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Chitosan/PVA/Starch Ternary Film With *Ilex paraguariensis* Hydroalcoholic Extract as an Antimicrobial Active Package for Meat

Filmes Ternários de Quitosana/PVA/Amido com Extrato Hidroalcoólico de Ilex paraguariensis como Embalagem Antimicrobiana Ativa para Carnes

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Abstract

The study investigated the development of a ternary active film incorporating *Ilex paraguariensis* extract as an antimicrobial agent. The film is composed of starch, polyvinyl alcohol, and chitosan solutions, combined with dry *I. paraguariensis* extract. Following preparation and drying, the films underwent antimicrobial and mechanical testing. The mechanical properties of the active films were promising, and the swelling index stabilized at 750%. To assess antimicrobial efficacy, tests were conducted using red meat samples contaminated with *Staphylococcus aureus*, comparing the performance of the active film to commercial polyvinyl chloride film as a control. The results demonstrated the superior antimicrobial properties of the ternary active film compared to the control. When used to wrap contaminated meat samples, the active film significantly reduced bacterial growth, showing a two-logarithmic-cycle difference in colony counts compared to the commercial polyvinyl chloride film. This substantial reduction in bacterial proliferation underscores the effectiveness of the *I. paraguariensis* extract as an antimicrobial agent within the film matrix. The study concludes that the developed ternary film exhibits both good mechanical properties and microbiological effectiveness, suggesting its potential for applications in food packaging, particularly in preserving meat products and extending their shelf life.

Keywords: Active Packaging. Antimicrobial Activity. *Ilex Paraguariensis*. Food Packaging. Natural Extracts.

Resumo

Este estudo explora o desenvolvimento de um filme ativo ternário incorporando extrato de *Ilex paraguariensis* como agente antimicrobiano. A composição do filme inclui soluções de amido, álcool polivinílico e quitosana, combinadas com extrato seco de *I. paraguariensis*. Após a preparação e secagem, os filmes foram submetidos a testes antimicrobianos e mecânicos. As propriedades mecânicas dos filmes ativos mostraram resultados promissores, e o índice de intumescimento atingiu um valor estável de 750%. Para avaliar a eficácia antimicrobiana, os pesquisadores conduziram testes usando amostras de carne vermelha contaminadas com *Staphylococcus aureus*, comparando o desempenho do filme ativo desenvolvido com o filme de cloreto de polivinilocomercial como controle. Os resultados demonstraram as propriedades antimicrobianas superiores do filme ativo ternário em comparação ao controle. Ao embalar amostras de carne contaminadas, o filme ativo reduziu significativamente o crescimento bacteriano, mostrando uma diferença de dois ciclos logarítmicos na contagem de colônias em comparação com o filme de cloreto de polivinilo comercial. Essa redução substancial na proliferação bacteriana destaca a eficácia do extrato de *I. paraguariensis* como agente antimicrobiano dentro da matriz do filme. O estudo conclui que o filme ternário desenvolvido apresenta boas propriedades mecânicas e eficácia microbiológica, sugerindo seu potencial para aplicações em embalagens de alimentos, particularmente para conservar produtos cárneos e prolongar sua vida útil.

Palavras-chave: Embalagem Ativa. Atividade Antimicrobiana. *Ilex paraguariensis*. Embalagem para Alimentos. Extratos Naturais.

1 Introduction

Films made from natural polymers incorporating antimicrobial compounds have garnered significant interest in food packaging due to their ability to release antimicrobial agents that control microbial flora in foods (Ibarguren *et al.*, 2015; Wen *et al.*, 2016). This enhances food stability and extends shelf life. Natural extracts and compounds with notable antimicrobial properties have been integrated into these natural polymer films (Muriel-Galet *et al.*, 2015; Zhang; Lu; Chen, 2014). Extracts from plant species known for their antimicrobial activity, such as *Ilex paraguariensis* A. St.-Hill. (Aquifoliaceae), commonly referred to as yerba mate, have been included in active antimicrobial films (Reis *et al.*, 2015).

Microbial growth is the leading cause of food spoilage, resulting in metabolites that impart off-flavors, rendering the product unsuitable for consumption. Consequently, efforts within industry and academia have been directed towards improving food preservation concerning microbial growth. Traditional methods employed for food preservation include thermal processing, drying, freezing, cooling, irradiation, and the use of salt or antimicrobial agents (Muriel-Galet *et al.*, 2015). One significant advantage of incorporating antimicrobial agents in food packaging is the reduction of food preservatives usage. Active polymers, which incorporate antimicrobial natural extracts into the polymer, can reduce, inhibit, or delay microbial flora growth, particularly on food surfaces.

Meat is particularly susceptible to microbial contamination and spoilage. Therefore, there is a concerted effort to develop new polymers with antimicrobial properties specifically for meat packaging (Lavoine *et al.*, 2015). Most new active polymers are created by blending biodegradable polymers (e.g., chitosan, gelatin, or starch) with synthetic biodegradable polymers (e.g., polyvinyl-alcohol) in various proportions and incorporating antimicrobial compounds (Lee *et al.*, 2015; Wen *et al.*, 2016). The advantage of blending natural and synthetic polymers lies in the enhanced mechanical and physical properties of the blend compared to individual polymers (Kanimozhi; Khaleel; Sugantha, 2016).

This study reports on the development and potential application of an active antimicrobial package for red meat. It involves a blend film of chitosan, polyvinyl alcohol (PVA), and starch with an extract from *I. paraguariensis*, known for its antimicrobial activity.

2 Material and Methods

2.1 Preparation of the Ilex paraguariensis hydroalcoholic extract

For the experiment, 100 g of toasted *I. paraguariensis* leaves, sourced from a local market, were infused in freshly boiled distilled water (150 mL) and ethanol (150 mL) for 20 min. The infusion was filtered through quantitative filter paper, and the volume was reduced to 50 mL under low pressure. Following volume reduction, the solution was subjected to freeze drying and lyophilized to obtain a dry extract.

2.2 Antimicrobial activity of the Ilex paraguariensis hydroalcoholic extract

The extract was diluted to three concentrations: 50, 100, and 150 mg/mL. Antibacterial activity was evaluated against strains of *Staphylococcus aureus* (NEWP 0023) and *Escherichia coli* (ATCC 25922). *In vitro* analysis of antimicrobial activity was conducted using sterile discs. The sensitivity test was performed as previously reported in existing studies (Bauer *et al.*, 1966). To evaluate the antibacterial activity of the extract, *S. aureus* and *E. coli* isolates were reactivated in brain heart infusion media at 37 °C for 48 h. Afterward, samples of each activated microorganism were spread uniformly on the surface of plates containing Mueller-Hinton Agar (Kasvi). Subsequently, the extract was placed on the surface of the plate with the aid of sterile forceps at equidistance positions. The same procedure was conducted with a control disc of chloramphenicol (CLO-SENSIDISC 30). The plates were incubated at 36 °C for 24 h. Antimicrobial activity was determined by measuring the zone of inhibition in millimeters.

2.3 Ternary film preparation

Ternary films were prepared using the casting technique. In summary, a 1% PVA hydrogel was obtained by dissolving 1 g of polymer powder (Sigma-Aldrich, Art. No. 363146, 99% degree of hydrolysis, MW 116,000–124,000) in distilled water (100 mL) under magnetic stirring at 70 °C until completely dissolved. A 2% starch solution was prepared by dissolving 2 g of corn starch in distilled water (100 mL) under magnetic stirring for 1 h at 90 °C. Additionally, 1 g of medium molecular weight chitosan (Sigma-Aldrich, Art. No. 448877) was dissolved in a 1% aqueous acetic acid (v/v) solution (100 mL) and stirred at room temperature for 24 h.

Blend films of chitosan, PVA, and starch were prepared following the preparation of hydrogels. Hydrogels were cast into Petri dishes (90 mm diameter) to prepare the PVA/chitosan/starch films without extract in a 4:4:2 ratio. This hydrogel ratio was selected based on preliminary tests. The active film was prepared by adding 1 g of dry extract to the final hydrogel blends. Films containing 100% PVA, chitosan, or starch were also prepared as control films. All films were dried in an oven for 72 h at 38 °C.

2.4 Film characterization

2.4.1 Mechanical properties

The tensile (stress/strain) properties were measured using a universal testing machine (WDW 300E, Time Group Inc., China) according to the ASTM D1708-10 method. Five samples of each film type were prepared. The initial grip separation and traction speed were set at 22 and 12.5 mm/min, respectively, with a 150 kgf load cell. All testing was conducted at room temperature (approximately 25 °C). Young's modulus (E), or modulus of elasticity, was determined in the linear region of the graphs using linear regression. Data were analyzed with the Origin Pro v. 8.1 software.

2.4.2 Swelling index

Initially, the samples were cut into slices with a 4.0 cm^2 area. The samples were then kept in a desiccator with silica gel for seven days. The samples were then weighed and immersed in 250-mL beakers containing distilled water at various time intervals (0.5, 1, 3, 5, 7, 10, 15, and 20 min) at room temperature (25 °C). The samples were removed, dried, and weighed after each interval. The swelling index was calculated using Equation 1:

$$S = \frac{FinalWeight - InitialWeight}{tialWeight} \cdot 100 \qquad (1)$$

All experiments were conducted in triplicate.

2.4.3 Antimicrobial activity of films with the Ilex paraguariensis extract

S. aureus isolates were reactivated in brain heart infusion media at 37 °C for 48 h. Subsequently, the activated culture (20 µL) was dissolved in a sterile saline solution (50 mL). Two sets of three red meat samples were cut into pieces measuring 1 × 0.5 × 3 cm (length × width × thickness) and weighed approximately 2.5 g each. The meat samples were contaminated with the prepared saline solution. One set of samples was wrapped with the active film, while the other set was wrapped with commercial polyvinyl chloride film as a control. The samples were incubated at 7 °C for 48 h as reported by Wu, Wang and Chen, (2010).

To assess the antimicrobial effectiveness of the films, after 48 h of incubation, the films were removed, and the meat samples were soaked in peptone water (225 mL; Acumedia) for 3 min. A 1-mL aliquot was added to peptone water (9 mL) to obtain the initial dilution (10^{-1}). Subsequently, this dilution (1 mL) was transferred into tubes containing peptone water (9 mL) to ensure a 10^{-2} dilution, repeating the process for further dilutions up to 10^{-7}.

To count *S. aureus* colonies, 0.1 mL of each dilution was deposited onto plates containing Baird-Parker agar (Acumedia-BP) enriched with egg yolk and 0.1% tellurite (Laborclin) and incubated at 37 °C for 48 h. The colonies were then counted, and the colony-forming units (CFU) were recorded.

All experiments were conducted in triplicate.

2.5 Statistical analysis

All experiments were conducted in triplicate, with results presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was performed, followed by Tukey's honest significant difference post-hoc test to determine pairwise differences between groups.

3 Results and Discussion

3.1 Mechanical properties

Mechanical tests were conducted on ternary blend and pure polymer films. For the pure PVA film, a maximum tensile stress (σ_{max}) of 41.67 MPa, failure stress (σ) of 36.67 MPa, specific strain (ε) of 196.98%, and Young's modulus (E) of 7.35 MPa were recorded. These values are consistent with those found in existing studies (Costa-Júnior *et al.*, 2009). For the pure chitosan film, a Young's modulus of 13.26 MPa, σ_{max} of 38.42 MPa, σ of 22.63 MPa, and ε of 11.89% were obtained. This behavior indicates that the elasticity of chitosan surpasses that of PVA, thus increasing the material's tension before it transitions past its elastic behavior. For pure starch films, σ_{max} was 20.95 MPa, σ was 19.05 MPa, ε was 3.41%, and the Young's modulus was 11.83 MPa. The produced ternary films exhibited a linear range corresponding to the elastic phase of the material, as the successively linear region of the curve corresponds to uniform plastic deformation. The active film displayed a σ_{max} of 25.31 MPa, σ of 14.06 MPa, ε of 12.81%, and E of 13.184 MPa. PVA acts similarly to a plasticizer by reducing the intermolecular forces between starch chains, generally decreasing strength and increasing flexibility as the concentration increases. Comparisons to blends with commercial packaging polymer (i.e., PVC), which typically has a Young's modulus of 25–1600 MPa, suggest that the blends exhibit similar strain values. Ultimately, the produced blend demonstrates intermediate mechanical properties relative to the pure components.

3.2 Swelling index

Table 1 presents the swelling index values of the ternary films. All samples exhibited a common trend: an initial, continuous increase in swelling to a maximum level reached in 5 min, followed by a decrease due to saturation and the release of water-soluble components, a result of the solubilization of the samples. The swelling behavior is significantly influenced by the proportion of PVA in the blend, with swelling exceeding 700% in distilled water and over 1000% in a buffer solution during the first 10 min. The degree of swelling in the formed films decreases with the incorporation of a hydrophobic component.

Table 1 – Swelling index (%) for the ternary films

Time (Min)	**Swelling (%)**			**Mean /± STD. DEV.**
0.5^{a}	333.97	479.90	371.49	395.12 ±± 75.78
1^{b}	590.68	704.45	622.51	639.21 ±± 58.69
3^{c}	769.51	730.80	842.81	781.04 ±± 56.89
5^{cd}	818.17	783.19	887.20	829.52 ±± 52.92
7^{cd}	807.03	795.64	871.23	824.63 ±± 40.75
10^{c}	787.60	760.99	789.85	779.48 ±± 16.05
15^{c}	721.72	689.05	780.67	730.48 ±± 46.43
20^{c}	721.95	682.72	770.26	724.98 ±± 43.84

$^{a\text{-}d}$Different lowercase letters in the same column under the same antimicrobial agent indicate significant differences ($p < 0.05$). Data are shown in mean ± standard deviation. ($n = 3$).

Source: the authors.

The analysis of variance (ANOVA) indicated statistically significant differences in the degree of swelling as a function of time ($F = 22.95$; $p < 0.0001$). The mean swelling value at 0.5 min was significantly lower than at all other times (group “a”). The 1 -minute time point formed group “b,” showing intermediate values.

The times of 3, 10, 15, and 20 min did not differ significantly from each other (group “c”), while the times of 5 and 7 min, which presented the highest mean values, were classified into group “cd.” These results suggest that swelling reaches a plateau after approximately 3–5 minutes and remaining stable up to 20 minutes.

The large high F value, relative to the critical F, further supports that time has a real and substantial influence on swelling behavior, with the process reaching a plateau after approximately 3–5 minutes and remaining stable thereafter.

3.3 Evaluation of in vitro antibacterial activity of the hydroalcoholic extract of mate tea

The averages mean of the inhibition halos for different *I. paraguariensis* mate tea concentrations are detailed in Table 2. The different extract concentrations did not inhibit the growth of microorganism *E.scherichia coli* growth, although they despite inhibiting the growth of *Staphylococcus aureus* as shown in Table 2., which This result is in accordance with the results previously corroborates founded by Prado Martins et al. (2013) which reported no inhibition for *E.coli* and inhibitory zone of 23.67 ±0.57 mm for *S. aureaus*.

Table 2 – Inhibition halos (mm^2) of *I. paraguariensis* mate tea extract and results of microbiological evaluation of the ternary film

Microorganisms	**5%**	**10%**	**15%**	**Chloramphenicol**
S. Aureus	10.5^{b}	13.5^{a}	14^{a}	13.1^{a}
E. Coli	0	5.5	7	12.7
Films (N = 5)	***S. Aureus***			
Control Film	3.5 10^4 ±2.7 10^3 CFU/G			
Active Film	9.3 10^2 ±2.8 10^1 CFU/G			

*Inhibition of halo with the same letter does not have significant differences between them.
Source: the authors.

The antimicrobial activity of extracts was evaluated against *S. aureus* and *E. coli*, demonstrated to be activity against *S. aureus*, and t. The mean inhibition zone diameters for each treatment are presented in Table 2. Statistical analysis was perfcormnducted using one-way ANOVA followed by Tukey’s post hoc test at a 95% confidence level.

The results indicated that the 15% and 10% active extract formulations, aslong well asith the chloramphenicol control, were not significantly different from each other ($p > 0.05$), forming group A. In contrast, the 5% active extract showed a significantly lower inhibition zone ($p < 0.05$) and was placed into group B (Table 2). Therefore, the extracts iat 10% and the15% concentrations of 10 and 15% exhibited antimicrobial activity against *S. aureus* compared able to the control (chloramphenicol) and were further evaluated using the active film.

3.4 Effectiveness of the Ternary Active Film effectiveness

After 48 hours of incubation, active films were removed from meat samples infected with *S. aureus* and colonies were counted (n = 5). The results are shown in; Table 2). The final results were 3.5 × 10^4 CFU/g for the meat sample involved with the control film and only 9.3 × 10^2 CFU/g for the meat sample involved with active film. This is a two-log reduction of the order two logarithmic cycles and proves the effectiveness of the active film. Raw red meat is very highly susceptible to microbial growth and oxidative reactions. Moreover, as *Ilex. paraguariensis* is a plant rich in polyphenols (Cheng *et al.*, 2024), the incorporation of natural antioxidants into starch-based films not only acts to combats microbial activity but also protects the meat from oxidative damage. This contributes to enhancing the sensory characteristics and nutritional value of the preserved meat, while preventing microbial degradation. Rajapaksha and Shimizu (2021) have found similar results for starch films incorporating spent black tea extract, which demonstrated uniform dispersion and functional interactions that enhance the film’s protective qualities against bacterial and oxidative degradation (Rajapaksha & Shimizu, 2021). Thus, an active packaging film that preserves meat products for a longer duration while ensuring that they are chilled prior to consumption is highly desirable.

4 Conclusion

Active films display intermediate mechanical effectiveness that is hampered by a high swelling index. The results obtained in the study indicate that the alcoholic extract of mate tea exhibits antimicrobial activity relative to pathogenic bacteria of importance in food (*S. aureus*). Additionally, the antimicrobial activity is not reduced when the extract is incorporated into the polymeric matrix. Furthermore, combining *Ilex paraguariensis* extract with biopolymers can correspond to an alternative while developing antimicrobial active food packaging.

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