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Original Article

Chemical composition and antibacterial activity of essential oils against pathogens often related to cattle endometritis

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Abstract

Introduction: Endometritis is a condition marked by inflammation of the endometrium that affects dairy cows from 21 days after parturition, causing damage to herd fertility and economic losses on farms. The use of active compounds obtained from plant sources has gained importance as disease treatment agents in farm animals due to the high resistance rates currently observed against traditional antibiotics commonly used. The study was carried out to examine the chemical composition and to investigate the antibacterial activity of rosemary, cinnamon, cloves, eucalyptus, lemon, oregano and thyme essential oils against the reference strain of *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286), *Trueperella pyogenes* (ATCC 19411) and *Staphylococcus aureus* (ATCC 29213), considered as typical bacteria causing endometritis.

Methodology: The chemical composition of the seven essential oils were analyzed by GC-MS and their antibacterial activity was evaluated by the disc diffusion method.

Results: Thirty-six components were identified in total using GC-MS analyzes. The main compounds were cinnamaldehyde (86.5% for cinnamon essential oil), eugenol (85.7% for clove essential oil), 1,8-cineol (80% for eucalyptus and 47.8% rosemary essential oils), limonene (65.5% for lemon essential oil), carvacrol (72.1% for oregano essential oil) and thymol (48.8% for thyme essential oil). The disc diffusion assay revealed that cinnamon, clove, oregano, and thyme essential oils showed the best results compared to the other three essential oils, showing the largest zone of inhibition against all bacteria evaluated.

Conclusions: These findings indicated that essential oils are a potential agent to be used as an alternative for bovine endometritis treatment.

Key words: Bovine endometritis; essential oils; antimicrobial activity; phytotherapy.

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Introduction

Uterine diseases cause profound economic losses in the dairy sector, mainly due to costs related to decreased milk production, increased use of medicines to treat diseases, discarding milk through antibiotics, and the damage caused by death or early culling of the cows [1,2]. Among uterine diseases, endometritis is one of the most important, being characterized by inflammation of the endometrium from 21 days after parturition [3-5], with purulent or mucopurulent uterine discharge [6].

The prevalence of endometritis reported in Brazil was 28.4% in 338 cows evaluated [6]. The bacteria most often described causing endometritis are *Trueperella pyogenes*, *Escherichia coli* and *Fusobacterium necrophorum* [1,7]. The use of antibiotics is the most used therapy against endometritis [1]. However, indiscriminate use of antibiotics may contribute to increased resistance of pathogenic bacteria, compromising the success of therapy, and may cause low efficacy of the drugs [8].

In this sense, the use of products of natural origin has become an alternative to reduce the use of antibiotics in dairy cows. Thus, essential oils are volatile substances naturally produced by plants as secondary metabolites, and are known for their

antibacterial, antifungal, and antiviral properties, among others [9]. They can be extracted from various parts of plants, such as roots, leaves, bark, seeds, and fruits [10,11]. Their components include two classes of separate biological origin: the prime group consists of terpenes and terpenoids, and the second of aliphatic and aromatic components [12]. According to Pauli and Schilcher, [13], the antimicrobial activity of essential oils can be witnessed by *in vitro* tests, being the most three important ones are the agar diffusion, the agar or broth dilution and the vapor phase test.

The use of essential oils in cattle has increased in recent years, and the action in the treatment of diarrhea in calves [14] and mastitis [11] has been reported. However, there is little information on the use of essential oils as a therapy for endometritis. In this context, the aim of the present study were to characterize the chemical composition and to investigate the antibacterial properties of rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum cassia*), clove (*Eugenia caryophyllus*), eucalyptus (*Eucalyptus globulus*), lemon (*Citrus limon*), oregano (*Origanum vulgare*) and white thyme (*Thymus vulgaris*) essential oils against four bacteria' strains causing endometritis.

Methodology

Essential oils

The essential oils of rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum cassia*), clove (*Eugenia caryophyllus*), eucalyptus (*Eucalyptus globulus*), lemon (*Citrus limon*), oregano (*Origanum vulgare*) and white thyme (*Thymus vulgaris*) were obtained from Ferquimica® (Vargem Grande Paulista, São Paulo, Brazil).

Gas chromatography/mass spectrometry (GC-MS) analysis

The essential oils chemical components were identified by gas chromatograph coupled to mass spectrometry (GC-MS). GC analyses were performed using a Shimadzu GC-2010 gas chromatograph, equipped with a GCMS-QP2010 Ultra mass spectrometer (Shimadzu, Suzhou, China). A split/splitless injector was used. Sample (1 µl) was injected into the injector with a split ratio of 1:10. Oven temperature was 40 ºC for 3 min, then programmed heating from 40 to 280 ºC at a rate of 8 ºC/min. Injector temperature was 250 ºC. Helium was used as carrier gas with 14 mL/minute flow rate. The volatile compounds were identified by comparison with mass spectra with those recorded in the National Institute of Standards and Technology database.

Bacterial strains

The evaluated bacterial strains in this study were *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286), *Trueperella pyogenes* (ATCC 19411) and *Staphylococcus aureus* (ATCC 25923). All microorganisms were cultured in BHI broth (Brain Hearth Infusion, Acumedia, Lansing, MI, USA), being incubated at 37 ºC for 24 hours (*E. coli* and *S. aureus* strains) or 48 hours (*T. pyogenes* and *F. necrophorum* strains).

Disc diffusion assay

After incubation period the cultures were diluted in sterile saline solution and the turbidity adjusted to the 0.5 standard McFarland scale (~10^8 CFU/mL). With the use of a sterile cotton swab, surface of plates containing Mueller-Hinton agar (MHA; Difco) were inoculated with the bacterial suspension. To test *T. pyogenes* strain, the MHA was supplemented with 5% sheep blood and to test *F. necrophorum* strain, the medium used was Brucella agar (Acumedia, Lansing, MI, USA), supplemented with 5% sheep blood, hemine (Interlab Difco, Sao Paulo, Brazil) and vitamin K1 (Interlab Difco, São Paulo, Brazil).

Paper disks with 6 mm diameter (Whatman nº 3) soaked with 20 µL of each pure essential oil were laid on the surface of inoculated agar. Discs of ceftiofur (30 µg, Cefar Diagnósticos Ltda., São Paulo, Brazil) were used as positive control. A paper disc soaked with 20 µL of solution consisting of phosphate buffered saline (PBS, Sigma, São Paulo, Brazil) with 0.5% (v/v) polysorbate 80 (Tween 80) was used as negative control and loaded in each tested plate.

The plates were incubated at 37 °C for 24 hours (*E. coli* and *S. aureus*) or 48 hours (*T. pyogenes*) in aerobic conditions, or 37 °C for 48 hours in anaerobic condition (*F. necrophorum*). Anaerobic conditions were maintained by using an anaerobic jar with anaerobic atmosphere generator (Anaerobac, Probac, São Paulo, Brazil). After incubation, the inhibition zone diameter (IZD) was measured in accordance with the Clinical and Laboratory Standards Institute guidelines [15] and all experiments were carried out in three independent replicates.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and the differences between the means and

standard error were tested by Tukey test. Statistical significance is considered as $P < 0.05$.

Results

Chemical composition of the essential oils

The volatile compounds for all studied essential oils are listed in Table 1. The major chemical constituent found in cinnamon essential oil was cinnamaldehyde (86.5%), and in clove essential oil was eugenol (85.7%). The eucalyptus essential oil was particularly rich in 1,8-cineol (80.0%), while the essential oil of lemon contained a high percentage of limonene (65.5%). In oregano essential oil the most abundant compound was carvacrol (72.1%), and in rosemary essential oil was 1,8-cineol (47.8%). Thymol (48.8%) and p-cymene (26.4%) were the main compounds identified in the thyme essential oil.

Antibacterial activity

The *in vitro* antibacterial activities of seven essential oils against several bacteria strains were qualitatively and quantitatively assessed by the measuring of IZD using the agar disc diffusion method as shown in Table 2.

The results obtained with ceftiofur against *E. coli* ATCC 25922 (27.86 mm of IZD) and *S. aureus* ATCC 25923 (29.00 mm of IZD) strains were within the expected values according to [15] (Table 2). These results revealed that the cinnamon oil presented the greater IZD that varied from 29.67 to 38.33 mm. The larger IZD was observed in *S. aureus* (38.33 mm), and

Table 1. Chemical composition (%) for seven essential oils obtained with GS-MS analysis.

Compounds	Cinnamon	Clove	Eucalyptus	Lemon	Oregano	Rosemary	Thyme
Benzaldehyde	2.40	-	-	-	-	-	-
Borneol	0.95	-	-	-	0.90	2.70	0.33
Bornyl acetate	-	-	-	-	-	0.90	-
δ-Cadinene	-	0.10	-	-	-	-	-
Camphene	-	-	-	-	-	4.50	0.92
Camphor	-	-	-	-	-	11.90	-
Carvacrol	-	-	-	-	72.12	-	2.88
α-Caryophyllene	-	1.81	-	-	-	-	0.11
β-Caryophyllene	-	11.50	-	-	3.03	3.50	1.21
Caryophyllene oxide	-	0.17	-	-	-	-	0.08
1,8-Cineol	-	-	80.04	-	-	47.80	-
p-Cymene	-	-	2.96	-	4.81	1.40	26.43
Cinnamaldehyde	86.50	-	-	-	-	-	-
Cinnamyl alcohol	0.91	-	-	-	-	-	-
Coumarin	2.11	-	-	-	-	-	-
Decanol	-	-	-	0.04	-	-	-
α-Farnesene	-	0.08	-	-	-	-	-
Eugenol	-	85.73	-	-	-	-	-
Geranyl acetate	-	-	-	0.12	-	-	-
α-Humulene	-	-	-	-	1.01	-	-
Isoborneol	-	-	-	-	-	-	0.29
Limonene	-	-	9.02	65.59	0.83	2.20	1.05
Linalool	-	-	-	0.13	3.03	-	4.51
Myrcene	-	-	-	1.55	-	1.50	1.04
Neral	-	-	-	0.60	-	-	-
Neryl acetate	-	-	-	0.21	-	-	-
α-Pinene	-	-	3.97	2.34	0.30	11.40	3.43
β-Pinene	-	-	-	15.06	-	7.80	0.54
Sabinene	-	-	-	1.76	-	0.10	-
Salicylaldehyde	1.85	-	-	-	-	-	-
Styrene	2.34	-	-	-	-	-	-
α-Terpineol	-	-	-	-	0.70	1.70	0.48
γ-Terpinene	-	-	4.01	7.93	4.81	0.70	6.05
γ-Terpineol	-	-	-	-	-	-	0.14
Terpinen-4-ol	-	-	-	-	0.83	0.90	-
Thymol	-	-	-	-	2.04	-	48.80

Table 2. Inhibition zone diameter identified by disc diffusion method with essential oils tested.

Bacteria	IZD - Inhibition zone diameter (mm)[a]								
	Cinnamon	Clove	Eucalyptus	Lemon	Oregano	Rosemary	Thyme	Positive control	Negative control
Escherichia coli ATCC 25922	32.33 ± 1.29^{a}	9.67 ± 1.29^{c}	8.33 ± 1.29^{c}	6.00 ± 1.29^{c}	20.67 ± 1.29^{b}	8.00 ± 1.29^{c}	26.67 ± 1.29^{a}	27.86 ± 1.29^{a}	6.00 ± 1.29^{c}
Trueperella pyogenes ATCC 19411	29.67 ± 0.31^{a}	15.00 ± 0.31^{d}	6.00 ± 0.31^{e}	6.00 ± 0.31^{e}	21.00 ± 0.31^{c}	6.00 ± 0.31^{e}	25.00 ± 0.31^{b}	21.33 ± 0.31^{c}	6.00 ± 0.31^{e}
Fusobacterium necrophorum ATCC 25286	32.00 ± 1.13^{a}	21.67 ± 1.13^{b}	6.00 ± 1.13^{c}	6.00 ± 1.13^{c}	22.00 ± 1.13^{b}	6.00 ± 1.13^{c}	24.67 ± 1.13^{b}	37.33 ± 1.13^{a}	6.00 ± 1.13^{c}
Staphylococcus aureus ATCC 25923	38.33 ± 0.75^{a}	15.33 ± 0.75^{c}	8.00 ± 0.75^{d}	6.00 ± 0.75^{e}	36.00 ± 0.75^{a}	10.67 ± 0.75^{b}	36.00 ± 0.75^{a}	29.00 ± 0.75^{b}	6.00 ± 0.75^{e}

[a] Inhibition zone diameter, values represent mean of three replicates ± standard error. Different letters in the same line represent statistical difference ($P < 0.05$) in the size of inhibition zones including diameter of disc 6 mm formed under the paper disc by each essential oil.

smaller IZD was observed in *T. pyogenes* (29.67 mm). Clove essential oil produced an IZD varying from 9.67 to 21.67 mm, being the smaller IZD observed against *E. coli* (9.67 mm) and the larger IZD observed against *F. necrophorum* (21.67 mm). Eucalyptus essential oil produced an IZD varying from 8 to 8.33 mm, the smaller IZD was observed against *S. aureus* (8 mm) and the larger IZD against *E. coli* (8.33 mm) and no effect were observed on *F. necrophorum* and *T. pyogenes*. The lemon essential oil presented no IZD against any strains tested. The IZD produced by oregano essential oil varied from 20.67 to 36 mm, being the smaller IZD observed against *E. coli* (20.67 mm) and the larger IZD showed against *S. aureus* (36 mm). Rosemary essential oil produced an IZD varying from 8 to 10.67 mm, the smaller IZD was observed against *E. coli* (8 mm) and the larger IZD was seen against *S. aureus* (10.67 mm) with no inhibition effects being observed against *F. necrophorum* and *T. pyogenes*. The inhibition zone produced by the thyme essential oil varied from 24.67 to 36 mm, the smaller IZD was observed against *F. necrophorum* (24.67 mm), whereas the larger IZD was seen showed against *S. aureus* (36 mm).

For *E. coli*, essential oil of cinnamon and thyme had the larger ($P < 0.05$) IZD compared to the other essential oils. For *T. pyogenes* and *F. necrophorum*, cinnamon essential oil had the larger ($P < 0.05$) IZD compared to the other essential oils. Against the *S. aureus*, essential oil of cinnamon, oregano and thyme had the highest ($P < 0.05$) IZD compared to the other essential oils and positive control.

Discussion

Goñi *et al.* [16] showed that the major components of the essential oil of cinnamon is cinnamaldehyde. The content of cinnamaldehyde (86.5%) identified for cinnamon essential oil in our results is similar to Li *et al.* [17] (66.2-81.9), higher than Lv *et al.* [18] (77.3%) and lower than Zhang *et al.* [19] (92.4%). Our results highlighted the significant higher activity ($P < 0.05$) of cinnamon essential oil when compared to ceftiofur for *S. aureus* and *T. pyogenes*. *T. pyogenes* is considered one of the most important pathogens causing endometritis in dairy cows and cephalosporin-based drugs are most commonly used as treatments in cows with endometritis [1,20]. In addition, it is important to emphasize that cinnamon essential oil showed significantly higher antibacterial activity ($P < 0.05$) than the other essential oils (clove, eucalyptus, lemon, oregano, rosemary and thyme) investigated in the present study against the pathogenic species of *F. necrophorum* and *T. pyogenes*. Studies regarding antibacterial activity of essential oils in relation to these two pathogenic species causing endometritis have not been found. This is the first study to show the *in vitro* activity of essential oils in potentially endometritis-causing bacteria. According to our results, it was demonstrated the promising potential of cinnamon essential oil as therapy in cows with endometritis.

Based on the results of the clove essential oil components, our results are in accordance with literature data, which show that eugenol (> 85%) is the major component identified [21,22]. 1,8-cineol (> 80%) was the main component identified in eucalyptus essential oil, corroborating the results of previous study [23]. According to the identification of the lemon essential oil components, the main constituents identified were limonene (65.5%) and β-pinene (15%), which is in agreement with the study by Hsouna *et al.* [24] (39.7% and 25.44, respectively). Carvacrol (>70%) was the major component of oregano essential oil, similar results were described by Ebani *et al.* [25] (65.90%) and Fratini *et al.* [26] (65.94%). The main constituent of the rosemary essential oil was 1,8-cineol (47%) and is similar to that described by Yang *et al.* [27] (46%). The main compound identified in thyme

essential in oil in this study was thymol (48%), our results are in agreement with those described by Sokovic *et al.* [28] (48%) and Ebani *et al.* [25] (52%). Different growing environments such as altitude, hours of sunshine, temperature, rainfall, and parts of the plant extracted for the supply of essential oil may contribute to the difference between the percentages of identified active components [29,30].

The results of this study shown that the essential oils tested have different activity against the bacteria evaluated considering the IZD observed. To date, there have been no reports in the literature on the use of essential oils against strains of *T. pyogenes* and *F. necrophorum* strains.

Several authors have reported the antibacterial activity of *Cinnamon cassia* essential oil [19,31-34]. Our results of IZD of cinnamon essential oil against *E. coli* (32 mm) are similar to those described by Nimje *et al.* [31] (32 mm), Melo *et al.* [32] (30 mm) and Zhu *et al.* [34] (30 mm), and larger than those described by Zhang *et al.* [19] (19 mm). Based on the results observed in the present study the IZD (38 mm) of cinnamon essential oil against *S. aureus*, our results are in agreement with those described by Melo *et al.* [32] (40 mm) and Cieslak *et al.* [33] (35 mm), and larger than those described by Zhu *et al.* [34] (29 mm), Zhang *et al.* [19] (28 mm) and Nimje *et al.* [31] (21 mm). The main component of *Cinnamon cassia* oil used in this study was cinnamaldehyde (86%). The antibacterial activity of *Cinnamon cassia* essential oil is mainly due to the cinnamaldehyde component, that have hydrophobic properties, and can react with bacterial cell membranes, contributing to damage the membrane, another action is the ability to inhibit bacterial peptide and protein synthesis, thus having gram-positive and gram-negative bacteria action [34,35].

The antibacterial effects of clove essential oil have been described in the literature [36]. Our results of IZD of clove essential oil against *E. coli* (9 mm) were smaller than those noted by Oulkheir *et al.* [37] (16 mm), Prabuseenivasan *et al.* [38] (17 mm) and Bartkiene *et al.* [39] (11 mm). The IZD of clove essential oil against *S. aureus* (15 mm) noted in this study were similar than those described by Prabuseenivasan *et al.* [38] (16 mm) and Bartkiene *et al.* [39] (16 mm). The main component of clove essential oil was eugenol (85%), this compound is responsible for the antibacterial effect of clove essential oil. The eugenol has the ability to denature proteins and react with cell membrane phospholipids, altering membrane permeability [36].

Our study showed least inhibitory activity of eucalyptus essential against *E. coli* (8 mm) and *S. aureus* (8 mm). Fratini *et al.* [26] also did not observe IZD results using eucalyptus essential oil against *S. aureus* and *E. coli*.

Hsouna *et al.* [24] using lemon essential oil noted IZD against the reference strain of *E. coli* (15 mm) and *S. aureus* (22 mm). However, in the present study no antibacterial activity was identified against the bacteria tested.

Previous studies showed the antibacterial activity of oregano essential oil [26,40]. Our results of IZD of essential oil of oregano against *E. coli* (20 mm) were smaller than those noted by Melo *et al.* [32] (38 mm), while against *S. aureus*, our results of IZD (36 mm) are larger than those described by Ebani *et al.* [25] (13 mm). The major constituent of oregano essential oil in this study was carvacrol (72%). The main mechanism of action of carvacrol against the bacterial cell is the collapse of the proton motor force, the depletion of the ATP pool, and may act on the phospholipid bilayer of the cell membrane, increasing the permeability and leakage of vital intracellular components, which can cause membrane disruption and contribute to cell death [25].

The antibacterial activity of rosemary essential oil has been previously reported [41]. Our results of IZD of rosemary oil against *E. coli* (8 mm) and *S. aureus* (10 mm) were smaller than those showed by Prabuseenivasan *et al.* [38] (17 mm and 12 mm, respectively). The differences might be related with distinct composition of the essential oils tested.

The high antimicrobial activity of thyme essential oil has been previously revealed [11]. Thyme essential oil showed a range of IZD of 24–36 mm in this study. These results are in agreement with those reported by Oulkheir *et al.* [37] that showed activity of thyme essential oil against *E. coli* (18 mm) and *S. aureus* (22 mm). Thymol (48%), the main compound of thyme essential oil, have been found to exhibit antimicrobial activity [42], acting on the membrane of bacteria, contributing to the release of lipopolysaccharides, increasing the permeability of the cell membrane, and increasing the loss of ATP and the leakage of vital intracellular constituents [25].

Conclusion

This study revealed that essential oils have antibacterial activity against the main bacteria tested causing endometritis. Therefore, essential oils have great potential as an alternative to be explored as endometritis therapy in dairy cows. Further *in vivo*

studies are recommended to evaluate the use in clinical applications.

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