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Evaluation of the Antibacterial Effect of Polylactic Acid Films Containing Oak Ethanolic Extract and Cinnamon Essential Oil against Foodborne Pathogens

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ABSTRACT

Background: Today, the prevalence of foodborne illnesses has led to the widespread use of medicinal plants. This study aims to investigate the antibacterial effect of polylactic acid (PLA) films containing oak ethanolic extracts incorporated with cinnamon essential oil against a range of foodborne pathogens.
Methods: The oak extract was obtained by soaking method and the cinnamon essential oil was extracted using a Clevenger apparatus. PLA films containing 2% concentration of oak extract and cinnamon essential oil were prepared, homogenized, cast, and stored at 4°C. Subsequently, the antimicrobial efficacy of various concentrations of the extract against pathogenic bacteria was examined using the disk diffusion method.
Results: The film containing 400 mg/mL of ethanolic extract along with 3% cinnamon, showed the largest non-growth zone (with a diameter of 31.5 mm against *Staphylococcus aureus*). Furthermore, the film with a concentration of 100 mg/mL of extract displayed the smallest non-growth zone against *Salmonella typhimurium*, measuring 13.54 mm in diameter.
Conclusion: Based on the obtained results, the combination of oak extract and cinnamon essential oil has a strong inhibitory effect against common foodborne pathogens. Consequently, these substances may be useful for enhancing the safety and extending the shelf life of various food products.

1. Introduction

Infectious diseases are widely prevalent worldwide, imposing a significant financial burden on human society. Over the past decades, chemical antibiotics have played a crucial role in treating infectious diseases. However, the emergence of microbial resistance has led to an increasing inclination toward the use of herbal medicines (Ateş & Erdogrul, 2003; Cowan, 1999). PLA is a biodegradable polymer derived from renewable sources. It is thermoplastic, non-toxic, and highly transparent, showing good potential for replacing conventional polymers such as polyamides, polyethylene, polypropylene, and polyethylene terephthalate. Corn starch, a constituent monomer of PLA, serves as one of the primary sources of lactic acid (Zhou et al., 2019; Tayel et al., 2018). The high strength and transparency of PLA acid have attracted significant industrial attention, particularly in the field of medical packaging and food applications (Shin et al., 2022). The oak tree belongs to the *Fagaceae* family and the genus *Quercus*. With a maximum height of 12 m and numerous scattered branches, oak trees exhibit distinctive features (Ghaderi et al., 2012; Özünlü et al., 2018). Oaks are protected trees, and cutting them is prohibited according to European laws; therefore, utilizing this valuable resource is highly important. The main habitat of oak trees is in the western and northwestern regions of Iran. As we move towards the southern regions of the Zagros mountains range, the diversity of other trees decreases, and oak species dominate in provinces such as Kermanshah, Lorestan, and Kohgiluyeh and Boyer-Ahmad

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(Masoudi Nejad & Rezazade Azary, 2003). Oak fruit is a rich source of carbohydrates, amino acids, fats, and various sterols. Research indicates that the chemical compounds found in oak fruit closely resemble those found in grains (Ghaderi et al., 2012). In addition, oak fruit contains high levels of biologically active compounds, including tannins, gallic acid, ellagic acid, proanthocyanidins, and flavonoids (Ghaderi et al., 2012). Plant extracts contain a variety of natural compounds that can have various beneficial effects. Oak extracts, in particular, are rich in substances such as tannins and phenolics, which contribute to many potential health benefits. Plant extracts can help combat free radicals in the body, thereby reducing oxidative stress and potentially lowering the risk of chronic diseases. Moreover, certain extracts exhibit properties that fight against bacteria, fungi, and other microbes. Plant extracts may help reduce inflammation, which is associated with various health conditions. Research suggests the potential for blood sugar regulation, cholesterol reduction, and even anti-cancer properties (Morales, 2021). The antimicrobial activity of essential oils (EOs) is attributed to their monoterpenoid compounds. However, in food materials, there is an interplay between food components and phenolic compounds, which reduces the antimicrobial activity of phenolic compounds. Therefore, the presence of essential oil in film formulations and food coatings stabilizes these compounds. Consequently, such films and coatings gradually release antimicrobial compounds, thereby reducing surface contamination of food materials (Buonocore et al., 2003). EOs are extracted from various parts of the cinnamon tree, such as leaves, bark, fruits, roots, flowers, and buds. With over 80 identified compounds, the main constituents of EOs and cinnamon extracts are cinnamaldehyde, eugenol, and phenol linalool. Cinnamon bark EO, in particular, has a higher cinnamaldehyde content (65-80%) and a lower eugenol content (5-10%) (Sabahi et al., 2022). Traditional plastic packaging raises significant environmental concerns due to its slow degradation and contribution to plastic pollution. Biodegradable packaging films made from PLA offer a promising alternative. Throughout history, various parts of the oak tree, from its bark to its acorns, have been used in folk medicine. Modern science is now beginning to explore the potential applications of these natural compounds. Several studies have reported strong antibacterial effects for some EOs. Among these EOs, the potential antibacterial effect of cinnamon has been repeatedly documented. Considering the aforementioned points, the present study aimed to design and produce a biodegradable packaging film based on PLA combined with two natural antimicrobial compounds: oak fruit extract and cinnamon essential oil. The study employed the disk diffusion method to evaluate the antimicrobial effects against four foodborne pathogens, using a modeling technique.

2. Materials and Methods

Granules of PLA with a density of 1.3 g/cm^3 and a molecular weight of 197,000 g/mol were obtained (FKUR Kunststoff, Germany). Oak fruit samples for the study were randomly collected in the spring season from the Rawansar area of Kermanshah Province, Iran. The collected oak fruit was then chopped (Moulinex grinder, France) and dried. Cinnamon sticks were purchased from an Herbal pharmacy and then ground (Moulinex grinder, France) and stored in a closed container away from sunlight until essential oil extraction (Sheerzad et al., 2024). The culture media Muller Hinton Agar and BHI (Merck, Germany) were prepared. Additionally, equipment such as a spectrophotometer (Jenway, England), a digital scale with a precision of 0.001 g (A&D, Japan), and a homogenizer (D15 W, Korea) were used in the laboratory of Food Hygiene of the Faculty of Veterinary Medicine, University of Tehran.

2.1 Preparation for oak extract

The extraction of oak extract was carried out using the soaking method and ethanol as the solvent. For different concentrations, 50 g of crushed oak fruit was mixed with 500 mL of 80% ethanol (Majlisi, Iran) at room temperature and kept for 3 days. Then, the resulting solution was filtered, and centrifuged at 400 rpm for 5min using a centrifuge (Funke Gerber, German). Finally, the concentrated solution was evaporated to dryness at 50 °C using an oven evaporator (Elektro Helios, Sweden). The powder obtained from this process was collected from the surface of the container and stored at 4 °C until further testing. For preparing different concentrations (0, 100, 200, and 400 mg/mL), the specific amount of the dried extract was dissolved in 1 mL of ethanol solvent (Masoudi Nejad & Rezazade Azary, 2003; Ebrahimi et al., 2012).

2.2 Preparation for cinnamon essential oil

After collecting cinnamon and confirming its scientific name, it was dried, milled, and then extracted using a Clevenger apparatus for 3h. The obtained essential oil was collected in laboratory tubes, shielded from light, and stored at refrigerator temperature for subsequent experiments. According to previous studies, a concentration of 3% cinnamon essential oil was used in each film (Rizki et al., 2023; Sheerzad et al., 2024). The main constituents of cinnamon essential oil measured by Gas chromatography-mass spectrometry (GS/MS) can be seen in Table 1.

Table 1. Constituents of cinnamon essential oil measured by GS/MS

Name	Cinnamaldehyde	delta-Cadinene	cis-Cinnamaldehyde
Cinnamon EO	82.29%	2.81%	1.53%

2.3 Preparation for the polylactic acid film

To prepare the PLA film 2%, 2 g of PLA was dissolved in 100 mL of chloroform (Qatran Shimi, Iran) and transferred to a stirrer (Karaazma, Iran) equipped with a magnet until the ingredients were stirred. Then, the desired amounts of oak fruit ethanol extract (0, 100, 200, 400 mg/mL) and 3% cinnamon essential oil were added to the mixture, which was then homogenized for 1 min at 12,000 rpm. The resulting material was poured into glass molds and the solvent was allowed to evaporate at room temperature. Next, the prepared films were separated from the molds and stored in the refrigerator at 4 °C (Shavisi et al., 2017). The compositions of PLA are listed in Table 2.

Table 2. Composition of PLA films

Name	PLA (%)	Oak extract (mg/mL)	Cinnamon essential oil (%)
Film No:1	2	0	٠
Film No:2	2	100	3
Film No:3	2	200	3
Film No:4	2	400	3

2.4 Preparation of bacterial inoculum

Four foodborne pathogenic bacteria, including *Listeria monocytogenes* (ATCC 35152), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli O157:H7* (ATCC 43888), and *Salmonella typhimurium* (ATCC 14028) were obtained from the microbiology laboratory of the Faculty of Veterinary Medicine, University of Tehran. These strains were prepared in BHI broth and refreshed twice before the disk diffusion assay at 37 °C. For the inoculum preparation, a suspension was poured into a sterile Spectro cell and its absorbance was measured at 600 nm using a spectrophotometer. The target absorbance was set at 0.5 (0.5 McFarland standards). A bacterial density of 1×10^8 CFU/mL was obtained at a wavelength of 600 nm.

2.5 Assessment of antibacterial activity

A bacterial culture containing 1×10^8 CFU/mL was inoculated onto Mueller-Hinton agar plates at a volume of 100 µL. Then, disks containing the mentioned films, each containing different concentrations of oak ethanol extract and 3% cinnamon essential oil, were placed in the center of the plates. The plates were then incubated at 37 °C for 24h in an incubator. The transparent zone surrounding the film was considered the inhibition zone. The inhibition zone was measured using Digimizer 6.3.0 software (MedCalc software). Additionally, *Gentamicin* antibiotic standard disks were used as positive controls in this study.

2.6 Statistical analysis

Each condition served as a sampling unit, and the desired experiments were conducted on these samples. The resulting data were recorded in Microsoft Excel and analyzed using SPSS version 26 software (IBM). Analysis of variance (ANOVA) was used to examine significant differences among the treatment and control conditions. The Tukey statistical test was used to compare the means at a significant level of 0.05. A significance level of $P < 0.05$ was considered statistically significant.

3. Results and Discussion

PLA has been widely studied as a potential carrier for active compounds in food packaging. Various EOs and their compounds, as well as other phenolic compounds, have been incorporated into PLA matrices, resulting in different changes in the functional properties of the film while imparting antioxidant or antimicrobial capabilities to them, depending on the processing conditions of the film and the nature (volatility, solubility, or thermal sensitivity) of the active agent (Ordoñez et al., 2022). Figure 1 shows the inhibition zones created by the active films and gentamicin.

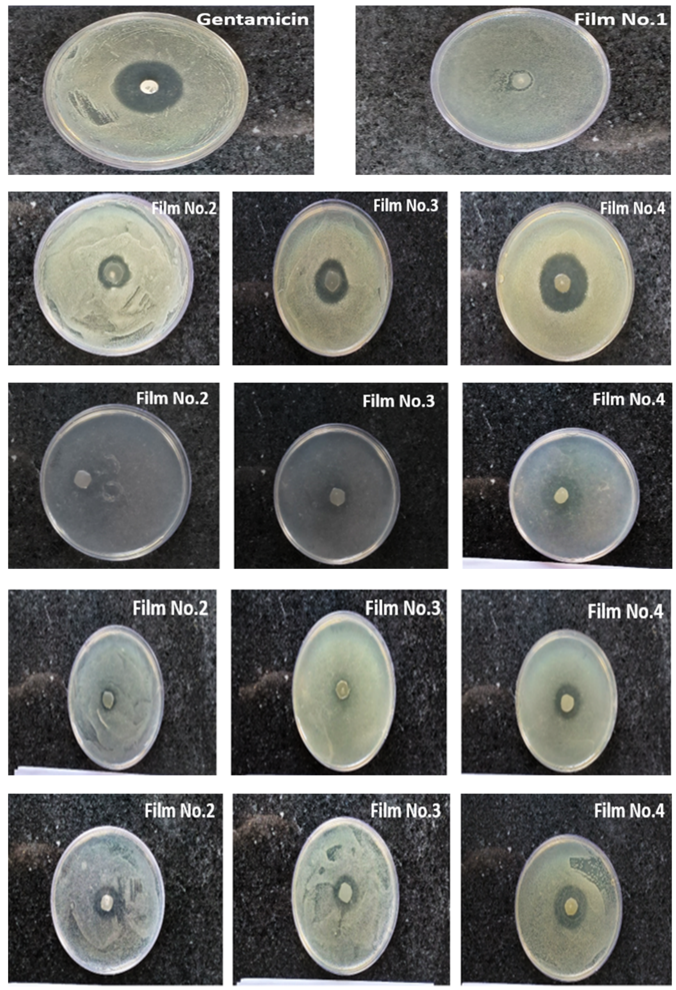

Figure 1. Comparison of the antibacterial effects of PLA films. From the left, related to gentamicin, film No 1 against *S.aureus*, films No 2, 3, 4 against *S.aureus*, films No 2,3,4 against *L.monocytogenes*, films No 2, 3, 4 against *S.Typhimurium*, films No 2, 3, 4 against *E. coli*.

Table 3 details the diameter of the *Gentamicin* inhibition zone against *S.aureus* as 27.80 mm, while the film with a concentration of 400 mg/mL of oak extract showed the largest inhibition zone against *S.aureus* with a diameter of 31.5 mm. Conversely, the film with a concentration of 100 mg/mL of the oak extract against *S.typhimurium* created the smallest inhibition zone with a diameter of 13.54 mm. In this study, the highest sensitivity was observed against *S.aureus*, followed by *E.coli O157:H7*, *L.monocytogenes*, and *S.typhimurium*, respectively. For *E. coli* and *L.monocytogenes*, significant differences were observed among other microorganisms ($P < 0.05$). Increasing the concentration of oak extract in the PLA film increased the size of the inhibition zone. Staphylococcus aureus demonstrated greater sensitivity to films containing oak extracts and essential oils compared to other bacteria. The release of active ingredients from the films into the surrounding agar and the creation of a zone of bacterial inhibition was observed in this study. Increasing the shelf life of food products is one of the main challenges in the food packaging industry. In recent years, active packaging has gained attention due to its ability to protect food against external factors, control moisture and oxygen, release various compounds, and incorporate sensors and indicators to determine shelf life (Vermeiren et al., 1999). Considering the large number of chemical groups present in EOs and extracts, their antimicrobial activity is not based on a specific mechanism but instead on multiple targets. The antimicrobial effect is enhanced by the presence of phenolic compounds, particularly thymol and carvacrol, in EOs (Pirbalouti et al., 2013). The biodegradable polymer PLA can be considered as a desirable base polymer for designing and developing modern antimicrobial films, especially in combination with plant-based antimicrobial EOs, as observed in this study (Rezaeigolestani et al., 2018). Among various extraction methods such as maceration, percolation, Soxhlet extraction, and boiling, the maceration method has been reported as the most effective method (Masoudi Nejad & Rezazade Azary, 2003). In this study, maceration was also used to obtain oak extract. Plant extracts generally exhibit greater activity against Gram-positive bacteria, which aligns with the findings of this study and previous research. Studies have shown that most medicinal plants, including the powdered oak leaf extract (Quercus ilex), demonstrate stronger antibacterial effects against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* compared to Gram-negative bacteria like *Escherichia coli* (Sánchez et al., 2022; Voravuthikunchai & Suwalak, 2008). This aligns with the findings of Hashim *et al.* (2013) who observed the greatest antibacterial activity of Quercus infectoria galls against *Bacillus subtilis*, followed by *Staphylococcus aureus* and then *E. coli*. This observed difference in susceptibility can be attributed to the structural differences between the outer membranes of Gram-positive and Gram-negative bacteria (Hashim et al., 2013). In addition to plant extracts, other natural materials also show promise against various bacteria. For example, Rezaeigolestani *et al.* (2018) demonstrated that PLA films containing rosemary extract significantly restricted the growth of foodborne pathogens, including both Gram-positive and Gram-negative bacteria. Combining rosemary extract with propolis extract also exhibited positive antibacterial effects, likely due to the hydrophobic nature of the active film components (Rezaeigolestani et al., 2018).

Table 3. Inhibition zone (mm) effect of PLA films containing various concentrations of oak extract and 3% cinnamon essential oil and gentamicin (Mean ± Standard Deviation)

Treatments (PLA films)	*E.coli*	*L.monocytogenes*	*S.Typhimurium*	*S.aureus*
Gentamicin	-	-	-	27/80 ± 0/64^{Cb}
Film No: 1	0	0	0	0
Film No: 2	19/20 ± 0/57^{Ab}	15/03 ± 0/83^{Ab}	13/54 ± 0/59^{Bc}	17/46 ± 0/27^{Cd}
Film No: 3	18/27 ± 1/03^{Ab}	17/86 ± 0/83^{Ab}	14/44 ± 1/57^{Bc}	22/40 ± 0/55^{Cc}
Film No: 4	25/01 ± 0/49^{Aa}	23/53 ± 2/9^{Aa}	20/35 ± 0/09^{Bb}	31/5 ± 0/34^{Ca}

4. Conclusion

Based on the results of this study, films containing the ethanol extract of oak fruit and cinnamon essential oil showed a significant inhibitory effect on the foodborne pathogens, comparable to the antibiotic *Gentamicin*. The positive effects of these two natural agents resulted in the formation of inhibition zones on the culture medium. Furthermore, the numerical analysis indicated that Gram-positive bacteria were more sensitive to the active ingredients inside the PLA film than Gram-negative bacteria. The findings of this study highlight the potential of using PLA films incorporated with oak extract and cinnamon essential oil in food packaging industries to enhance safety and extend the shelf life of food products.

Authors' Contributions

Kimia Bahraminejad: Investigation; Methodology; Writing the original draft. Ali Misaghi: Methodology; Resources; Visualization; Writing-review & editing. Abolfazl Kamkar: Data curation; Software; Formal analysis; Validation. Ali Khanjari: Conceptualization; Supervision; Project administration; Funding acquisition; Validation; Writing-review & editing.

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Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Ethical considerations

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured of the confidentiality of their information. Participants had the freedom to withdraw from the study at any time, and if desired, the research results would be made available to them. Written consent was obtained from all participants. This study was approved by the Ethics Committee of the University of Tehran. (Code 71485/76).

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