29 mg GAE/g film) and strong DPPH radical scavenging activity (87.15±1.14%), highlighting the effective encapsulation and retention of bioactive compounds within the polymer matrix. In terms of antimicrobial activity, the films showed selective inhibition against S. aureus (Gram-positive bacteria), with no observable effect on E. coli (Gram-negative bacteria). The OLE_2.5 and OLE_3.75 films exhibited inhibition zones of 18.50±0.15 mm and 17.50±0.25 mm, respectively, against *S. aureus*. The synergistic interaction between chitosan and oleuropein likely contributed to the enhanced antibacterial effect observed against Gram-positive bacteria.

The findings of this study suggest that oleuropein-loaded PEO/chitosan nanofiber films have significant potential for active packaging applications, particularly in extending the shelf life of perishable food products through their antioxidant and selective antimicrobial properties. Despite these promising results, challenges remain, including addressing the selective antimicrobial activity observed and improving the uniformity of fiber morphology. These challenges highlight areas for future research, such as optimizing oleuropein release profiles and expanding the range of microorganisms tested to ensure broader applicability. By tackling these limitations, the potential of these nanofiber films in food packaging and other active material delivery systems can be fully realized.

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