PAPER

ANTIMICROBIAL ACTIVITY STUDIES AND CHARACTERIZATION OF CELLULOSE ACETATE FILMS CONTAINING ESSENTIAL OILS

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ABSTRACT

This work aimed to evaluate the antimicrobial effectiveness of a biodegradable composite, cellulose acetate film with the incorporation of various combinations of cinnamon, oregano and sweet fennel essential oils (EOs) against *Escherichia coli*, *Staphylococcus aureus* and *Penicillium* spp. Moreover, thickness, the mechanical and water vapor barrier properties of these films were evaluated. The films with the incorporation of pure oregano EO and the combination oregano + cinnamon EOs presented the best results against all microorganisms tested. The water vapor transmission rate of the film was reduced with the addition of the EOs and the thickness of the film increased. The EO increased the flexibility of the films, and the film with pure oregano EO was the most resistant, showed 30,9 N of puncture strength. The results suggest that the EOs incorporated as natural antimicrobial agents can potentially be used in food packaging, reducing the risk of microbial contamination of food.

Keywords: active packaging, cinnamon, food packaging, food safety, oregano, sweet fennel

1. INTRODUCTION

Microorganisms may be responsible for a reduction in food quality, reducing shelf-life and causing economic losses. In addition, contaminated food can cause toxic infections. Microbial counts on fresh or processed solid or semi-solid foods are usually higher on the surface of these foods; therefore these surfaces require effective protection (MELO, 2003).
Thus, the development of materials that can serve as a protective barrier for food associated with other techniques, such as selection and control of raw materials, use of good manufacturing practices, and the use of additives that have been appropriately tested are of fundamental importance to ensure food safety, being able to provide consumers with guarantee of quality and safe food for consumption (DEVLIEGHERE *et al*., 2004; MASTROMATTEO *et al*., 2010).
The materials used for the production of active packaging include different biopolymer matrices, such as proteins, lipids and polysaccharides (MCHUGH *et al*., 1994; CHINNAN and PARK, 1995; ROJAS-GRAU *et al*., 2007; ATARÉS *et al*., 2010a). Such packaging must be environmentally acceptable, suitable for a wide range of food products and allow the incorporation of additives, including antimicrobial agents (ROSSI-MÁRQUEZ *et al*., 2009; SHOJAEE-ALIABADI *et al*., 2013).
The use of active antimicrobial materials for food packaging suggest that the product may have lower concentrations of preservatives or even be free of it, since the packaging with its additives will carry out this function. Thus, the consumer will have access to more natural products especially if the preservative used is also a natural product, such an essential oil (NAIDU, 2000).
Essential oils (EOs) can be used in the development of materials for antimicrobial active packaging because they have an excellent potential to act as a natural microbiological barrier (LAMBERT *et al*., 2001; ABDOLLAHZADEH *et al*., 2014). Several EO have been tested, such as: oregano, cinnamon, thyme, clove, satureja hortensis, lemongrass, ginger, anise, turmeric, guava leaf, nutmeg and lime (ROJAS-GRAU *et al*., 2007; ROSSI-MÁRQUEZ *et al*., 2009; ATARÉS *et al*., 2010a; MATAN, 2012; MANSO *et al*., 2011).
EOs are liquid, volatile, and rich in phenolic compounds. They can be synthesized by aromatic plants as their secondary metabolites (KAUL *et al*., 2003; RODRÍGUEZ *et al*., 2007). EOs have been widely used for their antibacterial, fungicidal, antiparasitic, insecticidal and medicinal applications (BAKKALI *et al*., 2008). In addition, EOs are approved for direct contact with food because they are considered to be GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (BURT, 2004).
EOs (each containing several components) when mixed in various combinations, may produce a synergic effect. This synergistic effect can increase the antimicrobial activity (ROMANO *et al*., 2009; GIBRIEL *et al*., 2013).
Antimicrobial active packaging must meet certain requirements such as: be effective against a large spectrum of microorganisms, show efficiency at low concentrations, not cause undesirable changes in the sensory characteristics of the product, and must be in accordance with the current legislation (SOARES and GONÇALVES, 2008).
The analysis of antimicrobial properties is important for predicting the behavior of antimicrobial films in food systems. Thus, this study aimed to evaluate the *in vitro* antimicrobial activity of cellulose acetate film incorporated with essential oils of cinnamon, sweet fennel and oregano, that are natural antimicrobial agents, against bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungus (*Penicillium* spp). The thickness, the mechanical and water vapor barrier proprieties in this cellulose acetate film were also analyzed for characterization them.

2. MATERIALS AND METHODS

2.1. Essential oils

Three EOs of plants were used: oregano, sweet fennel and cinnamom. The oils were acquired commercially from ®Ferquima (Ferquima Indústria e Comércio Ltda, Vargem Grande paulista, São Paulo-Br). In Table 1 the common name, plant variety, major components, and part of the plant material used for the extraction of the chosen EOs are presented. All selected EOs were produced by distillation and kept at room temperature in an amber glass bottle.

Table 1: Description of common name, plant variety, major components and part plant used to obtaining essential oils according to the supplier.

Common name	Plant variety	Major components	Part plant
Cinnamon	*Cinnamomum zeylanicum* Blume	cinnamaldehyde	Leaves
Sweet fennel	*Foeniculum vulgare* var. dulce (Mill.) Batt. & Trab.	Anetolhole	Seeds
Oregano	*Origanum vulgare* (L.)	Carvacrol	Leaves

2.2. Microorganisms

The microorganisms evaluated were the bacteria: *Escherichia coli* (ATCC 1122) (Gram-negative), *Staphylococcus aureus* (ATCC 6538) (Gram- positive) and the fungus *Penicillium* spp.
The microbial cultures were obtained from the American Type Culture Collection (ATCC) in the Universidade Federal de Viçosa, Minas Gerais, Brazil.
Bacterial cultures and fungus were pre-activated in brain heart infusion broth (BHI). Bacteria were grown on plate count agar (PCA) at 35°C/48 h and fungus was grown on Potato Dextrose Agar (PDA) at 25°C/5 days.
Microbial cultures were maintained at refrigeration temperatures as stock culture, in order to obtain freshly cultured microbial suspension. This microbial suspension, following, was used to microbial activity assay by disc diffusion method.

2.3. Preparation of active films

Cellulose acetate matrix films were obtained by casting according to MELO (2003) with same modifications.
Film-forming solutions were prepared by dissolving cellulose acetate (10% w/v) in acetone. Essential oils that were either pure or in combination (1:1 or 1:1:1) were added to the filmogenic solutions at a concentration of 50% w/v. Six different films were developed: 1) film incorporated with oregano + sweet fennel + cinnamon EOs; 2) film incorporated with oregano + sweet fennel EOs, 3) film incorporated with oregano + cinnamon EOs; 4) film incorporated with sweet fennel+ cinnamon EOs; 5) film incorporated with only oregano EO; and 6) negative control, film without any EOs.
Preliminary analysis by disk diffusion method using individually the oregano, sweet fennel and cinnamon EO, presented effective microbial activity for these oils, highlighting the oregano EO (data no shown).

The filmogenic solutions were cast on clean, flat glass plates with a glass rod to a pre-determined height. The films were dried at temperature room and then removed from the glass plates.

2.4. *In vitro* antimicrobial activity of the active films

The agar disk diffusion method was used to determine the antimicrobial activity (CLSI, 2003).
Microbial suspensions were prepared and adjusted in brain heart infusion broth (BHI). Inoculums of the bacteria were 100 μL of a suspension containing approximately 10^4 colony-forming units (CFU) per mL for bacteria, or 10^4 spores/mL for the fungus. The inoculums were spread on the surface of Mueller-Hinton agar plates. Then a sterile film disk (10 mm diameter) incorporated with the EO or EOs under test was placed in the center of the agar plate. A film disk without any EOs was used as negative control (ESPITIA, 2009). The 6 films were previously exposed to UV light (Prodicil, 110v, 254nm) for 15 minutes for sterilization.
The plates with bacteria were then incubated at 35±2°C for 48h, while plates with fungus were incubated at 25±2°C for 5 days. Following incubation, the total diameter of the inhibition zones (colony-free perimeter) was measured with a caliper. The data were expressed as inhibition zones (cm), including the disk area. Each experimented was done in triplicate.

2.5. Film characterization

2.5.1. Thickness

The films thickness was measured with a digital micrometer (Mitutoyo -Japan). The mean thickness was calculated from five measurements.

2.6. Water vapor transmission rate (WVTR)

The WVTR of films was measured gravimetrically, using the ASTM E96-95 standard test method. Diffusion cells containing anhydrous calcium chloride desiccant (0% RH) were sealed by the test film. The cells were stored in desiccators, each at a constant RH (75%) with a saturated salt solution and temperature of 25°C. The cells were weighed at 24 h intervals (over a 5 day period). Transported water vapor was determined from the weight gain of the diffusion cell at a steady state of transfer. The slope of the weight versus time plot (day) was divided by the effective film area (m^2) to obtain the WVTR using the equation below. At least 3 replicates were produced for each film type.

$$WVTR = \frac{\Delta W}{A}$$

Where WVTR is the water vapor transmission rate (g/d m^2) and A is the area of the exposed film surface (m^2).

2.7. Mechanical analysis

Puncture tests were performed to evaluate the mechanical strength of the film using a Texture Analyzer (TA.XT plus, Stable Micro Systems - Haslemere, England). For the puncture test, film discs (6.4 cm diameter) were fixed to a support with a circular opening

of 10 mm in diameter and 15 mm in depth. A cylindrical probe of 5 mm diameter was moved perpendicularly to the film surface at a constant speed of 1 mm/s until it passed through the film. The force-deformation curves were obtained and force (N) and deformation (mm) values at the puncture point were recorded to represent the puncture strength (N) and deformation (mm) of the film. Ten replications were performed for the mechanical tests.

2.8. Statistical Analyses

The experiment was carried out in a completely randomized design. All analyses were conducted in three replicates in triplicate. Differences between the results of the inhibition zones of the essential oils were evaluated by analysis of variance (ANOVA) using the *F*-test. The Tukey test was used for the antimicrobial analyze and the Duncan test was applied to films characterization. Differences were considered significant when $p < 0.05$, using the SAS® software 9.0 (SAS Institute Inc., NC, USA).

3. RESULTS AND DISCUSSION

3.1. Evaluation antimicrobial of active films incorporated with essential oils

Antimicrobial efficiency was observed in all formulations of the active film incorporated with EO. It was different of the control film (without EO), that not showed antimicrobial activity. The inhibition zones microbial of several films incorporated with OEs against the tested microorganisms are shown in Table 2.

Table 2: Inhibition zones (cm) formed by actives films incorporate with essential oils against bacterial strains *E. coli* and *S. aureus* and fungus *Penicillium* spp., analyzed at an ideal temperature for the microorganisms to develop: bacteria, 35 ± 2ºC/48 h and fungus 25 ± 2ºC/5 days.

Active films	Mean diameter of inhibition zones (cm)[+]		
	***Penicillium* spp**	***E. coli***	***S. aureus***
Oregano Pure	2.67[a]	0.99[ab]	3.75[a]
Oregano + Cinnamon	2.74[a]	1.14[a]	2.7[b]
Oregano + Sweet fennel	1.36[b]	0.50[c]	2.08[b]
Oregano + Cinnamon+ Sweet fennel	1.50[b]	0.73[bc]	1.11[c]
Cinnamon + Sweet fennel	1.15[b]	0.03[d]	0.4[c]

*In each column, different superscript letters are significantly different ($p < 0.05$), by Tukey test.
[+]No includes disc diameter (10 mm).

E. coli bacteria was more resistant than *S. aureus* and *Penicillium* spp. to the treatments with active films in this work. The lower antimicrobial activity against gram - negative bacteria, *i.e. E.coli*, can be attributed to the fact that they are in general more resistant due to the external lipopolysaccharide wall surrounding the peptidoglycan cell wall (ZINOVIADOU *et al*., 2009).
In relation to inhibition of the *S. aureus* microorganism, the active film with oregano EO was the most efficient ($p < 0.05$), followed by 2 different films: film incorporated with

oregano + cinnamon EOs and the film incorporated with oregano + sweet fennel EOs. There were no differences (p>0.05) found for these two latter films (Table 2).
The film with combination oregano + cinnamon + sweet fennel EOs and the film with combination sweet fennel + cinnamon EOs were both statistically similar (p> 0.05). They showed lower activity than the other films against the microorganism *S. aureus* (Table 2).
The films incorporated with oregano + cinnamon EOs and the film incorporated with oregano EO alone proved to be the most efficient against the microorganism *E. coli*. The film with the combination of cinnamon + sweet fennel EOs was the least effective (Table 2) against this bacteria.
Fungus *Penicillium* spp. was most sensitive to the film incorporated with oregano EO alone and to the film with combination of oregano + cinnamon EO (Table 2).
The films into oregano and the film with combination cinnamon + oregano presented the highest antimicrobial activity against bacteria and fungus tested . In the work of Ye *et al* (2013) was showed that the active components, cinnamaldehyde and carvacrol expressed high antibacterial activities both to *E. coli* and *S. aures*, besides of have synergistic antimicrobial activities for the bacterias.
The efficiency of the oregano EO against some microorganisms has already been reported by PEREIRA *et al*. (2008), PESAVENTO *et al*. (2015) and MARTUCCI *et al*. (2015). Positive results were also observed by EMIROGLU *et al*. (2010), when a combination of thyme EO with oregano EO was incorporated into Soy-based films. These authors showed an *in vitro* antimicrobial effect against *S. aureus* and *E. coli* 0157: H7.
According to preliminary studies (data no shown), the inhibitory effect of oregano EO and combinations of EOs when incorporated into the cellulose acetate films was lower than that found in pure oregano EO and combinations of EOs. The possible causes that would explain this result could be a partial loss of volatile compounds during film preparation. It may also be due to dilution of the EOs in the filmogenic solutions.
Various tests with EOs incorporated in films have obtained results that indicate a real potential to use such films in the food industry for packaging. EMIROGLU *et al*. (2010) evaluated the effect of a soy edible film incorporated with natural antimicrobial, thyme and oregano EOs on fresh ground beef patties; while OUSSALAH *et al*. (2007) used the Alginate based film incorporated with winter savory, oregano and cinnamon EO to wrap ham and bologna.

3.2. Film characterization

3.2.1. Thickness and water vapor transmission rate (WVTR)

Adding EOs causes an increase in the thickness of the films. There was a difference (p>0.05) between the thicknesses of the control film (without EO) and the active films incorporated with EO. However, the thickness of the different films after incorporating the EOs was similar, ranging from 29 to 44mm, as shown in Table 3.
The water resistance of the films improved with the incorporation of the EOs, *i.e.*, there was a reduction in the WVTR. The WVTR of the films with EOs presented a significant difference (p< 0.05) when compared to the control films (without EO). However, the WVTR films with EOs (Table 3) was same. Films incorporated with EOs showed a better barrier against water vapor. They had a lower permeability, and therefore are indicated for moisture control for moisture sensitive foods as they would give better protection.
The hydrophobic nature of EOs could potentially increase the hydrophobicity of cellulose acetate film. Studies on the incorporation of lipids as EOs into films have shown improvements in their water vapor barrier properties (ZINOVIADOU *et al*., 2009; BAHRAM *et al*., 2012).

Table 3: Water vapor transmission rate (WVTR) and thickness of differents active films incorporate with essential oils.

Active Films	Thickness (μm)	WVTR ($g.m^{-2}d^{-1}$)
Negative Control (without EO)	$29.0^{a} \pm 1.6$	$259.61^{a} \pm 69.46$
Oregano Pure	$42.8^{b} \pm 3.2$	$162.92^{b} \pm 27.23$
Oregano + Cinnamon	$38.5^{b} \pm 1.0$	$175.61^{b} \pm 14.54$
Oregano + Sweet fennel	$44.3^{b} \pm 4.7$	$192.10^{b} \pm 1.95$
Oregano + Cinnamon + Sweet fennel	$42.8^{b} \pm 3.2$	$197.86^{b} \pm 7.71$
Cinnamon + Sweet fennel	$40.0^{b} \pm 0.4$	$152.80^{b} \pm 37.35$

*In each column, different superscript letters are significantly different ($p< 0.05$), by Duncan test.

3.3 Mechanical analysis

The kind of EOs and its concentration affect the mechanical properties of films differently. The magnitude of such differences is dependent on the added compound (SHOJAEE-ALIABADI *et al*., 2014).
Puncture tests were performed to evaluate the mechanical strength of the film. Table 4 shows the influence of EO incorporations on the mechanical properties of cellulose acetate films. The presence of EO droplets in the film matrix affected both puncture strength and deformation at the break point of the films.

Table 4: Mechanical Analysis of different active films incorporate with essential oils Puncture tests: puncture strength (PS) and deformation at breaking point (DB).

Active films	DB (mm)	PS (N)
Negative Control (without EO)	$2.71^{a} \pm 0.76$	$23.42^{a} \pm 1.24$
Oregano Pure	$3.09^{b} \pm 0.38$	$30.94^{b} \pm 6.28$
Oregano + Cinnamon	$4.18^{c} \pm 0.71$	$26.53^{a} \pm 1.87$
Oregano + Sweet fennel	$3.28^{bd} \pm 0.19$	$24.37^{a} \pm 0.29$
Oregano + Cinnamon + Sweet fennel	$3.98^{c} \pm 0.51$	$24.65^{a} \pm 0.01$
Cinnamon + Sweet fennel	$3.58^{d} \pm 0.11$	$18.07^{c} \pm 6.59$

*In each column, different superscript letters are significantly different ($p< 0.05$), by Duncan test.

The puncture strength (PS) ranged from 18.1 to 30.9 N, for films for combinations of cinnamon + sweet fennel EOs and pure oregano EO, respectively. The film with pure oregano EO was the most resistant, thus, it had a greater force at breaking point.
Films incorporated with a combination of oregano + cinnamon + sweet fennel EOs and the oregano + cinnamon EOs were the most elastic, *i.e.* they had a higher deformation at the breaking point, while the control film presented the smallest deformation at breaking point (Table 4). Therefore, the presence of EOs in film matrices, makes them more elastic, resulting in more stretchable films, depending on the EO concentrations (SHOJAEE-

ALIABADI *et al*., 2013). It occurs because, the EO acted as a plasticizer and increased the flexibility of the polymer chains (ATEF *et al*., 2015).
The kind and amount of phenolic compounds present in oregano, cinnamon and sweet feenel EOs cause different binding and interactions between cellulose acetate molecules and essential oil (LEE *et al*., 2015). As resulted, the films containing these EO had different mechanical properties.
The work reported by Atarés *et al*. (2010a) who conducted tests using Scanning Electron Microscopy and surface morphology in addition to other tests, observed that films with ginger EO was less resistant and less elastic than those with cinnamon EO.
Atarés *et al*. (2010b) evaluated the use of cinnamon and ginger EOs in low proportions (less than 1:0.1 ratio of lipid to protein) in Sodium Caseinate based films. The authors observed that this quantity did not cause any impact on the mechanical properties and only caused a small impact on the water vapor permeability of the film.
When agents are incorporated into packaging materials, polymeric matrix physical and mechanical properties are changed, there are modifications specific to each combination of antimicrobial - polymer. Consequently when a new active packaging material is developed, it is born with new specific characteristics (SOARES and GONÇALVES, 2008).

4. CONCLUSIONS

Essential oils can be recognized as an appropriate natural food preservative, and may be used to replace the synthetic preservatives. Films incorporated with pure oregano EO and the combination of oregano + cinnamon EOs appear as an alternative to extend the commercial validity of foods. Therefore, there is the possibility to replace the synthetic chemical preservatives direct used in foods for antimicrobial active packaging incorporated with EOs for food preservation.
Active packaging based on cellulose acetate incorporated with EOs besides boosting food safety, maintaining their quality and safety, contributes to reducing the environmental impact of traditional packaging because it is based on renewable sources of raw materials.
The technology of active materials is an emerging and promising area of research and it can confer multiple benefits for a wide range of products. However, more research needs to be conducted for different applications in order to evaluate the technological, economic and safety potentials.

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