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Cassava-Starch-Based Films Supplemented with Propolis Extract: Physical, Chemical, and Microstructure Characterization

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Abstract: Considering the current trends in the development of biodegradable films and materials interacting with food packaging through the incorporation of active substances into the packaging material, the possibility of using propolis as a natural bioactive compound was evaluated in order to propose a bioactive packaging development technology. Cassava-starch-based films were supplemented with propolis extract (PE) at concentrations of 0, 30, and 60 g per 100 g of starch. The chemical profile and antioxidant and antimicrobial activities of the PE were evaluated. The effect of PE incorporation on the film's mechanical properties and the microstructure, the concentration of phenolic compounds, and the antioxidant activity were also evaluated. Artepelin C (10.957 mg/mL) was the highest compound identified in PE. The *S. aureus* was more susceptible to PE than *E. coli*. The PE incorporation into the cassava starch-based films improved their flexibility and extensibility while making them more homogeneous and less harsh. Cassava starch-based films include phenolic compounds and antioxidant activity from PE. In the present work, the developed film revealed its potential as active food packaging materials, reducing the number of synthetic antioxidants used for food preservation.

Keywords: green propolis; bioactive compound; antioxidant activity; active packging antimicrobial properties.

1. Introduction

Plastic films are used on large scales in food packaging because they can be produced in large quantities at low cost with good mechanical and barrier characteristics. However, the accumulation of synthetic plastics waste in the environment has driven the research into the development of biodegradable packaging as an environment-friendly strategy [1]. Moreover, biodegradable materials in plastics production only become viable if it is financially and functionally attractive compared to synthetic production routes. Agro-industrial raw materials can be used for the production of biofilms. The incorporation of bioactive compounds into the

films, such as the propolis extract, allows the elaboration of films to offer extra benefits when compared to conventional materials [2-4].

The strategy to control phytopathogens' growth during post-harvest of fruits is the use of active edible coatings [5]. Edible coatings are defined as a thin layer produced from proteins, polysaccharides, and/or lipids that cover food surfaces that act as a protective layer [6]. The choice of the material to be used in the films' formulation is crucial. This will depend on the interactions between the components of the material, which may react with the films' barrier and mechanical properties. Starch has been considered an excellent raw material for the production of polymers due to its ability to form a continuous matrix, low oxygen permeability, cyclic availability, low cost, and harmless to the environment and rapidly metabolized for soil microorganisms when disposed of in the environment [7-10].

Due to the need to avoid oxidative deterioration of packaged food, the interest in active packaging has increased [11]. Besides the conservation of food, this type of system can present additional functionality to support antioxidant substances. Thus, incorporating antioxidant compounds into biodegradable films would promote new ways to improve the safety and shelf-life of ready-to-eat foods [4,12,13]. A natural substance with a high potential to act as an additive in polymeric materials is the propolis extract [14-16].

Propolis, a natural resinous substance collected from plant resins by bees, has antibacterial, anti-fungal, anti-cancer, anti-inflammatory, antioxidant, antiviral, anesthetic, immunostimulant, and cytostatic effects [17-20]. Studies involving starch films with propolis extract (PE) as a source of bioactive compounds are still scarce in the literature. The present study's objective was to evaluate the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, the chemical profile, and the antioxidant activity of the Brazilian green propolis extract (PE), and the effect of supplementation of cassava starch-based films with PE.

2. Materials and Methods

2.1. Materials.

Caffeic acid (PubChem CID: 689043), p-coumaric acid (PubChem CID: 637542), 3,5-Di-O-caffeoylquinic acid (PubChem CID: 13604687), 4,5-Di-O-caffeoylquinic acid (PubChem CID: 6474309), aromadendrin (PubChem CID: 122850), drupanin (PubChem CID: 6440361), artepillin C (PubChem CID: 5472440), and baccharin (PubChem CID: 5358645) were acquired from Sigma Aldrich (St. Loius, MO, US).

Propolis was collected in the city of Nepomuceno (21º 12’ 17.79”S/45º 13’ 17.2” W) (Minas Gerais state, Brazil). Propolis extract (PE) was prepared, according to Bodini, Sobral, Favaro-Trindade, and Carvalho [14]. Briefly, a sample of 30 g of propolis was triturated with 100 mL of ethyl alcohol (80:20, etanol:water, v/v) under heating at 50 °C and stirring for 30 minutes. Then, the PE was then cooled, stored at 10 °C for 24 hours, and then filtered through a Whatman No. 4 filter paper.

2.2. Characterization of propolis extract (PE).

2.2.1. Antimicrobial activity.

The antimicrobial activity of the EEP was determined by the microdilution technique in 96-well microplates (NCCLS, 2003) against *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC25923). The initial microorganism suspensions were prepared

in saline solution (0.85%, w/v) with a turbidity of 0. 08-0.1 at 625 nm measured using a spectrophotometer. The initial suspension was diluted in Mueller Hinton Broth to the concentration of $5x10^5$ CFU/mL. An aliquot of the suspension (10 µL) was added to all wells with the PE (yielding a total of $5x10^3$ CFU/mL), and the plates were incubated at 36 ± 1 °C for 24 hours for further evaluation of cell viability. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of PE that shows no growth of microorganisms from an aliquot of the mixture (10 µL) inoculated on Mueller Hinton Broth incubated at 36 ± 1 °C for 24 hours, while minimum bactericidal concentration (MBC) was defined as the lowest concentration of PE that shows no growth of microorganisms from an aliquot of the mixture (20 µL) inoculated on Mueller Hinton medium at 36 ± 1 °C for 24 hours under anaerobic conditions.

2.2.2. Phenolic composition of propolis extract.

Chromatographic analysis of PE was performed using a High-Performance Liquid Chromatograph (HPLC) (Shimadzu LC-20AT Prominence Liquid Chromatograph) equipped with a diode array detector (HPLC-UV-DAD), automatic injector, oven, degasser, and quaternary pump. The Shim-Pack VP-ODS column (4,6 mm x 250 mm, a particle diameter of 5 µm) was used. The mobile phase consisted of a solution of methanol and 0.1 % formic acid in water. Elution was performed using a linear gradient from 25 to 100 % in 77 minutes at a flow rate of 0.8 mL. The temperature was maintained at 40 ºC, and the injection volume was 10 µL. The spectral data were collected at 275 nm, and identification was performed by comparison of retention times with those of standards. Patterns were used to quantify phenolic compounds using a calibration curve. The standards used were: gallic acid, caffeic acid, p-coumaric acid, aromadendrin, artepelin C, 3,5-dicafeoylquinic, 4,5-dicapheoquinquine, drupamine, and baccharin. The minimum detection limits were 0.12 µg/mL and 0.35 µg/mL.

2.3. Film development and characterization.

The filmogenic solutions were obtained by mixing 3 g of cassava starch and 100 mL of distilled water described by López *et al.* [21]. After the suspension was wholly dissolved, glycerol (20g/100 g of starch) was added, and the solution was heated at 70 °C and then cooled to 40 °C. Finally, PE in three concentrations (0, 30, and 60 g/100 g of starch) and ethyl alcohol (15 g/100 g of starch) were added to the mixture. The film solution was kept in an ultrasonic bath for 20 minutes to avoid bubble formation. 100 mL of the film-forming were then spread on each petri dish (15×15 cm^2) and dried in BOD chamber at40 ºC for 30 hours.

2.3.1. Film characterization.

The films were equilibrated at 23 ± 2 °C and 50% ± 10 relative humidity with a desiccator over 48 hours. The thickness of the films was determined using a digital micrometer (n=10) [22].

2.3.2. Mechanical properties.

The tensile strength, elongation at rupture, and Young's modulus were determined using a texture analyzer (Instron - Series 3367, Grove City, US) [23] (n=10). The films were cut into a piece of 10 cm x 1.5 cm and conditioned at 50 ± 10 % RH at 23 °C ± 2 for 48 hours. A 500 N load cell was used, and the tensile force was recorded during the extension of films to

0.2 mm/s until rupture. The tensile strength values, elongation at rupture, and Young's modulus were estimated from force deformation data.

2.3.3. Morphological properties.

Scanning electron microscopy analysis of the films was performed using an electron microscope. JSM-6610 series scanning electron microscope (Jeol®, Tokyo, Japão) equipped with EDS at 5 kV accelerating voltage. The films were placed on conductive carbon tape and sprayed with a thin layer of gold before the imaging.

2.4. Total phenolic content and antioxidant activity.

Total phenolic content and antioxidant activity of PE and films were realized. The extract from films was performed using a mixture of 11 mg and 6 mL of water heated to 50 °C for 50 minutes. 4 mL of ethyl alcohol (80%) was added, and the temperature was maintained for another 10 minutes with subsequent filtration.

Total phenolic content was realized as described by Al-Duais *et al.* [24] with adapted for micro volumes. Briefly, 20 µL of the extracts were mixed with 100 µL of the Folin-Ciocalteau reagent (10 %), and after 5 minutes, 75 µL of 4 % sodium carbonate (7.5 %) was added. After 40 minutes of the reaction, the absorbance was measured at 740 nm using a microplate reader Spectra-Max M3 (Molecular Devices, LLC, Sunnyvale, CA, US). The total content of phenolic compounds was expressed in gallic acid equivalent (GAE) calculated using a calibration curve (20 to 120 µg/mL).

The antioxidant activity was determined using the $ABTS^{\bullet+}$ scavenging method (2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)) as described by Al-Duais, Müller, Böhm, and Jetschke [24]. 220 µL of the $ABTS^{\bullet+}$ solution and 20 µL of Trolox or sample were mixed inside of each well of the microplate. Then, the plate was shaken and kept in the dark for 6 minutes. Absorption was measured at 734 nm using a microplate reader. The results are expressed in µmol Trolox equivalents calculated using a standard curve (12.5 to 200 µM).

2.5. Statistical analyses.

All tests were performed in triplicates with three replicates. Mechanical properties and thickness were carried out in ten replicates were run for each sample. Data are expressed as mean ± standard deviation, and statistical comparison between groups was carried out using analysis of variance (ANOVA) followed by Tukey's test ($p<0.05$).

3. Results and Discussion

3.1. Propolis extract characterization.

Table 1 shows the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of propolis extract. Although the PE showed activity against the two microorganisms tested, *S. aureus* was more susceptible to PE than *E. coli*.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of propolis extract (n=3).

Microorganisms		MIC (mg/mL)	MBC (mg/mL)
S. aureus (ATCC25923)	Gram-positive	0.017±0.00	0.161±0.00
E. coli (ATCC25922)	Gram-negative	0.161±0.00	1.288±0.00

These results corroborate what has been described in the literature [25,26], demonstrating propolis is more active against gram-positive than gram-negative bacteria [27,28]. Gram-negative bacteria have a chemically complex cell membrane. One of its constituents, the lipopolysaccharide, determines the antigenicity, toxicity, and pathogenicity of these microorganisms [29,30]. The bactericidal and bacteriostatic effects of propolis can result from the combined actions of cinnamic and flavonoid derivatives, increasing the permeability of the bacterial cell membrane by acting as ionophores, inhibiting the motility, and thereby contributing to the cytotoxic effect [28,31].

Figure 1 and Table 2 show the HPLC analysis of PE. The phenolic compounds identified were: caffeic acid, p-coumaric acid, 3,5-di-O-caffeoylquinic acid, 4,5-Di-O-caffeoylquinic acid, aromadendrin, drupanin, artepelin C, baccharin, and levels of prenylated phenolic acids, including artepelin C.

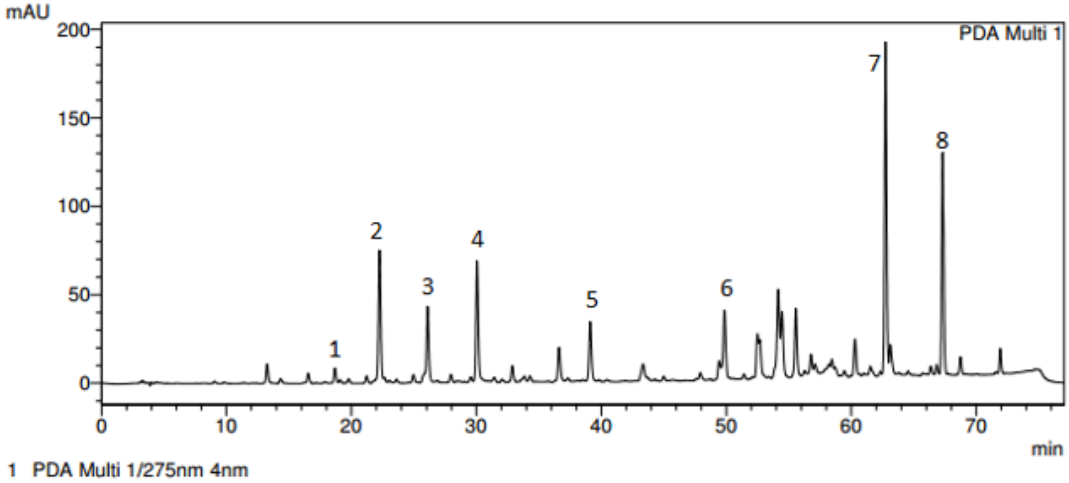

Figure 1. High-performance liquid chromatography (HPLC) of propolis extract detected at 2754 nm.

Table 2. Phenolic compounds of propolis extract determined using High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD).

Peak Number	Phenolic compound	Retention time (min)	mg/mL of PE
1	Caffeic acid	19	0.278
2	p-coumaric acid	23	1.494
3	3,5-dinitrocatechol	26	2.975
4	4,5-di-caffeoylquinic acid	30	4.761
5	Aromadendrin	39	1.623
6	Drupanin	50	2.017
7	Artepillin C	63	10.957
8	Baccharin	67	2.263

The main compound found in the present work was artepillin C (10.96 mg/mL), which had already been reported in the literature. The compound was present in greater quantity in Brazilian green propolis [32,33]. This is one of the most important biologically active compounds in Brazilian green propolis [34], previously reported for important biological activities including antitumor [35,36], immunomodulatory, and immunosuppressive effects [37], as well as induction of apoptosis and excellent scavenging of free radicals [38,39].

3.2. Characteristics of the cassava-based film supplemented with propolis extract.

3.2.1. Physical and morphologic characteristics.

Table 3 shows the thickness measurements and mechanical characteristics of the cassava-based films with propolis extract. There was no difference in the thickness after PE addition, indicating that the propolis extract did not alter the solids in the filmogenic solutions.

Table 3. Physical and morphologic characteristics of cassava-based film supplemented with propolis extract in the concentration of 0 (PE0), 30 (PE30), and 60 (PE60) g per100 g of starch.

Films	Thickness (mm)	Tensile strength (MPa)	Elongation at rupture (%)	Young's modulus (MPa)
PE0	0.07±0.00[a]	13.66±0.06[a]	1.61±0.03[c]	1196.21±52.71[a]
PE30	0.07±0.00[a]	6.47± 0.72[b]	20.58±2.21[b]	471.94±54.19[b]
PE60	0.07±0.00[a]	4.55± 0.33[b]	28.39±1.32[a]	277.41±7.41[c]

Different letters in the same column indicate a significant difference by the Tukey test (p>0.05).

When tensile strength and Young's modulus decreased with the addition of PE (concentration-dependent), elongation at rupture increased with the addition of PE. This increase in elongation at rupture resulted in more flexible films. We hypothesized that PE might have acted with plasticizing behavior due to its strong interaction with the starch's polymer matrix. The polar compounds of the PE may form molecular interactions with the hydroxyl groups, substituting the interactions previously formed by the starch molecules. This plasticizing effect of PE was related to biodegradable gelatin films with PE (5 to 200 g of PE/100 g of gelatin), resulting reduction in tensile strength and modulus of elasticity with an increase of PE concentration [14]. For applications such as packaging, tray coverings in food storage, or the like, the films developed in the present work can confer an advantage due to their high elongation capacity.

Figure 2 shows the surface micrographs of cassava-based film supplemented with propolis extract. The films' surface micrographs revealed the presence of insoluble particles in all treatments, characterized by residual starch dispersed in the film matrix. This indicates an incomplete dissolution of the starch molecules during the process. However, the amount of non-solubilized starch granules decreased on the surface of the films supplemented with PE.

The PE addition to the polymer matrix granted greater homogeneity to the films due to hydroxyl groups on the phenolic compounds present in PE. These hydroxyl groups form hydrogen bonds with hydroxyl groups of starch and, consequently, reduce the intermolecular interactions between the polymer chains, improving the film's homogeneity [40]. The PE concentration increase also made the film less harsh, reflecting the compounds present in the PE with the cassava starch.

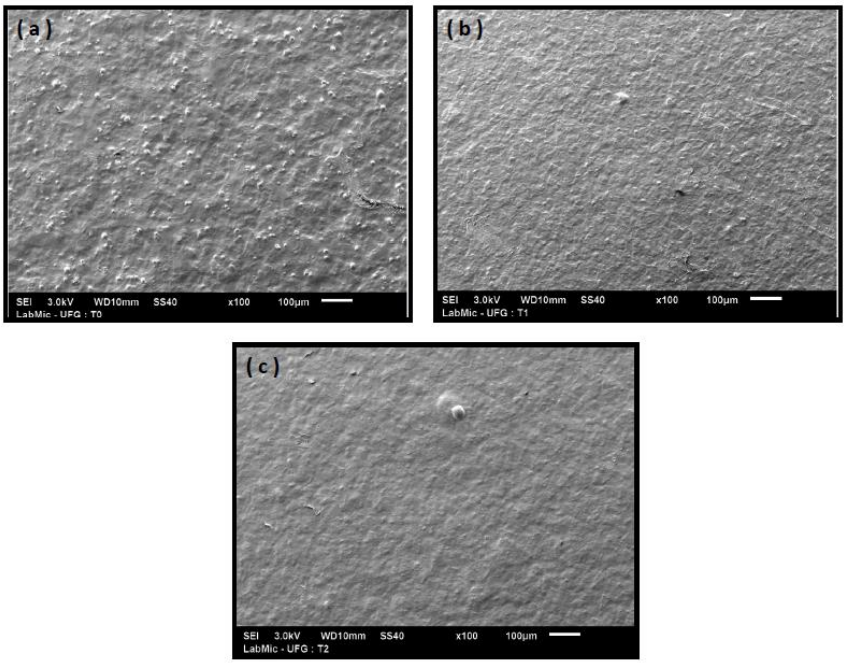

Figure 2. Surface image (100 x) cassava-based film supplemented with propolis extract: **(a)** PE0: film without propolis extract, **(b)** PE30: film with 30 g of propolis extract per 100 g of starch, and **(c)** PE60: film with 60 g of propolis extract per 100 g of starch.

In the present work, the film of matrix homogeneity results in diminishing the films' mechanical resistance, which was previously related by other authors [2,3]. Moreover, the films' maximum tensile strength decreased with the addition of PE and presented a significant difference in relation to the control (Table 3).

3.2.2. Total phenolic content (TPC) and antioxidant activity of cassava-based film supplemented with propolis extract.

Table 4 shows TPC and antioxidant activity of developed films. The propolis extract presented a TPC of ~39 mg GAE/g, close to that reported by da Silva *et al.* [41] for the propolis from Minas Gerais state (4.10 to 39.0 mg GAE/mL). The main components of propolis from São Paulo and Minas Gerais states are terpenoids and prenylated derivatives of p-coumaric acid [25], which also were found in the present study (Table 2).

Table 4. Total phenolic compounds and antioxidant activity of cassava-based film supplemented with propolis extract (PE) in the concentration of 0 (PE0), 30 (PE30), and 60 (PE60) g per100 g of starch.

Samples	Total phenolic compounds (mg GAE/g)	$ABTS^{\cdot+}$ (µmol TE/g)	$ABTS^{\cdot+}$ (mg TE/g)
PE	38.40 ± 0.36^{a}	845.21 ± 6.99^{a}	211.35 ± 0.4^{a}
PE0	0^{d}	0^{c}	0^{c}
PE30	4.18 ± 0.28^{c}	38.18 ± 1.33^{b}	9.55 ± 0.13^{b}
PE60	5.52 ± 0.21^{b}	38.62 ± 0.69^{b}	9.66 ± 0.60^{b}

GAE: gallic acid equivalent; TE = Trolox equivalent. Different letters in the same column indicate a significant difference by the Tukey test ($p>0.05$).

In the present work, propolis extract showed antioxidant activity close to propolis from Minas Gerais region (77.90 to 86.40 mg TE/mL using the ABTS method) [42] and propolis samples from the southern region of Brazil (0.29 to 1.24 µmol TE/mg using the ABTS method) [43]. Tiveron *et al.* [43] showed the high antioxidant activity of artepelin C when compared to other compounds such as caffeic acid, coumaric acid, and gallic acid also present in propolis extract. The highest concentrations of artepelin C already observed in natural products are those from the green propolis produced in the South and Southeast of Brazil, whose source is the plant species *Baccharis dracunculifolia* [44].

Although artepelin C is one of the main compounds responsible for the antioxidant activity, the compounds verified in the HPLC analysis such as caffeic acid and derivatives, aromadendrin, and baccharin also have proven antioxidant activities and therefore may also have been responsible for the antioxidant activity of the extract [45]. Although the total concentration of phenolic compounds or flavonoids is important for contributing to antioxidant activity, the chemical nature (molecule structure, presence, and nature of groups linked to the main molecule) and the presence of other compounds may also contribute total antioxidant activity of the sample as well [42].

The propolis extract contributed to the increase in TPC (dose-dependent) (4.18-5.52 mg GAE/g) and antioxidant activity (38.18-38.62 µmol TE/g) of cassava-based films. Our results are in the same range found by Zhao and Saldaña [46]. These authors produced films with potato by-products and demonstrated that when the potato peel concentration in the film is increased, the total phenolic content (0.3-6.1 mg GAE/g of the film) and antioxidant activity of the films (1.5-93.2 mg TE/g film using the ABTS method) is also increased. However, when gallic acid was added to the films, the film showed higher antioxidant activity (1.5-1974.0 mg TE/g of film using the ABTS method). Our results are in the same range found by these authors.

Due to the antioxidant properties found in cassava starch films supplemented with PE, it is expected that film to assist in the inhibition of lipid oxidation can occur in several types of foods and thereby help decrease the number of synthetic antioxidants used in food preservation.

4. Conclusions

The ethanolic extract of green propolis presented great potential for use as a bioactive compound with antioxidant properties in developing active packaging produced from cassava starch. The incorporation of the propolis extract into the starch biofilms resulted in homogeneous films, reflect on the compatibility of the compounds present in the propolis extract with the cassava starch. The films showed a decrease in mechanical strength and improved flexibility and extensibility, which can be appreciated by the packaging industry. Finally, according to the intended usage, the amount of propolis extract can be adjusted in complementary studies.

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Conflicts of Interest

The authors declare no conflict of interest.

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