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Original Research

Synergistic antimicrobial action and effect of active chitosan-gelatin biopolymeric films containing *Thymus vulgaris*, *Ocimum basilicum* and *Origanum majorana* essential oils against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: To replace synthetic preservatives and expand green consumption, several essential oils have been tested in foods and food packaging due to their antimicrobial properties. This study aimed to analyze the synergistic antimicrobial action of a chitosan-gelatin based active biopolymers with the addition of essential oils (EOs). The antimicrobial agents were tested against foodborne microorganisms *Staphylococcus aureus* and *Escherichia coli* strains. The antibacterial activity of *Thymus vulgaris*, *Ocimum basilicum*, *Origanum majorana*, and the synergistic interactions among them were assessed according to the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method. Chitosan-gelatin based active biopolymers were developed, and 23% (w/w) of each essential oil and combined oils were added. The antimicrobial effect of active films was measured using the disk diffusion method. Active films with the addition of essential oils have potential applications as active packaging agents, especially those that demonstrated inhibition zones. Combined EOs can be used to enhance the antimicrobial activity, ensuring reduced doses used in active packaging and decreasing the association with adverse sensory characteristics.

Key words: Antimicrobial activity; Pathogenic bacteria; Essential oil; Synergy.

Introduction

With the advent of clean-label foods and the demand for healthier and more sustainable products, the food industry has formulated and reformulated several products to meet consumer needs, replacing artificial coloring, flavoring and synthetic antimicrobial materials with natural options (1).

Outbreaks of foodborne diseases (FBD) have been reported regularly worldwide, representing a reason for concern to international and governmental bodies due to economic and public health issues (2). *Escherichia coli* and *Staphylococcus aureus* are some of the pathogenic bacteria that cause foodborne diseases. *Escherichia coli* belongs to the family Enterobacteriaceae and is one of the most important enteric pathogens. It is naturally present in the intestinal flora, but certain groups are foodborne pathogens and can cause severe gastrointestinal disorders (3). *Staphylococcus aureus* is associated with many causes of foodborne diseases worldwide (4) and many strains produce enterotoxins, which can cause food poisoning when ingested (5).

Essential oils are widely known for their antibacterial, antifungal, antiviral and antioxidant properties (6). They are a mixture of volatile, lipophilic, liquid and, in general, odoriferous organic substances found in roots, barks, seeds, leaves and fruits of aromatic plants (7). In plants, they are mainly present in cavities, excretory and epidermal cells and their roles are related to protection against dangers and internal or external stress (8). Because they are natural antimicrobials, they have potential applications in the food industry to fight against pathogens that cause FBDs and decay (9) and are generally recognized as safe (GRAS) in the United States, that is, they are safe to use (7). Essential oils like those obtained from *Thymus vulgaris* L., *Origanum majorana* L. and *Ocimum basilicum* L. are some oils that present antimicrobial activity. Terpenes, terpenoids and phenolic compounds are the major components of essential oils offering antimicrobial activity, which is determined by chemical concentration and composition (7). Usually, a large amount of essential oil should be added to food to obtain results similar to those from in vitro studies (6), but such addition to food is limited as it may cause sensory changes due to oil interaction with food components. Then, one of the challenges of using essential oil refers to finding a balance among all these factors: while applying it to food, its antimicrobial efficacy should be kept and the possibility of sensory changes and unwanted flavors should be minimized (10). Using combinations of essential oils and/or their components may increase their effectiveness in food due to possible

synergistic and additive effects (6), favoring smaller quantities of oil being applied to food.

Studies have demonstrated the possibility of using essential oils in the manufacture of active packaging. When added to these packages, their antimicrobial activity can eliminate or inhibit decaying and/or pathogenic microorganisms (11, 12), in addition to reducing waste and minimizing the risk of foodborne diseases.

A biopolymer film is defined as a package containing natural materials from agricultural and marine sources (12). Some studies have incorporated essential oils into biopolymer films that act as active packaging materials for food preservation (12, 13). Using essential oils in biopolymers has many advantages, such as reduction of unwanted flavors in food (10). The antimicrobial activity of biopolymer films will depend on factors such as the type of oil, oil concentration in the film, and oil interaction with the other ingredients of the biopolymer matrix (10).

Considering the above, this study aimed to evaluate the antimicrobial activity of the essential oils in question and their potential application as active biopolymer films.

Materials and Methods

Strain origin

This study used two wild-type strains of *Staphylococcus aureus coli* isolated from dairy producers of Minas cheese and two standard strains (ATCC 25923 and USA100). For *E. coli*, two wild-type strains isolated from dairy producers of Minas cheese, one EHEC (EDL933) and one ETEC (H10407) were used.

Essential oils and chemical composition

Commercial essential oils of *Thymus vulgaris* (WNF Essential Oils), *Ocimum basilicum* (BioEssência) and *Origanum majorana* (BioEssência) were used in this study. Their chemical composition was obtained from gas chromatography using an HP-6890 gas chromatograph coupled to an HP-5975 mass selective detector, according to the following conditions: HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), injector temperature of 220ºC, column temperatures of 60ºC, 3ºC/min, 240ºC, and detector temperature of 250ºC. The volume injected was 1.0 mL, helium was used as carrier gas, with flow rate of 1.0 mL/min and 40:1 split ratio.

Identification of the componets was performed by calculating the analyte retention indices, using a coinjection of a mixture of hydrocarbon standards (C8 to C24), comparison with the electronic equipment library (NIST-11) and data from the literature (14).

Minimum inhibitory concentration (MIC)

MIC was determined for the essential oils of marjoram (*Origanum majorana*), thyme (*Thymus vulgaris*), and basil (*Ocimum basilicum*) regarding the strains of *Escherichia coli* and *Staphylococcus aureus*. The strains were inoculated in BHI broth and incubated at 37ºC for 24 hours. The bacterial suspensions were standardized to obtain suspensions of around 10^8 CFU/ml and were subsequently diluted to 10^5 CFU/ml, based on the McFarland scale. This analysis used 96-well flat-bottomed cell culture plates containing 200 μL of Tryptone soy broth (TSB) and 0.5% Tween 80 in each well of the plate. The mixture of 360 μL of TSB with Tween 80 were inserted in the first column of wells, and right after that, it was homogenized with 40 μL of essential oil. Soon after that, the serial dilution was performed with 200 μL of the mixture transferred from the first well to the second well, and so on until the last column. Then 2 μL of bacterial suspension were inoculated, resulting in a final concentration of 10^3 CFU/mL in each well. A positive control (bacteria in TSB without essential oil) and negative control (just TSB) were prepared. The plates were incubated at 37°C for 24 hours. After incubation, 50 μL of 0.01% resazurin solution were added and five minutes later, blue color could be observed indicating microbial growth. The well with the lowest concentration of antimicrobial agent that remained with the blue color of resazurin was considered the minimum inhibitory concentration while the well whose color turned to pink indicated bacterial growth (15).

Minimum bactericidal concentration (MBC)

For the identification of MBC, 10 μL were removed from the well indicating MIC and from three previous wells, and were inserted in plates with nutrient agar using the droplet technique described by Knezevic et al., 2016 (16). The plates were submitted to incubation at 37°C for 24 hours. The sections without growth indicated bactericidal activity, while the sections with positive growth indicated bacteriostatic activity of the essential oil analyzed (16).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oil combinations

After MIC determination for the essential oils of marjoram (*Origanum majorana*), thyme (*Thymus vulgaris*), and basil (*Ocimum basilicum*) regarding the strains of *Escherichia coli* and *Staphylococcus aureus,* the mean values of minimum inhibitory concentration of each oil was calculated for each strain. The mean values of MIC for the essential oils were measured individually and a new MIC assessment was performed using combinations of two essential oils. MIC and MBC methodologies were the same as those adopted with individual essential oils.

Development of active biopolymers

Biopolymers were developed for individual oils and oil combinations as described by Gómez-estaca et al., 2010 (11) with modification (17). In this procedure, the amount of 1.5 g of gelatin was dissolved in 17.5 ml of distilled water, and 0.1 g of high molecular weight chitosan was solubilized in 2.5 ml of 1.5% acetic acid solution. Both solutions were mixed; then 0.24 g of glycerol and 0.24 g of sorbitol were added. The resulting mixture was heated until it reached 40ºC and stirred for 15 minutes. After that, the mixture was cooled down to room temperature and the essential oil was added at the concentration of 23% (w/w) with 0.2 g of soy lecithin. In the development of biopolymers containing two oils, the concentration of 11.5% (w/w) was used with each oil. The mixture was stirred in IKA T 25 ULTRA-TURRAX® using setting 5 for one minute, and the mixture was dispensed in Petri dishes coated with PVC film.

Then the plates were dried at 40ºC for 3 to 4 hours until a uniform film was obtained. The concentration of 23% (w/w) was selected after presenting more halo formation between the oil concentrations in previously tested films (17).

Antimicrobial activity of developed films

Films were cut into discs of 1.1 cm diameter and refrigerated to 8°C (+/-2°C) until use. The analysis was conducted through disk diffusion method, as described by Gómez-estaca et al., 2010 (11) modified (17), in which the suspension of analyzed cultures was inoculated on the surface of Mueller-Hinton agar plates at the concentration of 10^8 CFU/ml, based on the McFarland scale. After this stage, the discs were inserted on the surface of the plates and incubated at 37ºC for 24 hours. After incubation, halos were measured (17).

Disk diffusion test for antimicrobial sensitivity

The disk diffusion method was used to produce antibiograms. Suspensions had the addition of around 10^8 CFU/ml (Mc Farland scale) of the microorganism in question in Mueller Hinton agar plate. Then, paper filter discs impregnated with therapeutic agents at known concentrations were placed on the surface of the culture medium and incubated at 37ºC, for 18-24h. The results were expressed as R: resistant, I: intermediate, and S: sensitive, according to the reference tables after measuring the inhibition halos (18).

Statistical analysis

The results of MIC conducted for individual essential oils and oil combinations were analyzed in SISVAR 5.6, a software application developed by the Federal University of Lavras. Analysis of variance (ANOVA) and the Tukey's test were performed to assess the effects of each essential oil and each combination of essential oils on the microorganisms studied.

Analysis of the interaction between essential oils

The assessment of oil interaction was used for the determination of the fractional inhibitory concentration (FIC) index. This calculation used the values of MIC for individual oils and MIC for each oil in the combination. The following formulas were used in the calculation:

$$\sum \text{FICI} = \text{FIC(A)} + \text{FIC(B)}$$

Where

$$\text{FIC (A)} = \frac{\text{MIC (A) for oil combination}}{\text{MIC (A) for each oil}}$$

And

$$\text{FIC (B)} = \frac{\text{MIC (B) for oil combination}}{\text{MIC (B) for each oil}}$$

Results and Discussion

Chemical analysis of components of essential oils and mechanisms of action

Table 1 shows the results of the chemical analysis of essential oils.

The secondary metabolites of plants are the main agents promoting the antimicrobial activity of essential oils, and their respective components have different mechanisms of action in bacteria (8).

The antimicrobial activity of essential oils in microorganisms cannot be explained through a single mechanism due to a wide variety of major and minor components present in essential oils. Different biochemical and structural mechanisms are involved, which can occur in the cell membrane, cytoplasm, enzymes and proteins. The mechanism will vary with the essential oil and the target strains. Many authors (20, 21, 22) attribute the antimicrobial effect of oils to the harmful effects they can cause to the structures of microbial cell membranes and functions. Widely known mechanisms of action of essential oils include cell wall degradation, damage to plasma membrane, damage to membrane proteins, changes in protein synthesis and cytoplasm coagulation, increased cell permeability and its effect of reduced membrane potential, reduced ATP synthesis, change in ionic balance, damage to the electron transport chain, among others (8).

Essential oils are naturally hydrophobic liquids, allowing them to increase membrane permeability and penetrate microbial cells, causing changes in cell structure and functionality. With increased permeability, desaturase enzymes are dispersed, allowing oil action on the membrane (8). This fact contributes to stronger antimicrobial activities against strains of Gram-positive microorganisms, since the external membrane of Gram-negative microorganisms limits the penetration of hydrophobic components. It also shows the membrane of microorganisms to be the primary target of essential oils (8).

Many components of oils, such as carvone, thymol and carvacrol, the latter two found in the essential oil of *Thymus vulgaris*, increase the intracellular concentration of ATP, which is associated with destruction of the membrane in the microorganism. Oils with a high level of phenolic compounds can penetrate the phospholipid bilayer and bind to enzymes, preventing their proper function in cells. The essential oil of *Ocimum basilicum* has a high level of phenylpropanoid compounds, such as methyl-chavicol, also known as estragole.

Essential oils are mostly comprised of terpenoids and other smaller molecules of acids, alcohols, aldehydes, hydrocarbons, among others (8).

Table 1 presents the composition of each essential oil, showing their major and minor components. Although the major components ensure the antimicrobial activity of the oils, some studies show that essential oils present better activity than just a mixture of major compounds (23). According to Hussain et al., 2008 (24), the major components of *Ocimum basilicum* oil are methyl-chavicol, linalool and eugenol, while other studies report linalool as the main agent promoting the antimicrobial activity of this oil (25). Lachowicz et al. (26) show the pure oil of *Ocimum basilicum* is more effective than

Table 1. Chemical analysis of essential oils, their components and the percentage in each oil.

Essential Oils	Scientific names	Components	%
Marjoram	*Origanum majorana*	α-thujene	0.64
		α-pinene	0.79
		sabinene	7.39
		β-pinene	0.43
		β-myrcene	1.72
		α-phellandrene	0.36
		α-terpinene	7.76
		p-cymene	2.72
		limonene	3.84
		γ-terpinene	11.82
		cis-sabinene hydrate	4.73
		terpinolene	2.98
		trans-sabinene hydrate	17.94
		cis-p-2-menthen-1-ol	1.65
		trans-p-2-menthen-1-ol	1.01
		terpinen-4-ol	22.32
		α-terpineol	3.62
		cis-piperitol	0.47
		trans-piperitol	0.54
		trans-sabinene hydrate acetate	0.31
		linalyl acetate	2.49
		(*E*)-caryophyllene	3.05
		bicyclogermacrene	1.45
Thyme	*Thymus vulgaris*	camphene	0.69
		β-myrcene	0.96
		p-cymene	21.55
		1,8-cineole (eucalyptol)	1.10
		γ-terpinene	5.00
		linalool	5.41
		camphor	0.98
		M = 152	0.79
		iso-borneol	0.60
		endo-borneol	1.26
		terpinen-4-ol	1.02
		thymol	52.43
		carvacrol	3.56
		(*E*)-caryophyllene	0.83
		caryophyllene oxide	1.77
Basil	*Ocimum basilicum*	sabinene	0.57
		α-terpinene	0.32
		p-cymene	0.71
		gamma-terpinene	0.69
		n.i.	0.37
		linalool	18.76
		menthol	0.61
		terpinen-4-ol	0.82
		methyl-chavicol (estragole)	72.86
		trans-citral (geranial)	0.64
		trans-α-bergamotene	0.70
		α-humulene	1.55
		(*E*)-methoxycinnamaldehyde	0.76
		M = 164	0.64

its major components linalool and methyl-chavicol, together or separately. Santurio et al., 2014 (27) also show MIC and MBC of the essential oil of *Thymus vulgaris* is more efficient against strains of *E. coli* when compared to thymol only, its major component. Then, minor components are also important for the activity of an oil and many ensure a synergistic effect with other components of the same oil (7).

Terpenes are the most common chemical class present in essential oils; they consist of combinations of isoprene units (28). Terpenes include *p*-cymene, a major compound of the essential oil of *Thymus vulgaris*, terpinen-4-ol, *trans*-sabinene hydrate, and γ-terpinene, major components of the essential oil of *Origanum majorana*, and linalool, one of the major compounds of *Ocimum basilicum* oil. In vitro tests demonstrated that terpenes, when used alone, do not present effective antimicrobial activity (8). Its site of action is the cytoplasmic membrane, as well as phenolic compounds, which may lead to cell lysis and changes in electric potential

and inhibition of respiratory enzymes (29).

Terpenoids are modified terpenes whose methyl groups have been removed or oxygen molecules added (8). Their antimicrobial activity is related to their functional groups, such as the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons; for example, carvacrol and *p*-cymene. The chemical structure of both compounds is similar, except for the presence of a hydroxyl in carvacrol, which presents stronger antimicrobial activity when compared to *p*-cymene (20). Thymol, one of the major compounds of *Thymus vulgaris* oil and linalool, one of the major compounds in *Ocimum basilicum* oil, are some well-known terpenoids.

Like carvacrol, thymol interacts with the inner membrane of bacterial cells, not increasing membrane permeability and disintegration like other components, but it increases the permeability to ATP. *p*-Cymene has a high affinity for membranes and affects the membrane potential, as reported in studies (8), reducing the melting temperature of membranes. Thymol has a similar structure to carvacrol, with hydroxyls in different positions in the aromatic ring. It causes structural and functional changes in the cytoplasmic membrane and may damage membranes and interact with membrane proteins, leading to changes in membrane permeability (30, 31).

Phenolic compounds are also components of essential oils. According to some authors (32, 33), these substances promote the strongest antimicrobial activity in oils. Like terpenes, the target of phenolic compounds seems to be the cell membrane (34). Their effect on the microorganism is indicated by the presence of a hydroxyl group, which is associated with inactivation of microbial enzymes. Probably, the hydroxyl group interacts with cell membranes, causing removal of cell components and changes in fatty acids, phospholipids and alterations to the synthesis of genetic material (35). Methyl-chavicol, one of the major components of the essential oil of *Ocimum basilicum*, is a phenylpropanoid compounds. Some sources classify thymol, one of the major components of *Thymus vulgaris*, as a phenolic compound. Indeed, thymol is a phenol, which is a monoterpene derived from cymene (36).

Antimicrobial activity of essential oils

After finding the MIC values and performing the statistical analysis, data were inserted in Table 2, as follows.

MIC values identify the lowest concentration at which a compound inhibits the microorganism growth. From the results obtained, the best oils against the strains of *Staphylococcus aureus* and *Escherichia coli* were *Thymus vulgaris* and *Origanum majorana*, whose MICs did not present a significant difference; however, a significant difference was observed in *Ocimum basilicum* oil, as it presented a higher MIC when compared to the other two oils. According to Dormans and Deans, 2000 and Burt, 2003 (32, 37), the essential oil of *Thymus vulgaris* showed stronger antimicrobial activity against *E. coli*, among several oils. Results similar to this study were reported by Bussata et al., 2008 (38), who found a similar MIC for *Origanum majorana* oil against *E. coli.* Dormans and Deans (2000) (32), when testing the antimicrobial activity of essential oils against 25 Gram-positive and Gram-negative bacteria, showed that Gram-positive microorganisms are more susceptible to the antimicrobial action of essential oils than Gram-negative microorganisms. Burt (2004) (7) reports that many studies analyzing the antimicrobial activity of essential oils show superior susceptibility of Gram-positive bacteria to the antimicrobial action of oils than Gram-negative bacteria. It happens because the cell walls of Gram-positive bacteria are 90-95% peptidoglycan, allowing hydrophobic molecules, such as essential oils, to penetrate the cells, causing an effect of these molecules on cell wall and the cytoplasm. On the other hand, Gram-negative bacteria have a more complex cell wall consisting of a thinner layer of peptidoglycan and a lipoprotein outer membrane (8). This outer membrane has a double layer of phospholipids, which are linked with the inner membrane by lipopolysaccharides (LPS). LPS has lipid A and O antigen, which make Gram-negative bacteria resistant and less susceptible to essential oils by allowing less diffusion of hydrophobic compounds across the membrane, acting as a barrier to the penetration of these compounds (39). This way, hydrophilic components are able to penetrate the membrane, while for hydrophobic compounds, the membrane is partially permissive (40). In order to produce an effect on Gram-negative microorganisms, the oil has to cross the outer membrane barrier. The results of this study agree with expected results, as the MIC values for all oils for *S. aureus* were lower in relation to the MIC values of all oils for *E. coli.*

Regarding the minimum bactericidal concentration (MBC) for *Escherichia coli*, the most efficient oils according to this study were those of *Origanum majorana*, with MBC ranging between 0.725 and 2.99 mg/mL, and *Thymus vulgaris*, with MBC ranging between 1.43 and 11.46 mg/mL. For *Staphylococcus aureus*, the oil with the best MBC was *Thymus vulgaris*, ranging from 1.43 to 5.98 mg/mL, and only one strain of *S. aureus* showed minimal bactericidal concentration for *Origanum majorana*, ranging from 5.8 to 23.2 mg/mL. No MBC of *Ocimum basilicum* oil was obtained for all tested strains of *Staphylococcus aureus*.

Essential oils as antimicrobial agents have demonstrated very promising results in recent times, but this application involves some challenges. Many studies about the antimicrobial activity of essential oils have found obstacles in terms of reproducibility, due to factors such as lack of specification about the microorganism used in the study, lack of details regarding the amount of oil, type of methodology, variations in composition between batches of an essential oil from the same manufacturer. Regarding the characteristics of each oil, studies show that factors such as climate, soil, growing season, irrigation, extraction technique, and even genetic variations of the plant will change the gen-

Table 2. Results of minimum inhibitory concentration (MIC) of essential oils.

Essential oils	MIC values (mg/mL)	
	E. coli	*S. aureus*
Thymus vulgaris	2.44±1.31[a]	1.01±0.46[a]
Origanum majorana	1.81±0.85[a]	1.39±0.57[a]
Ocimum basilicum	7.72±5.91[b]	5.56±3.38[b]

*different letters show significance difference in each column.

eral chemical composition of the oils, with consequent impact on their antimicrobial activity (41).

Antimicrobial activity of essential oil synergism

Besides the use of individual essential oils in tests to analyze their antimicrobial activity and food applications, these oils can be combined with each other to promote interactions of components, which may increase or decrease their antimicrobial activity. These interactions of components of essential oils can produce the effects of addition, synergism, antagonism or indifference. The effect of addition occurs when the combined effect is equal to the sum of the effects of individual oils. Synergism happens when the effect of the combination is stronger than the sum of individual effects; antagonism occurs when the effect of one or both components is less strong when applied together than when applied individually; and an indifferent result refers to no interaction observed (7). Studies have also demonstrated that minor compounds are essential for the synergistic effect, making essential oil activity stronger than the mixture of major components (6).

Little is known about the mechanisms that would explain the occurrence of synergism in the combination of oils. However, some studies present some hypotheses, such as: each oil component has an effect on different targets of a microorganism; the effect is due to similar mechanisms, or the components interact and inhibit the microorganism together. A study conducted by Zhou et al., 2007 (42) proposed these mechanisms and presented two hypotheses to explain the synergistic effects of the components used in their study: combinations of cinnamaldehyde/thymol and cinnamaldehyde/carvacrol against *S. thyphimurium*. They suggest that thymol or carvacrol could increase the permeability of the cytoplasmic membrane, while cinnamaldehyde could be easily transported into the cell. Also, thymol or carvacrol could alter the number, size or duration of membrane pores resulting from the binding of cinnamaldehyde to proteins in cell membranes, thus, producing a synergistic effect.

Learning more about the synergism between antimicrobial agents, like essential oils, is very important, because smaller amounts of oil can be used to achieve the same antimicrobial activity, leading to reduced sensory impact on products, which is usually one of the factors limiting the use of oils (6).

The FIC (fractional inhibitory concentration) index is used to define the effect of additivity in most studies analyzing the combination of components against microorganisms. This index uses the Loewe additivity theory, which is based on the hypothesis that a drug cannot interact with itself; therefore, the effect of a combination of drugs is always additive, with an FIC index equal to 1. In this theory, an FIC index below 1 means synergism, and above 1, antagonism, since more or less of the same drug would be required to produce the effect of drugs separately. Due to factors such as double dilution and dilution error of the sensitivity methods, values of 0.5-4 were suggested for the FIC index by Meletiadis et al. (43). Some authors classify the limit values for the effects of addition, synergism, indifference and antagonism in different ways, with synergism classified as FIC index <0.9 (44).

After finding the MIC values for combinations of essential oils and performing the analysis of FIC index, data were inserted in Table 3, as follows.

Based on the results and parameters adopted for the FIC index in this study, an additive effect was observed for both species tested in the combination of *Origanum majorana* and *Ocimum basilicum*, oils that showed a significant difference in their antimicrobial activities. In the analysis of minimum inhibitory concentrations, the essential oils with better results for both *S. aureus* and *E. coli* according to the statistical treatment were those of *Thymus vulgaris* and *Origanum majorana*, for both species. However, only for *E. coli*, the combination of these two oils showed an additive effect, while for *S. aureus*, the treatment was indifferent.

Antimicrobial activity of developed films

In active packaging, the product and the atmosphere inside a pack interact with each other, extending the product shelf life, increasing product safety, and enhancing sensory properties, while maintaining product quality 45).

Edible films are one example of active packaging; they can extend shelf life, as they prevent dehydration by acting as a barrier to water and protection against light, oxygen and other gases. They also delay lipid oxidation (46) and control microbial growth. Several studies have used essential oils in the formulation of edible films (46, 47, 48, 49). Using essential oils incorporated in biopolymer matrices offers many advantages, such as reducing unwanted flavors and controlling the level of oil diffusion, promoting low sensory impact on food (10).

The antimicrobial activity of films containing essential oils will depend on factors such as the type of oil, amount of oil in films, and interaction of films with the

Table 3. Results of minimum inhibitory concentration for combinations of essential oils and FIC index.

Synergy between essential oils								
	E. coli				*S. aureus*			
Combinations	**MIC[1] (mg/mL)**	**MIC[2] (mg/mL)**	**FIC index**	**Interaction**	**MIC[1] (mg/mL)**	**MIC[2] (mg/mL)**	**FIC index**	**Interaction**
Origanum majorana[1] / *Ocimum basilicum*[2]	0.80±0.60	3.42±2.58	0.88	Additive	0.54±0.34	2.14±1.34	0.77	Additive
Origanum majorana[1] / *Thymus vulgaris*[2]	2.57±0.39	1.73±0.25	1.85	Additive	0.82±0.25	1.10±0.34	1.42	Indifferent
Ocimum basilicum[1] / *Thymus vulgaris*[2]	3.14±1.85	1.00±0.59	2.12	Indifferent	3.94±1.69	0.73±0.31	1.66	Indifferent

[1] Component A of oil combination. [2] Component B of oil combination.

Table 4. Results from measurements of diameters of inhibition zones in films.

Essential oils	Inhibition zone (mm)	
	E. coli	***S. aureus***
Thymus vulgaris	29.4±6.7[a]	48.1±4.1[a]
Origanum majorana	25.6±6.8[a]	0.0±0.0[b]
Ocimum basilicum	0.0±0.0[b]	0.0±0.0[b]
Combinations	*E. coli*	*S. aureus*
Origanum majorana/Ocimum basilicum	0.0±0.0[b]	0.0±0.0[b]
Origanum majorana/Thymus vulgaris	24.3±2.8[a]	29.9±4.8[a]
Ocimum basilicum/Thymus vulgaris	0.0±0.0[b]	0.0±0.0[b]

*different letters show significance difference in each column

biopolymer matrix (10).

Chitosan, used as an ingredient in the film developed in this study, is derived from chitin and has been analyzed in several studies about packaging and films, due to its ability to produce films (50) and coatings, or as a base for the addition of other ingredients, such as essential oils (51). Its mechanism of action is probably through the outer membrane of bacteria.

After developing films with the addition of individual and combined essential oils, the results of the antimicrobial activity, measured through the diameters of the inhibition zones, were inserted in Table 4, as follows.

Films with the addition of oils of *Thymus vulgaris* and *Origanum majorana/Thymus vulgaris*, which presented the development of halos for both microorganisms, had larger inhibition zones for *Staphylococcus aureus* when compared to *Escherichia coli*, supporting the MIC values for essential oils causing more susceptibility of Gram-positive microorganisms than Gram-negative microorganisms. In addition, in agreement with the MIC results for *S. aureus*, the oil of *Thymus vulgaris*, which had the lowest MIC (and therefore, the best result against the strain) also presented halo development, while for *Origanum majorana* and *Ocimum basilicum*, no halo was developed at the concentration tested in the analysis, not supporting the significant difference between the oils demonstrated by MIC. Regarding *E. coli*, as observed in MIC, the films with the addition of *Thymus vulgaris* and *Origanum majorana* individually presented antimicrobial activity demonstrated through the development of halos; however they did not differ significantly.

The oil concentration of 23% (w/w) was chosen after a viability test; it produced the largest halo formation, not necessarily linked with the MIC values.

In films with the addition of a combination of two oils, only the combination of *Thymus vulgaris*/*Origanum majorana* produced halos. Individual treatments with *Thymus vulgaris*, *Origanum majorana* and the combination of these two oils had no significant difference for *E. coli*. Although the combination had no significant difference, which would demonstrate stronger antimicrobial activity of the individual or combined oils, it is interesting for using lower amounts of both oils to obtain the same effect. It could represent minor adverse sensory effects when used as a protective film in foods, which are some factors limiting the application of oils to the films.

Some studies show that, despite an increase in antimicrobial activity with the addition of essential oils to films, such addition can affect physical and mechanical properties, that is, reduced permeability of water and steam, humidity and elongation, changes in film appearance, making them opaquer. For an effective application to foods, additional tests should be performed (10 – 51)

Figures 1 show the films developed in this study, cut as discs. Figure 2 shows the antimicrobial activity of the film with the addition of the essential oil of *Thymus vulgaris* against a strain of *Staphylococcus aureus*.

Sensitivity test to antimicrobial agents

Bacterial strains and species usually present various degrees of susceptibility to different antimicrobial agents, and such susceptibility may change over time, even during treatments of pathologies with antibiotics. Several tests can be performed to identify the best chemotherapeutic agent to fight pathogens, often used in cases of problems with antimicrobial resistance (52).

Antimicrobial resistance is a public health problem and inevitable even with new drugs; it is a major prob-

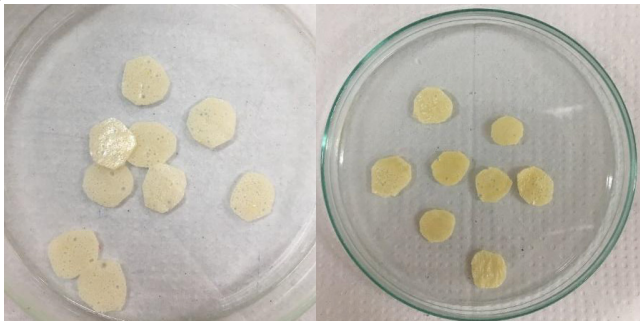

Figure 1. Dried films cut as discs of around 1.1 cm diameter.

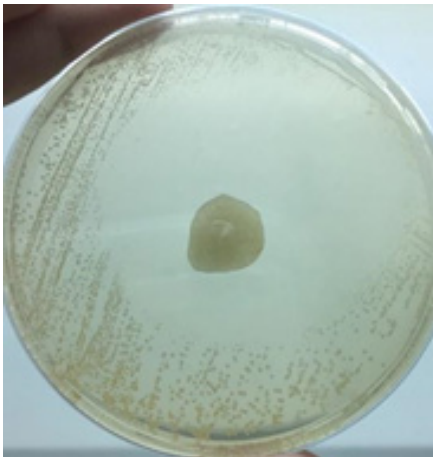

Figure 2. Antimicrobial activity of a film containing essential oil of *Thymus vulgaris* against *Staphylococcus aureus.*

Table 5. Results of antibiograms for the strains of *Escherichia coli*.

	Antibiotic agents							
Strain	**AMP**	**AMC**	**CAZ**	**CFO**	**GEN**	**TOB**	**NAL**	**IMI**
ETEC	I	S	I	I	S	S	S	S
EHEC	S	S	S	S	S	S	S	S
C5Q4	S	S	S	S	S	S	S	S
C6Q5	R	R	S	S	S	S	S	I

R: resistant, S: sensitive, I: intermediate. AMP: ampicillin, AMC: amoxacilin, CAZ: ceftazidime, CFO: cefoxitin; GEN: gentamycin, TOB: tobramycin, NAL: nalidixic acid, IMI: imipenem.

Table 6. Results of antibiograms for the strains of *Staphylococcus aureus*.

	Antibiotic agents						
Cepa	**CFO**	**CLI**	**ERI**	**GEN**	**TET**	**TOB**	**OXA**
C11F19	S	S	R	S	S	S	S
C6B21	S	S	S	S	S	S	S
ATCC25923	S	S	S	S	S	S	S
USA100	R	R	R	S	S	R	R

R: resistant, S: sensitive, I: intermediate. CFO: cefoxitin, CLI: clindamycin, ERI: erytromicin, GEN: gentamycin, TET: tetracyclin, TOB: tobramycin, OXA: oxacillin.

lem in the treatment of infections. One of the causes of antimicrobial resistance is the excessive exposure of bacteria to antibiotic agents, whether in a hospital, in farming activities, or in the community (53). Bacteria become resistant with antibiotic inactivation mechanisms through enzymes, changes in target site, in the metabolic pathway and membrane permeability, among other factors (54).

Disc diffusion tests, also known as Kirby-Bauer tests or antibiograms, are the most common tests with strains.

Tables 5 and 6 show the results of antibiograms for the strains used for this study.

Based on the results, for the two strains of each microorganism tested in this study, resistance was observed to at least one antimicrobial agent, that is, an intermediate result. Even a strain like USA100, resistant to 5 of all 7 antimicrobial agents tested for *S. aureus*, obtained satisfactory results of MIC, performed with essential oils. Many studies have analyzed essential oils and natural extracts as alternatives to antimicrobial drugs for use in several fields, including clinical uses. The approaches used in these studies replace the antibiotic agent with a tested oil or natural extract, or propose the application of both in a synergistic way, combining antimicrobial drugs with oils or extracts. Essential oils used in synergy with antibiotics have been effective in lowering the minimum effective dose for the treatment of infections, which reduces adverse effects of antibiotic agents, a common concern regarding antibiotics (53).

Using antibiotics associated with essential oils can be effective against resistant bacteria, considering that both agents may have different mechanisms of action. That is, oil components have different targets in the microorganism while most antimicrobial agents have only one target site (53).

Regarding the possibility of bacterial resistance to essential oils, few studies have addressed this issue and, in most cases, these studies refer to *Melaleuca alternifólia* oil. However, due to the numerous components presenting antimicrobial activity in essential oils, the chances of resistance are reduced.

This study concluded that *Thymus vulgaris*, *Origanum majorana* and *Ocimum basilicum* oils have antimicrobial activity against strains of *Escherichia coli* and *Staphylococcus aureus*, with *Thymus vulgaris* and *Origanum majorana* presenting superior antimicrobial activity for both species. In addition, the mixture of essential oils presented an additive interaction between the oils of *Origanum majorana* and *Ocimum basilicum* for both species, with oils presenting a significant difference in MIC in relation to individual oils. Also, for the application to active biopolymers, the oils presenting the best results were *Thymus vulgaris* for *S. aureus* and *Thymus vulgaris* and *Origanum majorana* for *E. coli*. For biopolymers developed with two oils, the only combination that produced results for both strains was the combination of *Thymus vulgaris/Origanum majorana*. Of note, the combination of *Thymus vulgaris/Origanum majorana* through the FIC index revealed an additive effect for *E. coli* and an indifferent effect for *S. aureus*, and even so, an inhibition halo was developed for it. In this case, new concentrations for MIC could be tested.

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Conflict of Interest

There is no conflict of interest.

Author's contribution

Souza, V. V. M. A., Iacuzio, R. carried out the experiment. Souza, V. V. M. A., Crippa, B. L. and Almeida, J. M. wrote the manuscript with support from Silva, N. C.C., and Silva, N. C.C. supervised the project.

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