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Inhibition of *Escherichia coli* from mastitic milk by copaiba oil

Inibição de *Escherichia coli* de leite mastítico pelo óleo de copaíba

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Abstract

The aim of this study was to evaluate the antimicrobial effect of two different copaiba oils against *Escherichia coli* obtained from mastitic milk. An antimicrobial assay was performed using an agar diffusion test, testing three solutions: a negative control and two solutions of different copaiba oils. This test was performed with 27 mastitic *E. coli* isolates. Eight isolates (29.63%) were inhibited by *C. langsdorffii* oil solution and seven by *C. officinalis* oil. The results of the present *in vitro* study suggest that copaiba oils may be potential sources of new and selective agents for the treatment of mastitis, pending further testing in *in vivo* models.
Key words: *Escherichia coli,* mastitis, copaiba oil, antimicrobial

Resumo

O objetivo deste estudo foi avaliar o efeito antimicrobiano de dois diferentes óleos de copaíba sobre isolados de *Escherichia coli* obtidos de leite mastítico. O ensaio antimicrobiano foi realizado utilizando o teste de difusão em agar, testando três soluções: um controle negativo e duas soluções dos diferentes óleos de copaíba. O teste foi realizado com 27 isolados de *E. coli.* Oito isolados (29.63%) foram inibidos pela solução com óleo de *C. langsdorffii* e sete pela solução com óleo de *C. officinalis.* Os resultados do presente estudo sugerem o óleo de copaiba como uma potencial fonte de novos e seletivos agentes para o tratamento da mastite, após posteriores testes em modelos in vivo.
Palavras-chave: *Escherichia coli,* mastite, óleo de copaíba, antimicrobiano

Introduction

Mastitis is one of the most important diseases in the dairy industry, including clinical and asymptomatic infections (MELCHIOR; VAARKAMP; FINK-GREMMELS, 2006). The social context of mastitis reflects its importance due the losses that can result in considerable milk disposal (more than 50%), the cost of medication and veterinary care, and due to the death and culling of animals with a loss of genetic material. In addition to these losses, there is still a potential risk to public health, since mastitis promotes the spread of pathogens that cause zoonosis and the toxins produced by them (MARTINS et al., 2010).

In Brazil, the mean prevalence of clinical and subclinical mastitis is 17.45% and 72.56%,

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respectively (LADEIRA, 2001). In many countries, the bacterial aetiology of mastitis has changed from contagious to environmental pathogens (SHUM et al., 2009) and programmes for control and prevention have not been totally effective for these types of pathogens (ERSKINE, 2000).

Escherichia coli has been considered as one of the main pathogens of environmental mastitis (MOREIRA et al., 2008), which can range from a simple local inflammation to severe systemic abnormalities such as ruminal atony, dehydration and toxemic shock resulting in the animal's death (WENZ et al., 2006). Despite intensive research, mastitis remains one of the most difficult diseases to control and treat in the dairy herd. Studies suggest that more than 4,7% of clinical mastitis cases caused by *E. Coli* may have originated from previous episodes of mastitis, from the same strain, where there was failure in the primary treatment (ALMEIDA et al., 2011).

With the increase of bacterial resistance to antibiotics, particularly by *E. coli,* there is considerable interest in investigating the antimicrobial effects of natural products such as propolis and essential oils against a range of bacteria, in order to develop other classes of natural antimicrobials useful against these pathogens (AL-REZA; RAHMAN; KANG, 2009). Propolis resin (GEBARA; LIMA; MAYER, 2002; NOGUEIRA et al., 2007; REZENDE; PIMENTA; COSTA, 2006; VARGAS et al., 2004) and a number of natural oils have presented activity against different bacteria (BETONI et al., 2006; PIERI et al., 2010a; PIERI et al., 2010b; SANTOS et al., 2008).

Thus, folk medicine has served as a source of compounds for use in the treatment of many diseases and combating pathogens. Copaiba (*Copaifera* sp.) has been highlighted among the natural products in Brazil; this large tree can reach up to 40 metres in height. Of the 72 species described worldwide by Index Kewensis, 16 are exclusive to the Brazilian flora (PIERI; MUSSI; MOREIRA, 2009).

An oil, called copaiba oil, is extracted from the copaiba tree, which has been used for more than 500 years in folk medicine with many potential properties including healing, anti-inflammatory, anti-microbial and anti-septic activity (CASCON; GILBERT, 2000; RIGAMONTE-AZEVEDO; WADT; WADT, 2004). The Food and Drug Administration (1972), the American regulatory body for drugs, approved the use of copaiba oil after it was subjected to tests for sensitisation and irritation using 25 volunteers; negative results were obtained for both assays.

Several studies have been conducted in order to identify microorganisms sensitive to copaiba oil as an antimicrobial, to further its use as a drug for treatment or for the prevention of diseases. Pieri et al. (2010b) evaluated the activity of copaiba oil against bacteria that cause throat and skin infections, periodontal disease and caries. Studies have presented data on the inhibition of Gram-negative bacteria, including *E. coli* (MENDONÇA; ONOFRE, 2009). However, other studies have presented the absence of inhibitory activity by copaiba oil on the growth of these bacteria (PACHECO; BARATA; DUARTE, 2006; PACKER; LUZ, 2007; SANTOS et al., 2008).

The aim of this study was to evaluate the antimicrobial activity of two copaiba oils obtained from different species of *Copaifera* against *E. coli* obtained from mastitic milk.

Materials and Methods

Plant material

The *Copaifera langsdorffii* oil used here was exuded directly from the trunk of one tree, according Pieri, Mussi and Moreira (2009). It was collected at Alfenas City, Minas Gerais State, Brazil in September 2008, and the tree was located at the geographic coordinates 21º 26' 33" S and 46º 0' 55" W. After collecting this material, it was stored in an amber bottle at a temperature of ± 4°C for subsequent analysis. *Copaifera officinalis* oil was obtained at a local drugstore in Alfenas City and

stored under the same conditions. Information about the extraction of this oil was not available.

Microorganisms

We used 27 *E. coli* isolates from mastitic milk obtained from the Veterinary Department of Federal University of Viçosa. The isolates were labelled from I-1 to I-27.

Solutions

Three solutions were used. The solution used as the negative control was prepared with 10 mL of Tween 80 (Vetec, Rio de Janeiro, Brazil) and 90 mL of distilled water. The first test solution consisted of a sterile negative control solution supplemented with copaiba oil from *C. langsdorffii* species, and the second test solution was supplemented with *C. officinalis* oil. Both test solutions were prepared to a final concentration of 10% copaiba oil.

Antimicrobial assay

Adapting the technique of diffusion in agar with modified orifice plating (ESMERINO et al., 2004), the bacteria were grown in Petri dishes containing a thin layer (10 mL) of bacteriological agar nº 1, and another layer above (20 mL) of Brain Heart Infusion (BHI agar) (Oxoid, Hampshire, United Kingdom). Three orifices were made in the BHI agar only and were evenly distributed on each plate.

An inoculum of 100 μL following the McFarland standard 0.5 was plated on the BHI agar surface. Next, each orifice was filled with one of the test solutions. The plates were incubated at 37°C for 24h.

The antimicrobial activity of each solution against bacterial isolates was identified by the presence or absence of an inhibition halo. The measurement of the halo diameter was used to compare the difference between the activities of the two different oils. The tests were performed in triplicate.

Statistical analysis

We compared both solutions for inhibition of all microbial strains and also the activities for each isolate. We also analysed the isolates for sensitivity to both oils. The data were analysed by an ANOVA and compared with a Tukey test or a Mann-Whitney test taking account if the data passed a normality test. Results were considered significant at $P<0.05$.

Results and Discussion

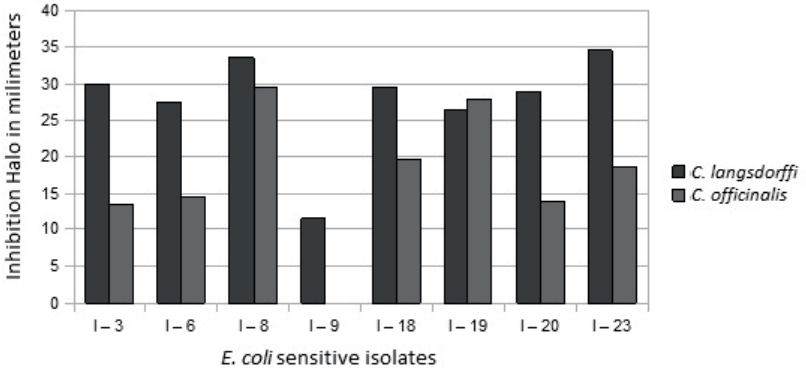
Eight isolates (29.63%) were inhibited by the *C. langsdorffii* oil solution and seven (25.92%) by the *C. officinalis* oil solution (Figure 1). The results showed that nineteen E. coli isolates (70.37%) possessed resistance to both copaiba oil antimicrobial substances with no inhibition halo.

The data show that *C. langsdorffii* was a more efficient antimicrobial in comparison to *C. officinalis*, as demonstrated by the number of sensitive isolates. An assessment of the data by ANOVA did not pass the normality test and the results were evaluated by the Mann-Whitney test. The results showed median inhibition halo values of about 29.25 mm and 16.5 mm to *C. langsdorffii* and *C. officinalis*, respectively, taking into account only the sensitive isolates, which was a statistically significant difference ($P<0.05$).

Variation in chemical composition is common among different species and even among individual trees (CASCON; GILBERT, 2000). Some research regarding their composition shows that even intraspecific genetic variation of plant species can alter the percentage of the active ingredient present in the oil. In addition, other factors such as climate, soil, timing and methods of planting, fertilisation, pesticide use, irrigation, and environmental conditions, the condition of the plant material

(fresh or dried), extraction techniques, botanical sources, planting, harvest and geographic patterns of variation (latitudes and longitudes) can affect the chemical composition of oils, causing changes in antimicrobial activity (NASCIMENTO et al., 2007). For example, resin oils from *C. reticulata* collected in Acre and Pará showed differences in activity against bacteria due to different chemical compositions (SANTOS et al., 2008).

Figure 1. Inhibition halo means of copaiba oils against eight sensitive isolates of *Escherichia coli* obtained from mastitic milk.



It has been suggested that these different results between our two oils may have occurred due to possible differences in composition, since they were obtained from different species of the genus *Copaifera* and originated from different regions. It is important to highlight that this suggestion could not be confirmed in this work, because we did not know the chemical composition of the oils.

In relation to the sensitive isolates, a different profile was presented in this assay. Isolate I-9 was considered resistant to *C. officinalis* oil and was less sensitive to the other oil. Isolates I-6, I-3, I-18, I-20 and I-23 presented an intermediate profile of sensitivity to both oils. Isolate I-19 presented halos without statistically significant differences to the intermediate isolates, except for isolate I-6 which was more resistant with smaller halos. Isolate I-8 was the most sensitive isolate, presenting halos with larger diameters those seen with all the other bacteria. All these comparisons presented a *P* value <0.05 which was considered a statistically significant difference.

Different studies have presented data without inhibition halos caused by copaiba oil using Gram-negative bacteria, including *E. coli* and *Pseudomonas aeruginosa* (PACHECO; BARATA; DUARTE, 2006; PACKER; LUZ, 2007; SANTOS et al., 2008). The present data do not concur with these works, because the data identified sensitivity in eight E. coli isolates (Gram-negative bacteria).

Pieri et al. (2009) and Mendonça and Onofre (2009) reported the inhibition of Gram-negative bacteria by copaiba oil, and both studies tested the oil against *E. coli.* The first study tested eight strains of *E. coli* with *C. langsdorffii* oil and obtained inhibition in two isolates (25%), while the other study showed the result of a test against only one ATCC strain. Our work presents additional data to confirm the potential of copaiba oil against these bacteria.

Gram-positive bacteria have been consistently described as sensitive to this phytotherapeutic by researchers in recent years (MENDONÇA;

ONOFRE, 2009; PACHECO; BARATA; DUARTE, 2006; PACKER; LUZ, 2007; PIERI et al., 2010a; PIERI et al., 2009; SANTOS et al., 2008). Santos et al. (2008) have presented results suggesting that the mechanism of inhibition against Gram-positive bacteria is mediated by activity against the bacterial cell wall, identified by electronic microscopy. However, we suggest that beyond this action, there may be compounds in the oil that act differently at the bacterial cell in view of the activity of the oils on the Gram-negative bacteria E. coli revealed in our research. This could result in a way to prevent or hinder the emergence of resistant bacteria, since this oil could contain different components acting synergistically on various structures and pathways in bacterial cells.

The different sensitivity profile of *E. coli* isolates presented in this work, as in the Pieri et al. (2009) study, may represent variability of the phenotypic profile of resistance in different strains of *E. coli.* This fact has been most worrying in terms of *E. coli* infections, since this strain of bacteria is known to possess a strong ability to acquire resistance, often to several antibiotics. Thus, the use of an herbal medicine could reduce the emergence of these resistant bacteria, given the possibility of multiple active ingredients acting on different sites or pathways in bacterial cells.

Conclusions

This study suggests that copaiba oils may be a potential source of new and selective agents against *E. coli*, based on this *in vitro* assay. Further laboratory and clinical studies of these oils are required in order to understand their antibacterial properties and how these agents could be used in animals for the treatment of mastitis.

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