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MICROBIOLOGY

Antimicrobial activity of essential oils against *Pasteurella* spp. isolated from the oral cavity of domestic cats

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ABSTRACT: *Pasteurella spp. have been identified predominantly in the oral microbiota of domestic cats. However, Pasteurella spp. was significantly more prevalent in cats with inflammatory oral disease; and consequently, it was considered as part of the etiology in this disease. In addition, in animals, Pasteurella spp. have become increasingly resistant to a large number of antimicrobials. Natural products, especially essential oils, could contribute to minimizing this issue. This study determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of six essential oils against Pasteurella spp. isolates from the oral cavity of domestic cats. Our results showed that essential oils with better antimicrobial effectiveness against most of the Pasteurella isolates were lemongrass, tea tree and clove, with inhibition values between 50 to 800 µg mL^{-1}. All essential oils showed bacteriostatic activity against the species of Pasteurella isolated from the domestic cats. These results suggested that lemongrass, tea tree and clove oils have potential to be used in products for oral hygiene and treatment of oral infections in domestic cats.*
Key words: *cats, oral hygiene, essential oils, antimicrobial agents, Pasteurella.*

Avaliação da atividade antimicrobiana de óleos essenciais frente a isolados de *Pasteurella* spp. oriundos da cavidade bucal de gatos domésticos

RESUMO: *O gênero Pasteurella spp., considerado um comensal da cavidade bucal de gatos domésticos, vem sendo, nos últimos anos, apontado como possível agente etiológico de quadros inflamatórios crônicos bucais em felinos. Ademais, em animais, as espécies de Pasteurella têm apresentado cada vez mais resistência a um grande número de antimicrobianos de uso rotineiro. Nesse contexto, os produtos naturais, como óleos essenciais com potencial antimicrobiano tem sido alvo de estudos e apontados como alternativa terapêutica. Neste estudo, objetivou-se determinar a Concentração Mínima Inibitória (CMI) e da Concentração Bactericida Mínima (CBM) de seis óleos essenciais frente a isolados de Pasteurella spp. oriundos da cavidade bucal de gatos domésticos. Dos óleos essenciais testados, o capim-limão, tea tree, cravo e a camomila romana apresentaram ação bacteriostática frente aos isolados de Pasteurella spp. Contudo, os óleos de capim-limão, tea-tree e cravo apresentaram os melhores resultados, com valores de inibitórios entre 50 a 800 µg mL^{-1}. Esses resultados sugerem que os óleos de capim-limão, tea tree e cravo têm potencial para serem utilizados como produtos para higiene bucal e para o tratamento de infecções da cavidade bucal de gatos domésticos.*
Palavras-chave: *gatos, higiene bucal, óleos essenciais, agentes antimicrobianos, Pasteurella.*

INTRODUCTION

The oral cavity of domestic cats has a microbiota rich in aerobic and anaerobic bacteria (KIL & SWANSON, 2011). Some differences have been detected between bacterial microbiota from healthy or diseased oral cavities (DOLIESLAGER et al., 2011; DOLIESLAGER et al., 2013; STURGEON et al., 2014). *Pasteurella* spp. have been identified predominantly in the oral microbiota of healthy domestic animals (ABRAHAMIAN & GOLDSTEIN, 2011). However, *Pasteurella* spp. was significantly more prevalent in cats with gingivitis than in healthy cats; and consequently, it was considered as part of the etiology in this disease (DOLIESLAGER et al., 2011). In addition, the genus *Pasteurella* has been the most prevalent genus in samples of human lesions caused by cat bites, resulting in cellulitis,

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lymphangitis, abscesses, and septic arthritis (HEY et al., 2012; GUSTAVSON et al., 2016).

Penicillin and tetracyclines have been chosen as the best drug for the treatment of *Pasteurella* infections (LION et al., 2006; FERREIRA et al., 2015a), but there are some β-lactams antibiotics that have no antimicrobial effect, especially if used alone against different species of *Pasteurella* (WILSON & HO, 2013). In human, antibiotic resistance has rarely been reported among *Pasteurella* spp. isolates. However, in animals, *Pasteurella* spp. have become increasingly resistant to a large number of antimicrobials (KEHRENBERG et al., 2001). In this sense, a study showed that 12.1% of *Pasteurella multocida* isolated from oral samples of domestic cats presented resistance to all tested antimicrobials, and 75.6% displayed resistance to sulfamethoxazole-trimethoprim and 60.9% to sulfisoxazole (FERREIRA et al., 2015b).

At present, multidrug resistant bacterial isolates have been frequently identified in small animals (GANDOLFI-DECRISTOPHORIS et al., 2013; LEITE-MARTINS et al., 2015; YUKAWA et al., 2017; PULSS et al., 2018). These findings are important as the population of domestic cats and the contact between these animals and humans has increased, allowing cross-transmission of these bacteria (LLOYD, 2007). Conversely, in the last years, the synthesis of new antimicrobials has diminished (RANA et al., 2019). Thus, new treatment options are necessary to overcome the advent of bacterial resistance and natural products have this potential, including plant essential oils (EOs), which are natural, volatile and complex products, originating from their secondary metabolism (LARA et al., 2016). These compounds present great therapeutic and pharmacological potential, especially antimicrobial activity (CHINSEMBU et al., 2016). This study evaluated the antimicrobial activity of six essential oils against fourteen isolates of *Pasteurella* spp. from the oral cavity of domestic cats.

MATERIALS AND METHODS

Pasteurella spp. isolates

The study was carried out with fourteen isolates of *Pasteurella* spp. from the Laboratory of Innovative Therapies, Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Pirassununga, Brazil.

Pasteurella spp. were isolated by rubbing a sterile cotton-tipped swab over the teeth, gums, and tongue and then placing the swab into a glass bottle containing 1 mL of phosphate buffered saline. The bottle was mixed thoroughly using a vortex mixer and the resulting bacterial suspension was inoculated onto ovine blood agar (5%) (Blood agar base, HiMedia Laboratories, Mubai, India) and chocolate agar (Blood agar base, HiMedia Laboratories, Mumbai, India). The media were incubated aerobically at 35 ºC (+/- 2 ºC) for 24-48 h and pure cultures obtained. The isolates were identified using standard microbiological methods (ZBINDEN, 2015).

Essential Oils (EOs)

The EOs tested were bergamot (*Citrus bergamia*), roman chamomile (*Anthemis nobile*), lemongrass (*Cymbopogon citratus*), copaiba (*Copaifera officinalis*), clove (*Eugenia caryophyllus*) and tea tree (*Melaleuca alternifolia*). All oils were obtained commercially (Arte dos Aromas Indústria e Comércio Ltda, Brazil), and included a technical report of the chemical composition determined by gas chromatography (Table 1). All EOs were obtained in sealed amber glass bottles.

Agar well diffusion test

The agar well diffusion test was performed according to a previously described methodology (DUARTE et al., 2005), with minor modifications. Briefly, the inoculum of *Pasteurella* species was prepared in Mueller Hinton Broth (MHB, HiMedia Laboratories, India) at 37 °C for 24h. *Pasteurella* cultures in the exponential phase of growth were diluted with MHB and adjusted to McFarland scale 0.5 to obtain a final concentration of 1 to 2 x 10^8 CFU/mL for use in the assays. Then, the inoculum was added to Mueller Hinton agar (MHA, HiMedia Laboratories, India) at 50 ºC and distributed in 150 mm Petri plates. After the agar solidified nine holes were bored in each plate with a sterile tip (1 mL), and in each of them 40 µL (1600 µg mL^{-1}) of the EO to be tested was added. All the EOs were diluted in 80% (v/v) ethanol (Sigma, EUA). The 80% ethanol was used as a negative control and gentamicin (25 mg mL^{-1}, Sigma, EUA) as an internal control in all plates. The plates were then incubated in a bacteriological incubator at 37 °C for 24 h. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (mm) surrounding bacterial growth. The zone of inhibition above 7 mm in diameter was taken as positive result. The tests were performed in duplicate.

Determination of minimum inhibitory concentration (MIC)

The determination of the MIC of essential oils from roman chamomile, lemongrass, clove,

Table 1 - Chemical composition of the roman chamomile, clove, tea tree, lemongrass, bergamot and copaiba oils.

Compound	RC[1] (%)	CL[2] (%)	TT[3] (%)	LG[4] (%)	BG[5] (5)	CP[6] (%)
Geranial	-	-	-	2.5-10	-	-
Limonene	-	0.01	-		45	-
Citronellal	-	-	-	≤2.5	-	-
Linalool	-	0.1	-	≤2.5	15	-
terpinen-4-ol	-	-	25-50	-	-	-
α-terpinene	-	-	10-25	-	-	-
isobutyl angelate	25-40	-	-	-		-
α -Pinene		-	-	-	2	-
β-Pinene		-	-	-	2	-
p-menta-1,3-dieno	-	-	10-25	-	-	-
1,8 cineol		-	≤2.5	-	-	-
Geranyl acetate	-	-	-	2.5-10	-	-
Citral	-	-	-	50-100	-	-
B-Myrcene	0.1	-	-	10-15	-	-
Linalyl acetate	-	-	-	-	35	-
Tocopherol	-	-	-	-	-	<0.1
trans-β-Caryophyllene	-	-	-	-	-	>50
Methil Eugenol		0.1				
Isoeugenol	-	0,5	-	-	-	-
Eugenol	-	92	-	-	-	-

[1]RC, Roman chamomile oil; [2]Cl, Clove oil; [3]TT, Tea tree oil; [4]LG, Lemongrass oil; [5]BG, Bergamot oil; [6]CP, Copaiba oil. Results expressed as %.

and tea tree was performed according to the broth macrodilution method previously described (DUARTE et al., 2005), with some modifications. Briefly, from the stock culture of *Pasteurella* isolates, the inoculum was prepared in MHB (HiMedia Laboratories, India) and incubated under shaking at 37 °C for 24 h. The inoculum was diluted with MHB (HiMedia Laboratories, India) and adjusted to 0.5 McFarland to obtain a final concentration of 1 to 2 x 10^8 CFU/mL. Subsequently, 1.95 mL of the bacterial inoculum plus 0.05 mL of the diluted essential oils were added into glass tubes, at the following concentrations: 1600 µg mL^{-1}, 800 µg mL^{-1}, 400 µg mL^{-1}, 200 µg mL^{-1}, 100 µg mL^{-1}, 50 µg mL^{-1}, 25 µg mL^{-1} and 12.5 µg mL^{-1}. All tubes were incubated under shaking at 100 rpm for 18 to 24 h at 37 °C. Four internal controls were used for the test: 1) Mueller Hinton broth (MHB, HiMedia Laboratories, India) alone; 2) Pure bacterial inoculum; 3) Gentamicin (25 mg mL^{-1}, Sigma, EUA); and 4) 80% ethanol (Sigma, EUA) (Figure 1). The analyses were performed in duplicate.

At the end of the incubation period, 0.3 mL of each tube was transferred to 96-well microtiter plates and the absorbance (620 nm) was recorded. In addition, after the spectrophotometric reading, 0.005 mL resazurin (3 mg mL^{-1}, Rezazurin sodium salt, Sigma, EUA) was added in each well. Then, the plates were placed under shaking at 37 °C for 30 min at 60 min (or until color change) and a new reading was carried out. The interpretation of the results was based on the coloring, with blue color interpreted as absence of bacterial proliferation and pink as the presence of bacterial proliferation (SARKER et al., 2007).

Determination of Minimum Bactericidal Concentration (MBC)

MBC is the lowest concentration of essential oils required to kill the inoculum, and it was determined in the wells with no visible bacterial growth in the MIC assay after 24 h of incubation (LARA et al., 2016). A 0.1 mL aliquot was transferred from these wells to the surface of Muller Hinton agar (MHA, HiMedia Laboratories, India) and incubated at 37 °C for 24 h. Subsequently, a visual inspection of the plates was performed. The interpretation of results was based on the presence of colonies (RADAELLI et al., 2016); the presence of them

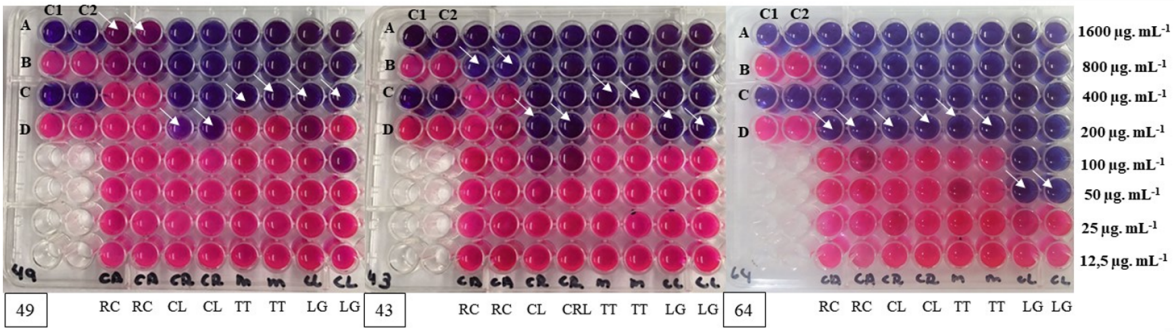

Figure 1 - Minimal inhibitory concentration (MIC, µg mL^{-1}) against isolates of *Pasteurella* spp. from samples of the oral cavity of domestic cats.

The white arrows indicate the MICs values. Disclosure by resazurin, blue color indicates inhibition of bacterial proliferation and pink color indicates bacterial proliferation. C1 and C2 = controls, A= Mueller Hinton broth (MHB, HiMedia Laboratories, India) alone; B= Pure bacterial inoculum; C= Gentamicin (25 mg mL^{-1}); D= 80% Ethanol; RC = Roman chamomile oil; CL = Clove oil; TT = Tea tree oil; LG = Lemongrass oil.

indicated that EO had bacteriostatic activity, whereas the absence of them indicated bactericidal activity of the EO tested. The assay was performed in duplicate.

RESULTS AND DISCUSSION

We tested the inhibitory and bactericidal effects of six essential oils on fourteen *Pasteurella* spp. isolates. Our results revealed the best bacteriostatic activity was achieved by lemongrass oil, with average of inhibition halo between 20 to 23 mm and with MICs and MBCs values ranging from 50 to 400 µg mL^{-1} (Figure 1, Tables 2 and 3). Lemongrass oil has been used for several purposes, as a natural antibiotic for quite some time (NAIK et al., 2010; BASSOLÉ et al., 2011; KORENBLUM et al., 2013; OLIVEIRA et al., 2013).

Table 2 - Diameter of inhibition zone expressed in mm of six investigated essential oils against isolates of *Pasteurella* spp. from samples of the oral cavity of domestic cats.

Pasteurella spp. strains	RC[1]	CL[2]	TT[3]	LG[4]	BG[5]	CP[6]	Gent[7]	ET[8]
1	11	14	11	21	0	3	25	0
2	11	14	12	20	0	0	28	0
3	12	15	11	22	2	4	29	0
4	13	16	14	23	3	3	26	0
5	13	16	14	20	0	0	25	0
6	13	14	13	20	0	0	26	0
7	11	15	13	23	2	3	28	0
8	12	15	12	21	2	0	27	0
9	13	15	14	22	0	0	29	0
10	13	14	14	23	0	0	25	0
11	11	14	11	21	0	0	28	0
12	12	15	12	22	0	0	27	0
13	12	16	13	21	0	0	26	0
14	13	16	14	20	0	0	28	0

[1]RC, Roman chamomile oil; [2]Cl, Clove oil; [3]TT, Tea tree oil; [4]LG, Lemongrass oil; [5]BG, Bergamot oil; [6]CP, Copaiba oil; [7]Gent, Gentamicin; [8]ET, 80% Ethanol Results expressed as mm.

Table 3 – MIC and MBC of four essential oils against isolates of *Pasteurella* spp. from samples of the oral cavity of domestic cats.

Pasteurella spp. strains	----------RC[1]----------		----------CL[2]----------		----------TT[3]----------		----------LG[4]----------	
	MIC[a]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC
	--------(µg mL^{-1})--------		--------(µg mL^{-1})--------		--------µg mL^{-1})---------		--------(µg mL^{-1})--------	
1	100	100	200	200	200	200	50	50
2	100	100	200	400	200	400	50	100
3	200	200	400	400	200	400	50	50
4	200	800	400	400	400	400	100	100
5	ND	ND	800	800	400	400	200	400
6	1600	ND	400	400	400	400	50	50
7	800	1600	800	800	200	400	100	100
8	800	800	200	400	200	400	100	200
9	200	800	400	800	200	200	100	200
10	100	100	200	200	400	400	50	200
11	100	400	800	400	200	200	100	200
12	400	ND	200	200	200	400	100	100
13	400	800	200	400	200	400	200	200
14	ND	ND	800	800	400	400	400	400

[1]RC, Roman chamomile oil;[2]CL, Clove oil; [3]TT, Tea tree oil; [4]LG, Lemongrass oil.
[a]MIC, Minimum inhibitory concentration; [b]MBC, Minimum Bactericidal Concentration.
ND, Not determined.

Several chemical compounds, such as citral, β-myrcene, dipentene, linalool, geranial, citronellol, among others have already been identified in the lemongrass oil composition (BASSOLÉ et al., 2011). Nevertheless, its antimicrobial activity has been attributed to the major presence of citral (PRABUSEENIVASAN et al., 2006; BASSOLÉ et al., 2011). In agreement, our results reinforce this fact, as the chemical compound with the highest concentration in the lemongrass tested was citral (50 to 100%, manufacturer's report). In the study of MAYAUD et al. (2008), the authors demonstrated that lemongrass displayed antibacterial activity against *P. multocida*. The chemical composition of the lemongrass oil tested by these authors had high concentrations of citral, which strengthens the theory cited above.

The tea tree oil also had antimicrobial efficacy against isolates of *Pasteurella* spp. with average of inhibition halo between 11 to 14 mm and MICs and MBCs values ranging from 200 to 400 µg mL^{-1} (Tables 2 and 3). The antimicrobial activity of tea tree oil has been attributed to the terpinen-4-ol, which is reported as the major compound, comprising approximately 40% of the composition of the EO (OLIVEIRA et al., 2013). The tea tree oil used in the present study also presented a high concentration of terpinen-4-ol (25 to 50%, manufacturer's report) in its composition, highlighting that perhaps this chemical compound is the main agent with antimicrobial activity. It is important to note that the tea tree oil has been used commercially in oral antiseptics.

The average of inhibition zone from the clove oil was between 14 to 16 mm against all the isolates *Pasteurella*. spp. MAYAUD et al. (2008) tested the sensitivity of *P. multocida* isolated from humans with clove and obtained a MIC value of 0.47% (v/v). In our study the MICs and MBCs values was 0.09% (v/v) (200 to 800 µg mL^{-1}). The discrepancies between results may be attributed the techniques used, as well as the form of dilution and incorporation of EO to the culture medium. In addition, differences between the concentrations of eugenol, the main chemical compound with antimicrobial activity, may explain the discrepancies between our results and another study (MAYAUD et al., 2008), which were 92% and 75.52%, respectively.

The least active oil was roman chamomile, with generally lower bacteriostatic activity (MICs and MBCs = 100 to 1600 µg mL^{-1}), since some isolates of *Pasteurella* spp. were resistant. In the agar diffusion test, the *Pasteurella* spp. isolates showed inhibition zone between 11 to 13 mm in the presence of roman chamomile oil. In previous study, the antimicrobial activity of roman chamomile oil against Gram-

negative bacteria was mainly due to the presence of isobutyl and methylbutyl angelate and isobutyl isobutyrate, with an inhibition halo between 9 to 19 mm and with MICs values ranging from 60 to 600 µg mL^{-1} (BAIL et al., 2009). One important observation which may explain the differences in results is that only isobutyl angelate was present in the composition of the roman chamomile oil tested by us.

Furthermore, the copaiba and bergamot oils failed to inhibit any of the tested isolates. The absence of antimicrobial activity of copaiba against the isolates of *Pasteurella*, a Gram-negative bacterium, was similar to another study, which had demonstrated that this oil possibly does not possess activity against Gram-negative bacteria (SANTOS et al., 2008). It could be explained by the intrinsic tolerance of some Gram-negative bacteria to plant volatile compounds, mainly due to the composition of their cell wall (COX & MARKHAM, 2007).

In addition, the absence of antimicrobial activity of bergamot was in contrast with other studies (FISHER & PHILLIPS, 2006; MANDALARI et al., 2007), which reported antimicrobial activity compared to different genera of Gram-negative bacteria. It should be noted that, to date, there are no published studies that have studied the sensitivity of *Pasteurella* spp. to three EOs (bergamot, copaiba, and roman chamomile). At the same time, this difference in our results may be due to the chemical composition of the oils tested. Apart from the different concentrations of the compounds and the synergism among them, other possible explanations for discrepant results between scientific studies are the phytogeographic origin, the season of the year and the mode of cultivation of the plant used to obtain the extract of the EOs. It has already been shown these factors affect the composition of the EO (BURT, 2004) and consequently their activity.

In conclusion, only the EOs of lemongrass, clove and tea tree displayed acceptable antimicrobial activity against *Pasteurella* spp. isolates from the oral cavity of domestic cats. Nevertheless, the results suggested that these three EOs have potential to be used in products for oral hygiene and maybe treatment of oral infections caused by *Pasteurella* spp. in domestic cats. However, further studies are necessary to demonstrate this potential, especially with respect to toxicity tests *in vitro* and *in vivo*.

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BIOETHICS AND BIOSSECURITY COMMITTEE APROVAL

The Bioethical Committee of the FZEA - Universidade de São Paulo, Pirassununga, SP, Brazil has approved this study under the protocol number 14.1.1500.74.6. All animals were handled according to the National Institutes of Health Guide for the Care and Use of the Laboratory Animals.

DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare with respect to the research, authorship and/or publication of this article.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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