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Development and characterization of wheat mill by-product films enriched with commercial rosemary extract

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ABSTRACT. This study aimed to develop and characterize an active film from a wheat mill by-product little explored, named Glue Flour (GF) enriched with a commercial rosemary extract (RE). First, RE was characterized by its antioxidant and antimicrobial properties. Subsequently, films were elaborated by the casting technique and characterized by the thickness, moisture content, solubility, mechanical and barrier properties, optical characteristics, and their bioactive activities. The RE concentrations tested in films were 1, 5, 10, and 20% ($v \cdot w_{water}^{-1}$). RE showed antioxidant potential and antimicrobial activity against *Staphylococcus aureus* and *Aspergillus brasiliensis*; however, it did not inhibit *Escherichia coli* growth. Film solubility and moisture were approximately 12 and 35%, respectively. The addition of RE weakened the films mechanical and barrier properties. As the color of RE is green, the films tended to this coloration, lost luminosity, and the opacity increase was proportional to the RE concentration. Low luminosity indicates better barrier properties against UV light. From Pearson's correlation test ($p < 0.05$), the films concentration of RE, antioxidant activity, and inhibition zone (IZ) demonstrated a positive and significant correlation. This study demonstrated a by-product and a commercial RE potential in developing active biodegradable films.

Keywords: agro-industrial by-product; antimicrobial activity; antioxidant activity; active packaging; biodegradable films.

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Introduction

Packaging plays a vital role in the handling and preservation of food products. Most materials currently used for packaging are derived from petrochemical sources, the use of which has increasingly been associated with environmental concerns. As a result, recent studies involving packaging development have focused on the use of natural polymers (Cazón, Velazquez, Ramírez, & Vázquez, 2017; Oliveira Filho et al., 2019).

An increase in demand for foods with a longer shelf life has encouraged the industry to develop new packaging systems (Abdollahzadeh, Nematollahi, & Hosseini, 2021). Food packaging no longer has a passive role in protecting and marketing food products. New concepts, such as active packaging (AP) and intelligent packaging (IP), have taken on an essential role, offering many innovative solutions for the extension of shelf life, as well as the maintenance, improvement, and verification of food quality and safety (Ganiari, Choulitoudi, & Oreopoulou, 2017). The market for both AP and IP is currently on the rise.

In this new context, biopolymer-based films that incorporate natural substances exhibiting antimicrobial activity for scientific and technological applications have generated a great amount of interest (Yong & Liu, 2021). Additives with antioxidant and antimicrobial properties, such as herbal and spice extracts, oils, and some agro-industrial by-products, are exciting alternatives for direct use in foods and food packaging (Ganiari et al., 2017; Göksen, Fabra, Ekiz, & López-Rubio, 2020). Several researchers have evaluated their potential for application in biodegradable films (Hanani, Yee, & Nor-Khaizura, 2019; Martínez, Castillo, Ros, & Nieto, 2019; Pieretti, Pinheiro, Scapim, Mikcha, & Madrona, 2019; Pluta-Kubica, Jamróz, Kawecka, Juszczak, & Krzyściak, 2020). According to Ziani, Fernández-Pan, Royo, and Maté (2009), the use of eco-friendly polymers with antimicrobial properties is on the rise as a result of the need to minimize the harmful effects of conventional materials on both the environment and human health.

Rosemary (*Rosmarinus officinalis*) extract (RE), considered a natural food preservative, because exhibits high levels of antioxidant and antimicrobial activity (Xie, VanAlstyne, Uhlir, & Yang, 2017). Although several

studies have explored the use of RE as a food additive, only a few have dealt with its application in food packaging materials (Piñeros-Hernandez, Medina-Jaramillo, López-Córdoba, & Goyanes, 2017). However, according to the knowledge of the authors, studies involving the potential of food-grade RE already available on the market and its application in biodegradable films are scarce. In view of the above, and in addition to the desirable organoleptic characteristics of RE and the ready availability of a commercial extract, RE was chosen for this study.

A complementary approach to the development of AP and IP is the use of underutilized natural resources, by-products, or waste from food production (Arifin, Adzahan, Abedin, & Lasik-Kurdyś, 2023). One such by-product is Glue flour (GF), a term used to denote wheat flour that does not meet required validation and the quality standards for the technical regulation of identity and quality and as a result is usually used in the glue manufacturing industry (Frantz, Paludo, Stutz, & Spier, 2019). Its high starch concentration (64.81%), as reported in a study by Peron-Schlosser, Carpiné, Jorge, and Spier (2021), makes it a promising alternative for use in biodegradable film production.

One particular challenge related to the development of such products involves the techniques used to measure antimicrobial activity in films. Although various protocols are available, few studies involving biodgradable films perform more than one. According to Abdollahzadeh et al. (2021), two of the most frequently used techniques are disk diffusion and cell viability. It is equally challenging to find studies that test antifungal activity in films.

Therefore, the aim of the current study was to evaluate the antimicrobial and antioxidant potential of commercially available RE and incorporate it into a little-explored film matrix, previously optimized by Peron-Schlosser et al. (2021), in order to develop active biodegradable films. In doing so, this study also contributes to current research involving antimicrobial analysis in films.

Material and methods

Materials and microorganisms

Commercial ethanolic RE (food grade) was kindly supplied by HEIDE Natural Ingredients (Pinhais, Paraná State, Brazil). Glue Flour (GF) was provided by a wheat mill located in Curitiba (Paraná State, Brazil). Sorbitol 70% (Dinâmica, São Paulo State, Brazil) was used as a plasticizer. Broth and Brain and Heart Infusion (BHI) Agar (Biobrás, Minas Gerais State, Brazil), Potato Dextrose Agar (PDA) (Kasvi, Spain), Muller Hilton Agar (MH) (Biobrás, Minas Gerais State, Brazil) were used for antimicrobial tests. *Escherichia coli* (LB 25922), *Staphylococcus aureus* (LB 25923), and *Aspergillus brasiliensis* (ATCC 16404) were provided by culture collection from laboratories in Curitiba (Paraná State, Brazil). Reagents for determining antioxidant activity were acquired from Sigma-Aldrich (St. Louis, USA).

Films preparation

Films were made using a modified form of the method described by Peron-Schlosser et al. (2021). An aqueous suspension with 8.0 ($w \cdot w^{-1}$) of GF and 4.0% ($w \cdot w^{-1}$) sorbitol 70% was stirred for 1 min., after which it was heated to 80°C and maintained under magnetic stirring for 10 min. in a stirring and heating plate (Fisatom, São Paulo State). Agitation was kept at approximately 200 rpm to prevent the formation of air bubbles. The resulting suspension was then cooled to 40°C, after which (based on previous tests and studies) 1, 5, 10, and 20% ($v \cdot w_{water}^{-1}$) of RE was added to the filmogenic suspension and spread on acrylic plates (90 x 10 mm). The films were subsequently dried in an air circulation oven at 35°C for 16 h. Before characterization, the films were removed from the plates and stored at 25°C for 7 days in a desiccator containing a saturated solution [magnesium nitrate ($MgNO_3)_2$] (58% relative humidity). A film to which no RE had been added was used as a control.

Antibacterial and antifungal activity of RE

The well diffusion method was used to evaluate the antibacterial activity of RE against *S. aureus* and *E. coli*. Before analysis, the microorganisms were cultured overnight in BHI agar at 35°C for 24 h. A bacterial suspension was then performed in 0.85% saline solution ($w \cdot v^{-1}$) and compared to turbidity using the 0.5 McFarland Standard (~10^8 $CFU \cdot mL^{-1}$). The pour plate technique was used to inoculate microorganisms. After agar solidification, agar wells (Ø 5 mm) were prepared, into which approximately 20 µL of undiluted RE and

antibiotic [chloramphenicol 3% ($w \cdot v^{-1}$) was used as positive control], were added. The plates were incubated at 35°C for 24 h. The resulting halo diameters were measured, and the inhibition zone (IZ) was calculated according to the difference between the external diameter (D_{ext}) formed by the extract activity under the microorganisms and the internal diameter (D_{int}) occupied by the extract. The results were expressed in mm. All tests were conducted in triplicate.

For antifungal activity, the *A. brasiliensis* inoculum (10^7 spores$\cdot mL^{-1}$) was prepared according to Spier, Woiciechowski, Vandenberghe, and Soccol (2006). PDA medium was prepared according to the instructions provided by the manufacturer, after which it was autoclaved 20 min. at 121°C. The medium was then cooled to approximately 50°C, at which point the RE was added and homogenized in the medium. The RE test concentrations used were 1, 5, and 10% ($v \cdot v^{-1}$). Approximately 10 mL of culture medium was placed on the Petri dishes (Ø 60 mm). After the agar solidified, 5 µL of *A. brasiliensis* inoculum was added to the center of the plates and then incubated at 28°C until radial growth was observed. Plates containing PDA without extract were used as a growth parameter. Antifungal activity was estimated by quantifying the colony diameter over several days. Each treatment, was performed in triplicate.

Phenolic compounds and antioxidant activity of RE and films

Total phenolic compounds (TPC) were obtained based on the reducing capacity of the Folin-Ciocalteau reagent, according to Singleton and Rossi (1965). Antioxidant activity was determined using through 2,2-diphenyl-1-picrilhydrazil (DPPH) according to the method described by Brand-Williams, Cuvelier, and Berset (1995), as well as 2,2-azinobis (3-ethyl benzothiazolin-6-sulphonic acid) (ABTS), according to the method proposed by Re et al. (1999). The FRAP method, which analyzes electron transfer based on the ability to reduce iron, was used as described by Benzie and Strain (1996). The RE used in the analysis was diluted in water 200 times for the DPPH method and 10 times for the ABTS, FRAP, and TPC methods. Extract solutions for film analysis were prepared according to Piñeros-Hernandez et al. (2017). Supernatant aliquots were collected, and free radical capture was evaluated using DPPH and ABTS.

Films characterization

Film thickness was determined using a digital micrometer (0.001 mm resolution) (Zaas Precision, Amatools, São Paulo State, Brazil). The mean thickness was obtained by averaging at least ten measurements repeated at different points on the film.

The moisture content of the films was determined using the gravimetric method. The samples were dried at 105°C for 24 h (Association of Official Agricultural Chemists [AOAC], 1998; Peron-Schlosser et al., 2021). Solubility in the water of the films was measured according to the method suggested by Gontard, Duchez, Cuq, and Guilbert (1994). Film WVP was evaluated using a modified form of the method E96/E96M-16 (American Society for Testing and Materials [ASTM], 2016).

Films color was performed using a colorimeter MiniScan XE Plus (HunterLab, Reston, VA, EUA) operating in the CIELab system (L*, a *, b*). Film opacity was determined using a UV-VIS spectrophotometer (Q898DPT, Quimis, São Paulo State, Brazil). Opacity was considered to be the ratio between film absorbance at 600 nm and film thickness (mm) (Nouraddini, Esmaiili, & Mohtarami, 2018).

Mechanical properties were evaluated using a slightly modified form of the standard method D882-18 (American Society for Testing and Materials [ASTM], 2018). A texture analyzer (Brookfield CT3, Brookfield Engineering, USA) equipped with the TA-DGA probe (Double Grip Assembly, Brookfield) was used.

Chemical interactions between film components were verified using FTIR. The analysis was conducted using an Alpha FTIR spectrometer (Bruker, USA) equipped with an Attenuated Total Reflectance (ATR) accessory with a zinc-selected crystal. The spectral range was 500-4000 cm^{-1}, and the resolution was 4 cm^{-1}.

The surface and cross-section morphology of the films were analyzed using a Scanning Electron Microscope (SEM) (JEOL, model JSM 6360-LV, Japan). Before analysis, the films were kept for 7 days in a silica-containing desiccator. Samples were then fractured using liquid nitrogen, fixed on supports with copper tape, and metalized with a thin gold layer (Balzers Union, model FL 9496). A voltage of 10 kV was used to analyze the images, with a magnification of 250x and 1500x.

Antimicrobial activity of the films

For the disc diffusion method, Petri dishes containing MH agar were inoculated on the surface with 0.1 mL of the bacterial suspension (~10^8 CFU$\cdot mL^{-1}$ - Antibacterial and antifungal activity of RE). The films were cut

into discs (Ø 2 cm), and sterilized for 1 h under UV light before being added to the plates. The plates were then incubated for 24 h at 35°C. Halo diameters were then measured and the IZ was calculated as explained in Antibacterial and antifungal activity of RE.

A viable cell count *in vitro* was performed using a slightly modified form of the method described by both Tsai, Yang, Ho, Tsai, and Mi (2018), and Zhang, Shu, Su, and Zhu (2018). Erlenmeyer flasks containing 50 mL of BHI broth and pieces of film (1 × 1 cm per 10 mL of the medium) were inoculated with 10^5-10^6 CFU·mL^{-1} of *S. aureus* and incubated in a shaker (TECNAL, model TE-421, Brazil) at 36°C, 100 rpm for 24 h, after which serial dilutions were prepared and 0.1 mL was distributed onto plates containing BHI agar. The plates were then incubated at 36°C for 24 h, and the cells were counted. Microbial counts were expressed in terms of colony-forming unit per mL (CFU·mL^{-1}). The percentage of cell growth inhibition was calculated according to Equation 1.

$$Inhibition(\%) = \left(1 - \frac{CFU_{sample}}{CFU_{blank}}\right) \times 100 \quad (1)$$

where:

CFU_{sample} represents the CFU·mL^{-1} incubated in the presence of film, and CFU_{blank} represents the CFU·mL^{-1} set in absence of film.

Antifungal activity was evaluated as described by Ziani et al. (2009). Inoculum preparation was carried out as described in Phenolic compounds and antioxidant activity of RE and films. *A. brasiliensis* inoculation was performed on the surface of plates with PDA agar, and 0.1 mL of spores were added to the dishes. Films containing RE were cut into squares (2x2 cm), sterilized for 1 h in UV light, and placed onto the plates. A plate containing no film was used as the control.

Statistical analysis

The results were analyzed using Statistica® 14.0.0.15 (1984-2020 TIBCO Software Inc.), and the means were compared using a Tukey test ($p < 0.05$). Pearson's correlation test ($p < 0.05$) was used to correlate the film properties.

Results and discussion

Antioxidant and antimicrobial activity of RE

Table 1 shows the results of the RE antioxidant and antibacterial tests. Due to its potent antioxidant an antimicrobial capacity, RE is a natural food preservative (Xie et al., 2017). These properties, in addition to its polyphenolic profile, have been described extensively in the scientific literature (Moreno, Scheyer, Romano, & Vojnov 2006; Nieto, Ros, & Castillo, 2018; Martínez et al., 2019). Both oil-soluble and water-soluble RE compounds can be used for different food applications (Xie et al., 2017). According to Aziz et al. (2021) the antioxidant properties of rosemary are a result of the compounds naturally present in its oils and extracts. The main constituents responsible for antioxidant activity are carnosic acid and carnosol. The abundance of phenolic hydroxyl groups in their structures allows these substances to capture free radicals by the donation of phenolic hydrogen atoms.

Table 1. Inhibition zone (IZ) from antibacterial activity of various dilutions of commercial RE against *S. aureus* in concentrations of 10^8 and 10^4 CFU·mL^{-1}, as well as its total phenolic compounds (TPC), and antioxidant activity as determined by DPPH, ABTS, and FRAP.

Antibacterial activity			Antioxidant activity	
Dilution*	Inhibition zone (mm)		Method	Quantification
	10^8 CFU·mL^{-1}	10^4 CFU·mL^{-1}		
100:0	9.00 ± 1.00^a	21.50 ± 1.05^a	DPPH (µmol TE g^{-1} dry extract)	3814.41 ± 46.90
80:20	8.50 ± 1.64^a	18.83 ± 0.75^b	ABTS ((µmol TE g^{-1} dry extract)	380.77 ± 2.01
50:50	6.20 ± 0.84^b	14.83 ± 1.83^c	FRAP (µmol TE g^{-1} dry extract)	1.92 ± 0.08
30:70	3.33 ± 0.52^c	8.33 ± 0.82^d	TPC (mg GAE dry extract^{-1})	123.45 ± 2.36

Average ± standard deviation; *(extract: water); TE: Trolox equivalent; GAE: gallic acid equivalent; dilutions of RE antioxidant analysis: 1:10 for ABTS, FRAP and TPC, and 1:200 for DPPH. Different lowercase letters in the same column indicate a significant difference between samples ($p < 0.05$).

Tests were conducted to evaluate antibacterial activity against two concentrations of *E. coli* and *S. aureus*, 10^8 and 10^4 CFU·mL^{-1} est. RE exhibited antibacterial activity only for *S. aureus*. Even when diluted to a ratio of 30:70 (extract: water), RE retained its antibacterial potential (Table 1).

The study conducted by Del Campo, Amiot, and Nguyen-The (2000) corroborates the results found in the present study. Commercial RE exhibited no antibacterial effect against the *E. coli* (gram-negative) bacteria. On the other hand, it inhibited the growth of *S. aureus* and other gram-positive bacteria such as *L. monocytogenes, B. cereus, L. mesenteroides,* and *S. mutans*. Moreno et al. (2006) evaluated the antimicrobial activity of RE obtained using various solvents. RE obtained using acetone and methanol solvents exhibited good inhibition for *E. coli,* whereas the water-based extract exhibited antimicrobial activity only for *S. aureus*. Therefore, the methanol extract exhibited a higher activity for *S. aureus* than for *E. coli*. Andrade, Ribeiro-Santos, Bonito, Saraiva, and Sanches-Silva (2018) observed antimicrobial activity of commercial RE powder against *S. aureus*, *L. monocytogenes*, *C. perfringens*, and *E. coli*. However, they also observed that RE did not exhibit any activity against *E. coli*. Martínez et al. (2019) also detected antimicrobial activity against these microorganisms for commercial RE enriched with rosmanic acid.

Rosemary contains rosmanic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol in its composition. These compounds are responsible for the inhibitory effect of RE. Changes in genetic material, nutrients, electron transport, and fatty acid production occur when these compounds interact with the cell membrane of microorganisms. Their interaction with the protein-membrane results in the loss and destruction of its functionality (Nieto et al., 2018).

The commercial RE evaluated in this study did not exhibit an antimicrobial effect against *E. coli*. This observation may be related to the difference in the cell wall of gram-positive and gram-negative bacteria. Gram-negative bacteria have an extra external membrane around the cell wall that restricts the diffusion of hydrophobic compounds through the lipopolysaccharides layer, making them more resistant to antimicrobial agents (Akhter, Masoodi, Wani, & Rather, 2019; Sarıcaoglu & Turhan, 2020).

One of the functions of the cell membrane of microorganisms is to maintain the stability of the intercellular solution and the regular activity necessary for the life of the cell. The mechanism of antimicrobial action in essential oils (EO) and plant extracts is usually an alteration in the plasma membrane that impairs its function, allowing intracellular substances to be exuded through the membrane walls (Zhang & Wang, 2019).

Figure 1 shows the monitoring and diameters of *A. brasiliensis* growth over 172 h in media containing 0 (control), 1, 5, and 10% RE. It may be observed that the fungus exhibits no growth for the 10% RE concentration. For the 5% extract, spore development was discontinuous. Comparison with the control medium reveals that the 1% RE concentration delayed fungus growth. After 24 h of incubation, the control medium exhibited a diameter of approximately 1.3 cm, whereas the medium with 1% RE exhibited a diameter of 0.9 cm. The medium containing 5 and 10% RE showed no growth during this same period. After 172 h, the medium with 10% RE did not exhibit *A. brasiliensis* development, whereas the control medium and media with 1 and 5% RE, exhibited diameters of approximately 4.5, 3.6, and 1.9 cm, respectively.

According to Özcan and Chalchat (2008), the efficacy of rosemary EO against fungal growth is probably due to the presence of substances such as timol, carvacrol, and menthol. Using techniques to determine minimum inhibitory concentration and fungicide, Bouddine et al. (2012) observed weak inhibition of rosemary EO against *A. niger*. In contrast, Ferdes, Al Juhaimi, Özcan, and Ghafoor (2017) observed antifungal activity for rosemary EO against *Aspergillus oryza*, *Aspergillus niger,* and *Fusarium oxysporum*. For Brito et al. (2015), the action mechanism of EOs against fungi is similar to the mechanism employed against bacteria. Moreover, both microscopic and macroscopic morphological changes can be observed in fungi. These alterations may be related to interference by the chemical constituents of the EO in the enzymes responsible for cell wall biosynthesis or maintenance, which affects fungal growth and morphogenesis.

Accordingly, in the assay with 5% RE it was possible to observe macroscopic morphological changes in *A. brasiliensis*, suggesting that the action mechanism of commercial RE against *A. brasiliensis* was similar to that of the EO reported by Brito et al. (2015).

Films characterization

Thickness, moisture, water solubility, and WVP

Thickness is an important parameter that affects the mechanical and barrier properties of films (Laureanti et al., 2021). The thickness of the films ranged from 0.159 (F1RE) to 0.211 mm (F5RE), the thickness of the control film (FCC) was 0.163 mm (Table 2). Although the masses of the filmogenic solutions added to the acrylic plates (Ø 15 cm) were all similar (~ 40±2 g), the thickness exhibited variations among the various formulations. According to Adilah, Jamilah, Noranizan, and Hanani (2018) such differences are to be expected

in the elaboration of films using casting technique. Thickness may be influenced by the mass added to the plate, the matrix components, and the drying conditions.

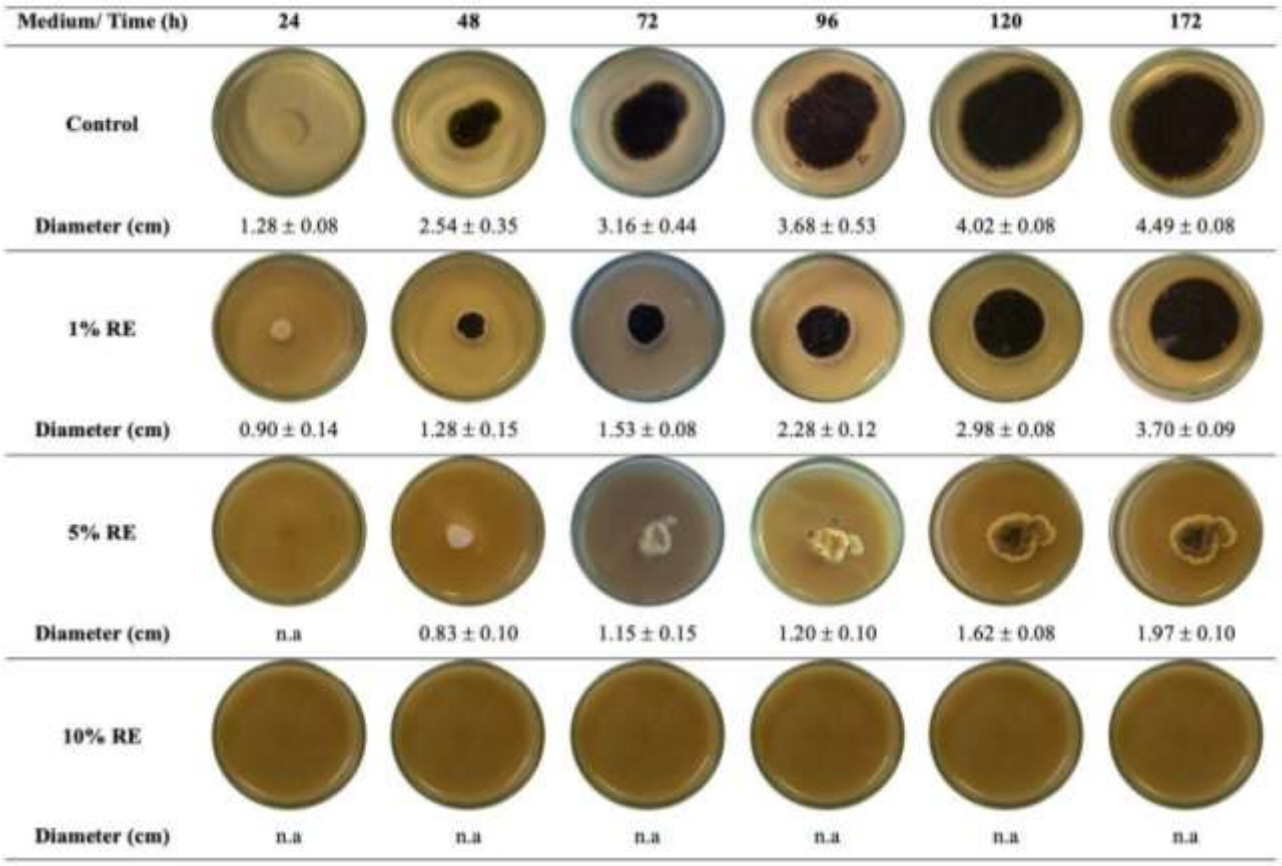

Figure 1. Stages and diameters of *A. brasiliensis* growth over 172 h in media containing 0, 1, 5, and 10% of a commercially available RE.

Table 2. Thickness, moisture, solubility in water, water vapor permeability (WVP) and mechanical properties of the control film (FCC) and the films with incorporated commercial RE.

Film	Thickness	Solubility	Moisture	WVP	TS	ELO
	(mm)	(%)	(%)	(x 10^{-10} g·m^{-1}·s^{-1}·Pa^{-1})	(MPa)	(%)
FCC	0.163±0.015^{b}	35.50±2.30^{a}	12.10±2.60^{a}	5.36±0.41^{c}	1.64±0.24^{b}	62.18±6.38^{ab}
F1RE	0.159±0.020^{b}	33.13±0.95^{a}	12.72±2.43^{a}	7.19±0.22^{b}	2.39±0.19^{a}	63.85±5.59^{bc}
F5RE	0.211±0.015^{a}	34.31±2.34^{a}	12.61±2.28^{a}	8.74±0.41^{a}	1.57±0.14^{b}	76.58±7.51^{a}
F10RE	0.199±0.016^{a}	35.00±2.40^{a}	11.63±1.08^{a}	7.29±0.01^{b}	1.56±0.11^{b}	39.92±3.35^{d}
F20RE	0.190±0.018^{a}	33.21±0.22^{a}	11.57±2.13^{a}	6.78±0.16^{b}	1.08±0.07^{c}	55.42±7.08^{c}

Average ± standard deviation; Different lowercase letters in the same column indicate a significant difference between samples ($p < 0.05$).

Studies in the literature involving films with added plant extracts reported thicknesses similar to those found in the current study. Films based on cassava starch with added yerba mate (*Ilex paraguariensis*) extract exhibited thicknesses of approximately 0.25 mm (Jaramillo, Gutiérrez, Goyanes, Bernal, & Famá, 2016). Piñeros-Hernandez et al. (2017) made films from cassava starch with added RE whose thicknesses were approximately 0.20 mm. An increase in film thickness with the incorporation of plant extracts into the polymer matrix was observed by Adilah et al. (2018) in films with added mango bark extract, and by Laureanti et al. (2021) in cassava starch films with added pink pepper extract.

Film moisture and solubility are shown in Table 2. The presence of RE did not significantly ($p > 0.05$) affect film moisture and solubility as compared to the control. The values obtained for moisture and solubility were approximately 12 and 35%, respectively. It may be observed that the film with 20% RE exhibited lower solubility, although there was no significant difference as compared to the control film. Other studies (Adilah et al., 2018; Laureanti et al., 2021) also demonstrated that the incorporation of plant extracts into films reduced solubility. This behavior may be related to intermolecular interactions between the starch network and polyphenolic compounds. These interactions reduce the availability of hydrogen bonds and the formation of hydrophilic bonds with water, which in turn reduces affinity with water.

Mir, Dar, Wani, and Shah (2018) reported significant reductions in water barrier properties when plant extracts were added to biopolymer films. The same may be observed in the present study (Table 2). The lowest permeability (5.36 x 10^{-10} g·m^{-1}·s^{-1}·Pa^{-1}) was observed for the FCC, which differed statistically ($p < 0.05$) from the others. The WVP of F1RE, F10RE, and F20RE exhibited no significant ($p > 0.05$) differences. Moreover, the F5RE, which had a higher WVP, differed statistically ($p < 0.05$) from the others.

Optical properties

The addition of plant extracts to films normally alters their color properties. Figure 2 contains images of the films placed above a surface upon which the word 'Transparent' is written to prove the film transparency. As with the films obtained by Piñeros-Hernandez et al. (2017), all films were transparent enough to be used as see-through packaging. As expected, the films exhibited greenish color. The increase in color intensity was directly proportional to the RE concentration, and differences were observable to the naked eye.

The results of the color analysis are shown in Table 3. All films tended toward green (a* negative) and yellow (b* positive) coloration. Green tones (with variations in brightness) are seen in all films (Figure 2). According to Mir et al. (2018), plant extracts are a rich source of polyphenols, which leads to various types of interactions with biopolymers, resulting in a variety of color properties. An increase in RE concentration in films results in a decrease in L* parameter values, thus indicating a reduction in film luminosity. The films exhibited a significant difference ($p < 0.05$) for the parameters L*, a*, and b*.

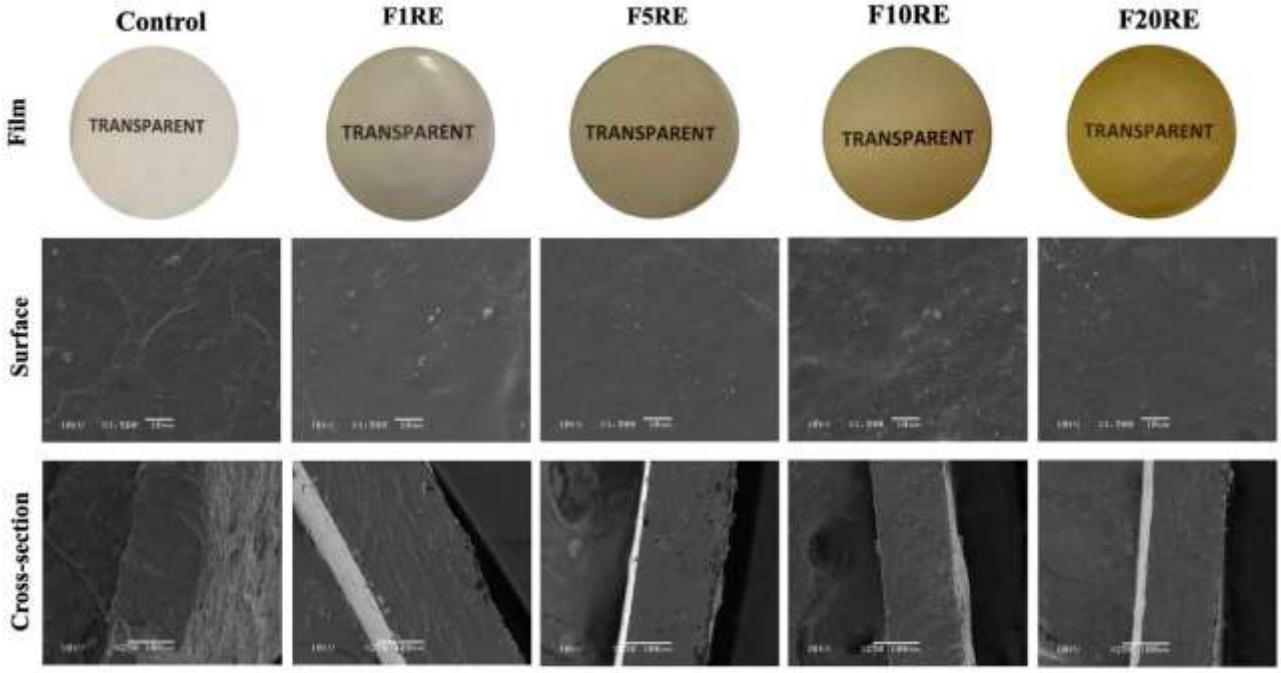

Figure 2. Images from control and the RE films on a surface upon which is written 'Transparent'. Scanning electron micrographs from the surface (1500 x) and cross-section (250 x) of the control film and films with RE.

Table 3. Color parameters (L*, a*, b*, ΔE*) and opacity of the control film (FCC) and the films with incorporated commercial RE.

Film	L*	a*	b*	ΔE*	Opacity (A_{600})
FCC	89.87 ± 0.14^{a}	-1.39 ± 0.06^{b}	6.05 ± 0.23^{e}	-	1.99 ± 0.10^{b}
F1RE	85.89 ± 3.17^{a}	-1.55 ± 0.11^{b}	14.24 ± 2.14^{d}	9.49 ± 1.21^{d}	2.07 ± 0.23^{b}
F5RE	78.92 ± 1.53^{b}	-2.16 ± 0.28^{a}	25.73 ± 2.38^{c}	22.54 ± 2.86^{c}	2.17 ± 0.01^{b}
F10RE	75.15 ± 0.94^{b,c}	-1.82 ± 0.22^{b,a}	32.69 ± 1.01^{b}	30.45 ± 1.08^{b}	2.33 ± 0.04^{b}
F20RE	70.94 ± 0.40^{c}	-0.56 ± 0.12^{c}	38.69 ± 0.52^{a}	35.75 ± 0.50^{a}	4.18 ± 0.18^{a}

Means with different lowercase letters are significantly different as determined by a Tukey HSD test ($p < 0.05$).

Film opacity was also affected by the addition of RE (Table 3). F20RE exhibited higher opacity and differed statistically ($p < 0.05$) from the other formulations. Adilah et al. (2018) also reported an increase in film opacity when mango bark extract was added. Packaging films should protect food, especially from UV radiation. The incorporation of plant extracts in films provides an adequate barrier that is essential to prevent the degradation of light-sensitive components (Mir et al., 2018). Moreover, opaque films can be used for

products that do not require transparent packaging to convince consumers to buy them. Films produced with added RE may be ideal for products such as oils in order to avoid oxidation.

Mechanical properties

Mechanical properties are affected by a variety of factors: thickness, film formation technique, compatibility among additives, chemical composition, and cohesive structure (Laureanti et al., 2021). The mechanical properties (TS and ELO) of the FCC and RE films are shown in Table 2. The TS of the films ranged from 1.08 (F20RE) to 2.39 MPa (F1RE). From the FCC, there was an increase, and subsequent decrease in value as RE was added. The Tukey test revealed that the TS of the F1RE exhibited a significant difference in value ($p < 0.05$) as compared to the other formulations. The FCC exhibited TS values that were statistically similar ($p < 0.05$) to those of F5RE and F10RE. The TS value for F20RE was lower and significant different ($p < 0.05$) than the other formulations.

Elongation of films with incorporated RE ranged from 36.92 (F10RE) to 79.69% (F5RE), except for F5RE ($p < 0.05$), which also exhibited a decrease as compared to FCC. Other studies corroborate these results. Yan, Zhang, Dong, Hou, and Guo (2013), who worked with starch and sodium alginate films, observed that TS and ELO values decreased as RE concentration increased. Laureanti et al. (2021) observed a decrease of approximately 75% in TS of films when pink pepper extract was added. Wang, Wang, Tong, and Zhou (2017), who made films from chitosan with incorporated honeysuckle flower extract, also observed a decrease in mechanical properties when the extract concentration was increased from 0 to 30%.

Piñeros-Hernandez et al. (2017) observed a statistically significant reduction ($p < 0.05$) of 60% in the ELO of films with RE as compared to starch films. The authors attributed this behavior to a heterogeneous structure and the weakening of glycerol-starch interactions due to the presence of RE. Pluta-Kubica et al. (2020) observed a reduction in elongation after yerba mate extract was added to the films. They stated that film mobility was possibly reduced due to stronger interactions between polyphenolic compounds and the polymer chain.

In light of the above, the presence of RE as an additive probably led to the development of a heterogeneous structure in the films, resulting in decreased TS and ELO. Moreover, according to Yan et al. (2013), RE can weaken the strong intermolecular interaction between polymers in composite films. Furthermore, the density of intermolecular interaction decreases in the materials while the free volume between the polymer chains increases, resulting in the wear of mechanical properties.

Identification of functional groups and morphology

ATR-FTIR spectra of the control film (FCC) and films with incorporated RE are shown in Figure 3. All films exhibited bands at 3280 (O-H stretching), 2923 (C-H of alkyl groups), and 1149 cm^{-1} (C-O stretching), which are characteristic of starch (Pavia, Lampman, Kriz, & Vyvyan, 2010). The presence of hydroxyls that interact with hydrogen bonds is observed at 3280 cm^{-1}. This band can be attributed to the interactions between GF proteins and starch, water, and plasticizer (sorbitol) (Peron-Schlosser et al., 2021).

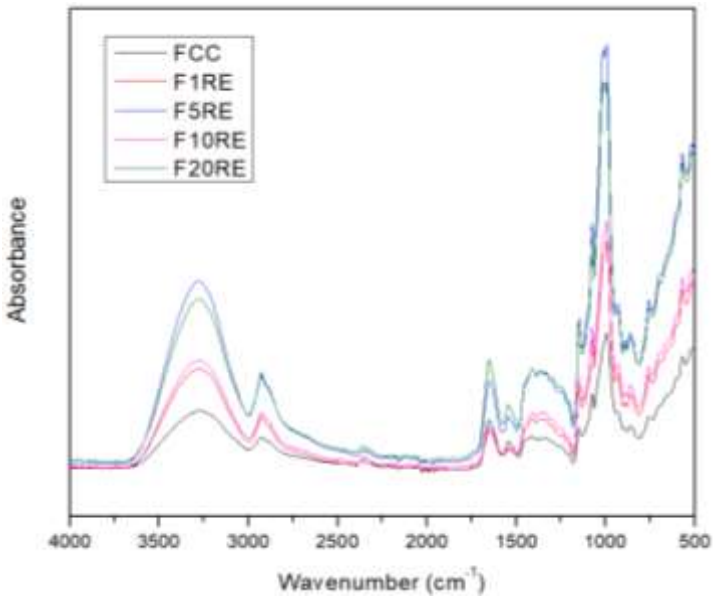

Figure 3. ATR-FTIR spectra of the control film and films with RE.

According to Orsuwan and Sothornvit (2018) bands at ~1654 cm^{-1} suggest the presence of water molecules bound to starch. As reported by Peron-Schlosser et al. (2021), the protein content of GF may be responsible for the bands at the 1640-1550 cm^{-1} (N-H primary and secondary amines and amides) and 1350-1000 cm^{-1} ranges (can be C-N amines) (Pavia et al., 2010). The band at ~1150 cm^{-1} may be associated with the starch hydrogen bonds, and the one at ~927 cm^{-1} is characteristic of glycosidic bonds (Sanches et al., 2021). The interaction of the starch present in GF with the sorbitol plasticizer is probably responsible for the band observed at ~1074 cm^{-1} (Jafarzadeh, Alias, Ariffin, & Mahmud, 2018).

Spectral profiles of films exhibited analogous absorption bands. Both the control film and the films with RE showed the same functional groups. In conclusion, the incorporation of RE into the matrix of the films did not generate new functional groups.

Figure 2 shows the micrograph (surface and cross-section) of the control film and the films with RE. From the micrographs, it may be observed that the films presented a cohesive surface; therefore, there was a positive interaction among the components of the film matrix. It is possible to observe that the control film exhibited a rough surface and some cracks. The other formulations also exhibited a rough surface and cavities in the cross-section images. The roughness on the film surface may be associated with GF particles that did not undergo complete gelatinization. This feature may also be related to the GF fibers and polyphenols present in the extract, which can migrate to the surface during the drying step.

Antioxidant and antimicrobial activity of films

The antioxidant activity of the films is shown in Table 4. As expected, a significant increase ($p < 0.05$) was observed in antioxidant capacity as measured by DPPH and ABTS, which was directly proportional to the increase in RE concentration in the films. It may be seen that when compared to FCC, F20R exhibited an antioxidant activity that was 89 times higher with regard to the DPPH radical and 24 times higher with regard to the ABTS radical. The differences among the antioxidant activities of the films with regard to the ABTS radical were statistically different ($p < 0.05$). Upon analysis using DPPH, the FCC control sample and the sample with 1% extract (F1RE) exhibited no significant difference ($p > 0.05$) when compared to each other. However, they did differ ($p < 0.05$) from the other formulations.

Piñeros-Hernandez et al. (2017) prepared films from cassava starch with the addition of 5, 10 and 20% RE. In their evaluation of the composition of total phenolics and antioxidant activity of the films using DPPH, these authors observed the same results that were obtained in the present study: increased RE concentration in the films resulted in increased phenolic compounds content and the antioxidant activity in the samples. Kurek et al. (2018), who developed chitosan films with added blackberry and blueberry bagasse extracts, observed a significant increase in antioxidant activity in samples containing the added extracts. Laureanti et al. (2021), who developed cassava starch films with added pink pepper extract, observed the same increase in antioxidant activity. In an acid medium, cassava starch films with 20% incorporated yerba mate extract exhibited an antioxidant activity of 15.8 μmol TE·g^{-1} (Ceballos, Ochoa-Yepes, Goyanes, Bernal, & Famá, 2020). This value was similar to that obtained for the F10RE in the present study.

Plant extracts represent a promising additive for biodegradable food packaging materials, mainly due to their natural origin and their being a source of antioxidant. It is known that free radicals can cause oxidative degradation of food, in addition to a loss of nutritional quality. In view of the above, one of the most promising applications of active packaging is its use in curtailing oxidative processes (Domínguez et al., 2018). Therefore, these antioxidant films with free radical scavenging activity may potentially be applied in active packaging of food such as meat, fish, and oils (López-Córdoba, Medina-Jaramillo, Piñeros-Hernandez, & Goyanes, 2017).

The antibacterial activity of the films against S. aureus was evaluated at concentrations of 108 and 104 CFU·mL^{-1} (Figure 4A). Table 4 shows the IZ (mm) obtained for each film with both microbial concentrations. It may be seen that F5RE, F10RE, and F20RE exhibited antimicrobial activity against S. aureus at concentration of 104 CFU·mL^{-1}. On the other hand, only the F20RE formulation exhibited antimicrobial activity (IZ: 0.92 mm) at 108 CFU·mL^{-1}. As expected, the same effect observed in Table 1, in the evaluation of diluted extract occurred in the films. The increase in RE concentration in the formulations was directly proportional to the antimicrobial efficacy and IZ. The IZ of F20RE for 104 CFU·mL^{-1} was 1.51 and 1.22 times greater than that of F5RE and F10RE, respectively.

Table 4. Antioxidant properties, and inhibition zone (IZ) from the antibacterial activity against *S. aureus* for the control film (FCC) and the films with incorporated commercial RE.

Film	ABTS	DPPH	IZ (mm)	IZ (mm)
	(μmol TE·g dry extract^{-1})	(μmol TE·g dry extract^{-1})	10^8 CFU·mL^{-1}	10^4 CFU·mL^{-1}
FCC	2.10±0.15^e	0.27±0.06^d	-	-
F1RE	6.12±2.08^d	0.44±0.10^d	-	-
F5RE	13.91±1.84^c	5.55±0.16^c	-	4.50±0.71^b
F10RE	28.78±0.80^b	13.24±1.68^b	-	5.25±0.61^b
F20RE	50.92±1.49^a	24.01±0.47^a	0.92±0.20	6.42±0.38^a

Average ± standard deviation; TE: Trolox equivalent; Different lowercase letters in the same column indicate a significant difference between samples ($p < 0.05$).

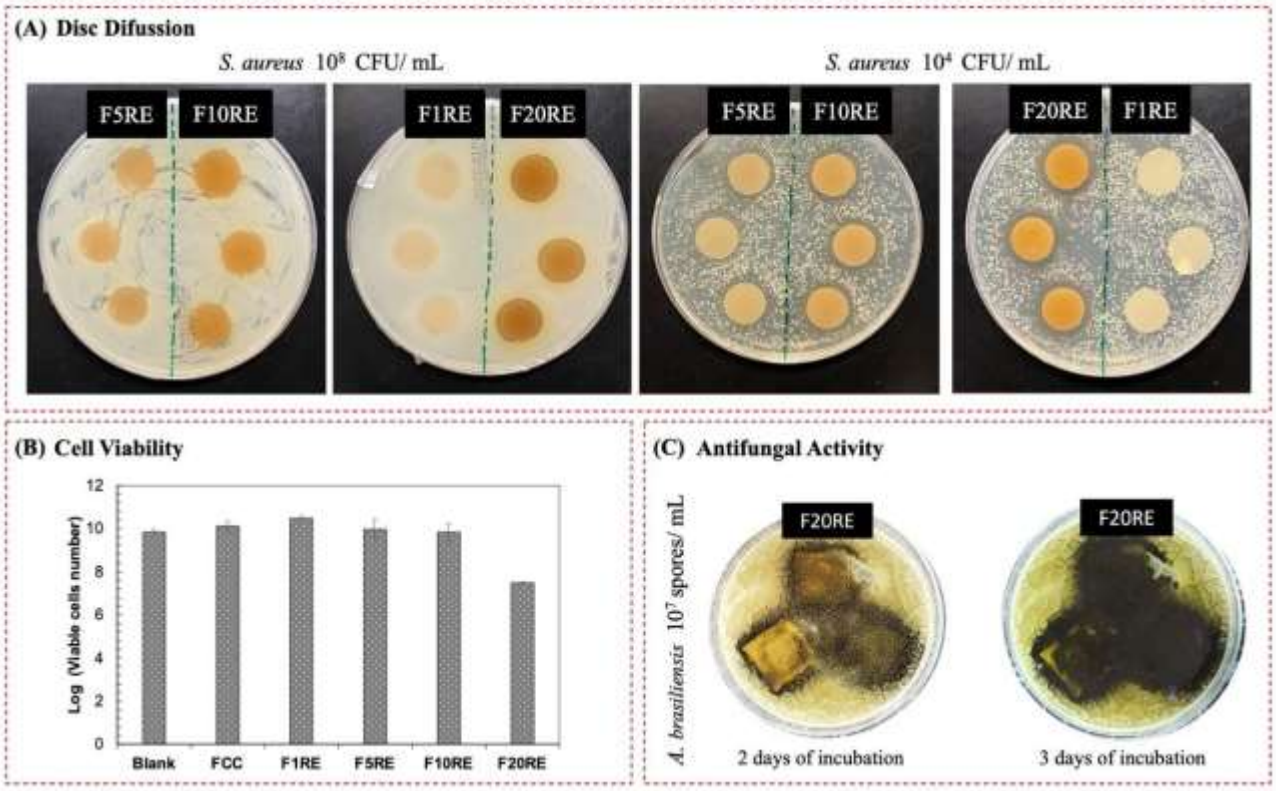

Figure 4. Antibacterial activity against *S. aureus* of the films with RE by (A) disc diffusion and (B) cell viability method, as well as (C) antifungal activity assay against *A. brasiliensis* of the film with 20% RE.

Andrade et al. (2018) developed films from soy protein isolate with 3 and 5% RE and evaluated the antimicrobial potential against *S. aureus* and *L. monocytogenes*. Active films were effective for both microorganisms as compared to the control (without extract). These results corroborate the observations of the current study. Yan et al. (2013) obtained films with incorporated RE that exhibited activity against *E. coli*. With results similar to those from the present study, they also observed that the IZ was proportional to extract concentration. Films made with rosemary OE have exhibited high levels of inhibition activity against gram-positive bacteria (*B. subtilis, S. aureus, L. monocytogenes*), which was attributed mainly to phenolic diterpenes in rosemary OE (Sarıcaoglu & Turhan, 2020).

Therefore, the results obtained in the present study show that films with 20, 10, and 5% RE may protect food from contamination by *S. aureus*. Table 5 presents Pearson's correlations between the RE concentration, antioxidant activities, and IZ from the antibacterial activity. The RE concentration showed a positive correlation with antioxidant and IZ from antibacterial activity, indicating that an increase in antioxidant activity and IZ is associated with an increase in RE concentration. Moreover, antioxidant activities as determined by DPPH and ABTS exhibited a positive correlation with IZ from antibacterial activities; that is, antimicrobial activities are directly related to antioxidant potential, and vice versa. Films with high antioxidant potential may also exhibit high antimicrobial potential.

The antibacterial properties of the films were also evaluated quantitatively using the cell viability method. FCC, F1RE, F5RE, and F10RE did not exhibit any antibacterial activity, which may be seen from the change in the number of viable bacteria in Figure 4B. However, the bacterial population of the F20RE was significantly

lower than the others. A 2-log reduction (*S. aureus*) in viable bacteria was observed within 24 h of exposure to F20RE. The IZ of the F20RE against *S. aureus* was 99.53%. Although this study demonstrated that commercial RE exhibits antifungal activity against *A. brasiliensis* (Antioxidant and antimicrobial activity of RE), the films prepared with RE were not capable of inhibiting the growth of *A. brasiliensis* (Figure 4C).

Table 5. Pearson's correlation between RE concentration, antioxidant activity, and inhibition zone for 10^8 UFC mL^{-1} and 10^4 UFC mL^{-1} of *S. aureus* for the films.

Parameters	RE	DPPH	ABTS	IZ 10^8	IZ 10^4
RE	-	0.997*	0.998*	0.876**	0.891*
DPPH	0.997*	-	0.998*	0.851**	0.899*
ABTS	0.998*	0.998*	-	0.859**	0.890*
IZ 10^8	0.876**	0.851**	0.859**	-	0.602
IZ 10^4	0.891*	0.899*	0.890*	0.602	-

RE: rosemary extract concentration (% $v \cdot w_{water}^{-1}$); *($p < 0.05$); **($p < 0.10$).

Conclusion

This study developed active films with RE, which altered their optical properties, making them tend toward green and reducing their luminosity, thus increasing their capability to block UV radiation, but also weakening their mechanical and barrier properties. This suggests potential application of RE as an antioxidant and antimicrobial agent in the development of films that protect food from contamination by *S. aureus*. Future industrial applications should focus on F5RE, which exhibits superior active and mechanical properties and lower production costs than F10RE and F20RE. Further studies should explore food applications (fruits and vegetables) to validate the potential of the film.

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