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# ANTIMICROBIAL ACTIVITY OF *COPAIFERA* SPP. AGAINST BACTERIA ISOLATED FROM MILK OF COWS WITH MASTITIS

# *ATIVIDADE ANTIMICROBIANA DE COPAIFERA SPP. FRENTE ÀS BACTÉRIAS ISOLADAS DE LEITE DE VACAS COM MASTITE*

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**Abstract**

The antimicrobial activities of the oleoresin (OR) and the essential oil (EO) of *Copaifera* spp. were checked against microorganisms isolated from milk samples of cows diagnosed with grade III subclinical mastitis. The OR had good antimicrobial activity (MIC ≤ 100 µg/mL) against samples of coagulase-positive *Staphylococcus,* coagulase-negative *Staphylococcus*, *Streptococcus* of groups C, F, and G, and *Corynebacterium* spp. Meanwhile, the EO had good antimicrobial activity (MIC≤100 µg/mL) against coagulase-negative *Staphylococcus* and *Corynebacterium* spp. The OR and the EO were inactive and weak to inactive, respectively, against *Escherichia coli*. Overall, the OR had better antimicrobial activity than the essential oil against the 55 bacterial isolates ($p$<0.0001). The GC/MS analysis identified sesquiterpenes in EO and by the ESI FT-ICR MS method, the identification of diterpenic acids in OR was possible. Therefore, this raw plant material is promising for the development of phytotherapeutic drugs against bovine mastitis.

**Keywords**: bovine mastites; ESI FT-ICR MS; essential oil; minimum inhibitory concentration; oleoresin.

**Resumo**

A mastite bovina é um problema sanitário que afeta o gado leiteiro em todas as áreas produtoras do

mundo, sendo de difícil controle e erradicação. A atividade antimicrobiana da oleorresina e do óleo essencial de *Copaifera* spp. foi verificada frente aos micro-organismos isolados de amostras de leite de vacas diagnosticadas com mastite subclínica grau III. A oleorresina de *Copaifera* ssp. apresentou boa atividade antimicrobiana (CIM≤100 µg/mL) frente às amostras de *Staphylococcus* coagulase positivo, *Staphylococcus* coagulase negativo, *Streptococcus* do grupo C, F, G e *Corynebacterium* spp. Já o óleo essencial de *Copaifera* spp. apresentou boa atividade antimicrobiana (CIM≤100 µg/mL) frente aos *Staphylococcus* coagulase negativo e *Corynebacterium* spp. Em relação à *Escherichia coli*, a atividade antimicrobiana da oleorresina e o óleo essencial foi inativa e fraca à inativa, respectivamente. Conclui-se que, frente às 55 bactérias isoladas, a olerresina apresentou melhor atividade antimicrobiana do que o óleo essencial de *Copaifera* spp. ($p<0,0001$). A análise por CG/EM identificou sesquiterpenos no óleo essencial e pelo método ESI FT-ICR MS, foi possível identificar ácidos diterpenos na oleorresina de *Copaifera* spp. Por fim, sugere-se que a oleorresina de *Copaifera* spp. é uma matéria-prima vegetal promissora no desenvolvimento de medicamento fitoterápico para o tratamento de mastite bovina.

**Palavras-chave**: concentração inibitória mínima; ESI FT-ICR MS; mastite bovina; óleo essencial; oleorresina.

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# Introduction

Bovine mastitis, or mammitis, is a sanitary problem that affects dairy cattle throughout the world and is hard to control and eradicate[1]. Mastitis is an inflammatory process of the mammary gland, usually infectious. Different etiological agents may cause the disease, although bacteria represent the most common origin[2].

Depending on its manifestation, this disease can be classified as clinical or subclinical. In clinical mastitis, there are visible signs of inflammation in the udder and macroscopic abnormalities in the milk[3]. If the manifestation is subclinical, the inflammatory reactions are milder, and there are no visible alterations in the mammary glands and milk of the animal[4]. In this case, diagnosis depends on complementary exams, such as the *California Mastitis Test* (CMT). Depending on the level of reaction, the mastitis may be classified into grades I, II, or III[5].

Mastitis can also be classified as contagious or environmental, based on the type of pathogen. Microorganisms in genera *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Mycoplasma* cause contagious mastitis. In contrast, the microorganisms present in the environment occupied by the animal[4], such as coliform bacteria (*Escherichia coli*), *Streptococcus uberis, Actinomyces pyogenes, Pseudomonas* spp., fungi, algae, and viruses[6] cause environmental mastitis. One of the main etiological agents, both in Brazil and in other countries, is *Staphylococcus aureus*[7].

Etiological agents are becoming resistant to the antibiotics used to treat bovine mastitis, and cure rates from conventional treatments are low. As a result, alternative methods are increasingly being sought to circumvent this problem[8, 9].

There are many medicinal plants in the Cerrado biome that are used traditionally and are scientifically proven to have antimicrobial activity. Among those we can find copaiba trees, which are native to tropical regions of Latin America and West Africa and include 72 species, 16 of those endemic to Brazil[10]. Copaiba trees belong to genus *Copaifera*, family Leguminosae, subfamily Caesalpinioideae. The oleoresin, extracted sustainably from the trunk, is an exudate composed of resin acids (diterpenes) and volatile compounds (sesquiterpenes), which are responsible for its antimicrobial, wound healing, and anti-inflammatory pharmacological properties[10-13].

Previous studies have shown that the copaiba exudate inhibits the growth of the bacteria *S. aureus, E. coli*, and *Pseudomonas aeruginosa*[14], in addition to bacteria in genus *Streptococcus*[15]. According to Santos et al.[16], the oleoresin acts on the cell wall of Gram-positive bacteria, causing ruptures, loss of cytoplasmic compounds and even loss of cell wall, altering cell morphology and reducing its volume. However, Deus et al.[17] demonstrated the essential oil of *Copaifera multijuga* had better antifungal properties against *Aspergillus flavus* and *Candida parapsilosis* than the oleoresin.

Furthermore, the study developed by Bulgacov et. al.[18] to test copaiba oil creams effectiveness against *Staphylococcus* spp. isolated from cases of subclinical mastitis in 79 teats of lactating cows verified positive results in 94.93% (75/79) of samples.

The goal of the present study was to evaluate the antimicrobial activity of the oleoresin and the essential oil of *Copaifera* spp. against microorganisms isolated from grade III subclinical bovine mastitis and identify diterpenic acids and sesquiterpenes in oleoresin (OR) and essential oil (EO) of *Copaifera* spp., respectively.

# Material and Methods

1. *Plant material:* The unique OR pool of *Copaifera* spp. sample was purchased in June 2012 from Nutragyn Indústria de Alimentos Ltda (Goiânia, GO, BRA) and stored in amber vials kept at room temperature.

2. *Cultivation media:* All cultivation media (mannitol salt agar, MacConkey agar, Sabouraud agar, and agar base) were obtained from ISOFAR Indústria e Comércio de Produtos Químicos Ltda (Duque de Caxias, RJ, Brazil).

3. *Identification of diterpenes present in OR of copaifera spp. by electrospray ionization of fourie transformed ionic cyclotron resonance mass spectrometry (ESI FT-ICR MS):* The OR of *Copaifera* spp. sample was diluted to ≈ 0.25 mg/mL in water:methanol (1:1) that contained 0.1 % v/v of $NH_4OH$ for electrospray (ESI) in negative mode, ESI (-). The resulting solution was directly infused at a flow rate of 5 µL/min into the ESI source. The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany) was set to operate over a mass range of *m/z* 150-500. The ESI source conditions were as follows: a nebulizer gas pressure of 3 bar, a capillary voltage of 3.5 kV, and a transfer capillary temperature of 250 °C. The ions were accumulated in the hexapolar collision cell with time of $5.10^{-3}$s followed by transport to the analyzer cell (ICR) through the multipole ion guide system (another hexapole). The time-of-flight in the hexapole was of 0.7ms. Each spectrum was acquired by accumulating 32 scans of time-domain transient signals in 8 mega-point time-domain data sets. All mass spectra were externally calibrated using NaTFA. A resolving power, $m/\Delta m_{50\%} \cong 500\,000$, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height of $m/z \cong 400$ and a mass accuracy of < 1ppm provided the unambiguous molecular formula assignments for

singly charged molecular ions. The mass spectrum was acquired and processed using Data Analysis software (Bruker Daltonics, Bremen, Germany). The MS data was processed, and the elemental compositions of the compounds were determined by measuring the *m/z* values[19]. The proposed structures for each formula were assigned using the chemspider (www.chemspider.com) database and also following the study by Veiga Júnior and Pinto[10].

4. *Extraction and chemical analysis of the volatile fraction:* The volatile fraction was obtained from the OR of *Copaifera* spp. by hydrodistillation in a Clevenger-type apparatus for 4 hours. We used a Shimadzu QP 5050A instrument with a fused silica capillary column CBP-5 (30 m x 0.25 mm x 0.25 µm) for gas chromatography coupled with mass spectrometry (GC/MS) analyses. Helium was used as carrier gas at a constant flow rate of 1.0 mL/min and initial temperature of 60 ºC for 2 minutes, followed by heating at 3 °C/min up to 240 °C, 10 °C/min up to 280°C, and constant temperature of 280 °C for 10 minutes. The ionization energy was 70 eV. The injection volume was 1 µL of the diluted sample of dichloromethane (Vetec Química Fina LTDA, Duque de Caxias, RJ, Brazil) at a 1:5 ratio. Individual components of the EO were identified by comparison of mass spectra, and retention indices were calculated from a linear sequence of n-alkanes C8-C36[20].

5. *Phenotypic characterization of microorganisms isolated from grade III subclinical bovine mastitis:* The study was approved by the ethics committee of Universidade Federal de Goiás (UFG) under the protocol number 063/12. Milk samples were collected from farms near Goiânia in August 2012 and February 2013. Among the three properties, two of them performed mechanical milking and one manual milking, twice a day, with an average interval of 10-12 hours. It is noteworthy that animal productivity was 30 L/day on average. Before milking, teats were washed with water, and a strip cup was used to test the animals for clinical mastitis. Mammary quarters without clinical mastitis were subjected to the CMT test (Tadabras Indústria e Comércio de Produtos Agroveterinários Ltda, Bragança Paulista, SP, Brazil) to detect subclinical mastitis[5]. After the evaluation by the CMT test and the antisepsis with 70% alcohol (Vetec Química Fina LTDA, Duque de Caxias, RJ, Brazil), the first three squirts of teats diagnosed with grade III subclinical mastitis were discarded, and 10 mL of milk were collected in sterilized glass tubes and immediately transported to the Bacteriology Laboratory of the Tropical Pathology and Public Heath Institute (IPTSP) of UFG. Samples were plated on mannitol salt agar, MacConkey agar, Sabouraud agar supplemented with 0.05% chloramphenicol, and agar base supplemented with 5% defibrinated horse blood, following Winn et al.[21]. Morphotypes were separated based on morphology and staining traits, isolated and identified following Baron et al.[22], Brasil[23], and Winn et al.[21]. Standard strains of *S. aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, and *E. coli* ATCC 8739, obtained from IPSTP/UFG, were used as controls. After identification, microorganisms were added to Eppendorf tubes containing tryptic soy broth (TSB) and 20% glycerol (Vetec Química Fina LTDA, Duque de Caxias, RJ, Brazil) and stored at -20 ºC.

6. *In-vitro antimicrobial activity:* The minimum inhibitory concentrations (MIC) of the OR and the EO of *Copaifera* spp. against isolated microorganisms were determined using microdilution in Mueller-Hinton broth (MH), following the protocol of the Clinical and Laboratory Standards Institute[24]. Sterile U-bottom 96-well microplates were used. We added 200 µL of OR with a concentration of 2000 µg/mL to the first column. Next, 100 µL were extracted from the first column and transferred to the second. This step was repeated for each successive column to obtain a serial microdilution. The final concentration of the last column was 1.95 µg/mL. The same procedure was used for the EO. Afterward, the inoculants were added. They were prepared to a turbidity of a 0.5 McFarland standard ($10^7$ bacterial cells), obtained by reading a spectrophotometer at 625 nm (0.08 to 0.10 absorbance) and diluted at 1:10 for the broth microdilution procedure. Inoculations were carried out in duplicate. The MH broth of fastidious organisms was supplemented with 5% lysed horse blood, added before the microorganism was inoculated to avoid additional dilution of the antimicrobial agents in the microplates. Microplates were incubated at 37 ºC, and the MIC was read after 24 hours using 0.5% triphenyl tetrazolium chloride (TTC) (Vetec Química Fina

LTDA, Duque de Caxias, RJ, Brazil). The MIC was defined as the lowest concentration of OR or EO of *Copaifera* spp. that inhibited microbial growth. Following Holetz et al.[25], the antimicrobial activity was considered good for MIC values ≤100 µg/mL, moderate for 100<MIC≤500 µg/mL, weak for 500<MIC≤1000 µg/mL, and inactive for MIC values above 1000 µg/mL.

7. *Statistical analysis:* We analyzed frequency data from the phenotypic characterization of microorganisms and from the antimicrobial activities of OR and EO against bacterial isolates. A Wilcoxon non-parametric test was used to compare the activities of the OR and the EO, using the NPAR1WAY procedure in SAS at a significance level of 5%.

# Results

By the ESI FT-ICR MS method, we identified the diterpenic acids in OR of *Copaifera* spp. Figure 1 shows mass spectrum, in the negative mode-ESI (-), of compounds present in OR samples from *Copaifera* spp. Table 1 contains molecular formula estimated in the ESI FT-ICR MS and diterpenes present in them.

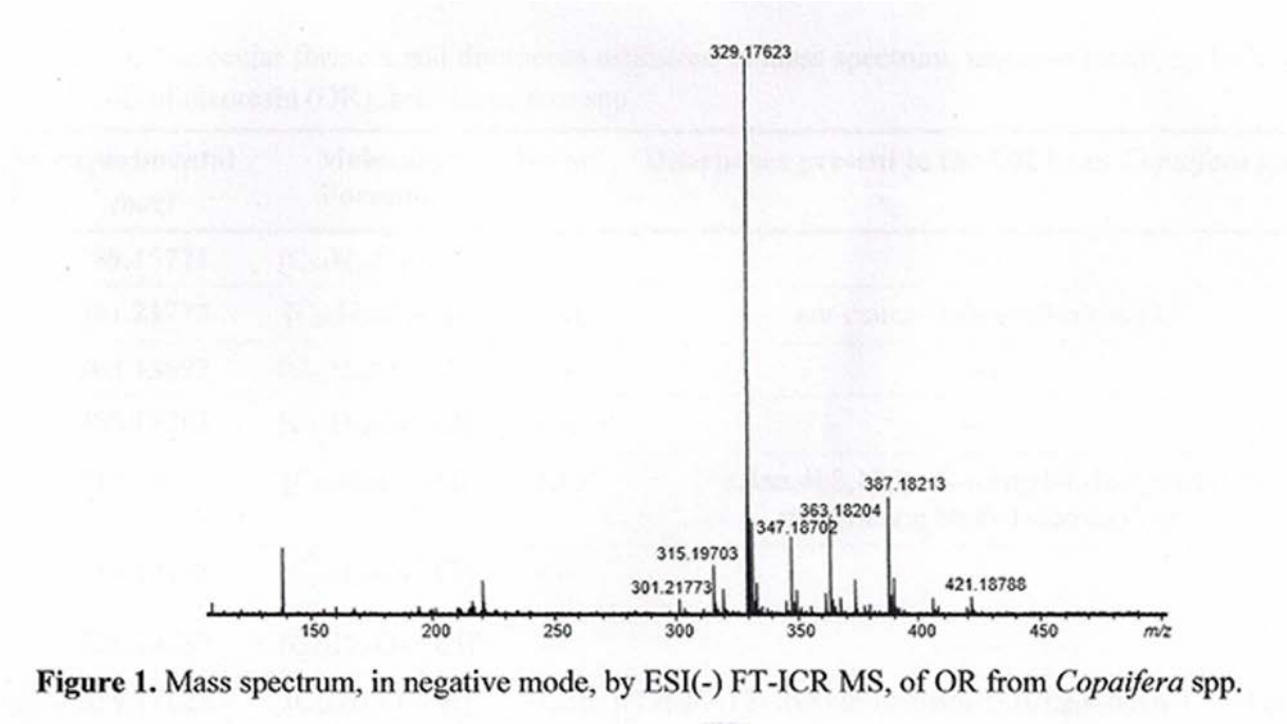


**Figure 1.** Mass spectrum, in negative mode, by ESI(-) FT-ICR MS, of OR from *Copaifera* spp.

**Table 1.** Molecular formula and diterpenes estimated in mass spectrum, negative mode, by ESI(-) FT-ICR MS of oleoresin (OR) from *Copaifera* spp

| Experimental (m/z) | Molecular Formula | Error | Diterpenes present in the OR from *Copaifera* spp. |
|---|---|---|---|
| 289.15771 | $[C_{15}H_{26}O_3+ Cl]^-$ | ---- | ---- |
| 301.21773 | $[C_{20}H_{30}O_2- H]^-$ | -1,41 | *ent*-caura-16-eno-19-oic acid |
| 303.13697 | $[C_{15}H_{24}O_4+ Cl]^-$ | ---- | ---- |
| 305.15263 | $[C_{15}H_{26}O_4+ Cl]^-$ | ---- | ---- |
| 315.19703 | $[C_{20}H_{27}O_3 - H]^-$ | -1,43 | (1α,4aα,4bβ,10β)-10-formyl-1,4a-dymetyl-8-methylenegibban-1-carboxylate |
| 319.13188 | $[C_{15}H_{24}O_5+ Cl]^-$ | ---- | ---- |
| 321.14757 | $[C_{15}H_{26}O_5+ Cl]^-$ | ---- | ---- |
| 329.17623 | $[C_{20}H_{25}O_4 - H]^-$ | -1,22 | [(17β)-3,17- dyhydroxyestra-1(10),2,4-trien-17-yl] acetate |
| 333.11122 | $[C_{15}H_{22}O_6 +Cl]^-$ | ---- | ---- |
| 333.20727 | $[C_{20}H_{30}O_4 - H]^-$ | ---- | *ent*-8(17),13-labdadiene-15,19-dioic acid (*ent*-agatic acid) or 13-clerodene-15,16-olydeo-18-oic acid |
| 347.18702 | $[C_{20}H_{27}O_5 - H]^-$ | -1,08 | (4aR,5S,7R,8aS,9aS)- 8a-hydroxy-3,4a, 5-trymetyl-2-oxo-2,4,4a,5,6,7,8,8a,9,9a-decahydronaphtho[2,3-b]furane-7-yl (2E)-2-metyl-2-butenoate |
| 363.18204 | $[C_{20}H_{27}O_6 - H]^-$ | -2,02 | 1-formyl-3,7-dyhydroxy-4a-(hydroxymetyl)-1-metyl-8-metylenegibbane-10-carboxylate |
| 367.18151 | $[C_{20}H_{28}O_4 +Cl]^-$ | -0,54 | 3,13-clerodadyene-15,16-olydeo-18-oic acid (patagonic acid) or *ent*-15,16-epoxy-7β-hydroxy-3,13(16),14-clerodatryene-18-oic acid (7-hydroxy-hardwyckyyco acid) |
| 373.20276 | $[C_{22}H_{29}O_5 - H]^-$ | -2,91 | ---- |
| 383.16329 | $[C_{20}H_{28}O_5 +Cl]^-$ | -0,57 | (5β, 7α, 8α, 9β, 10α, 13α, 14R) -7,14-dyhydroxy-15-oxokaur-16-en-19-oic |
| 387.18203 | $[C_{22}H_{27}O_6 - H]^-$ | -2,10 | ---- |
| 399.15823 | $[C_{20}H_{28}O_6 +Cl]^-$ | -0,59 | (1β, 5β, 9ξ, 10α, 11α, 13α, 14S) -1,11,14,20-tetrahydroxy-7,20-epoxykaur-16-en-15-ona |
| 421.18788 | $[C_{22}H_{29}O_8 - H]^-$ | -2,59 | ----- |

The EO of *Copaifera* spp. analyzed by GC/MS had many sesquiterpenes in its chemical composition; 96.16% of the identified components were sesquiterpene hydrocarbons, with β-caryophyllene as the major component (35.03%), followed by α-copaene (33.61%) (Table 2).

**Table 2.** Chemical composition of the essential oil (EO) of *Copaifera* spp. analyzed by gas chromatography coupled with mass spectrometry (GC/MS).

| Constituents | KI (Kovats Index) | Relative percentage (%) of constituents of the essential oil (EO) |
|---|---|---|
| **Sesquiterpene hydrocarbons** | | |
| δ-elemene | 1338 | 0.27 |
| α-cubebene | 1348 | 3.80 |
| α-copaene | 1376 | 33.61 |
| β-cubebene | 1388 | 1.44 |
| β-elemene | 1390 | 2.14 |
| Cyperene | 1398 | 0.21 |
| β-caryophyllene | 1419 | 35.03 |
| γ-elemene | 1436 | 0.60 |
| α-trans-bergamotene | 1434 | 1.33 |
| α-humulene | 1454 | 4.33 |
| Aromadendrene | 1460 | 2.23 |
| γ-muurolene | 1479 | 0.61 |
| cadina-1(6),4-diene<cis> | 1463 | 1.11 |
| α-muurolene | 1500 | 0.34 |
| β-bisabolene | 1505 | 0.40 |
| γ-cadinene | 1513 | 2.46 |
| δ-cadinene | 1523 | 4.18 |
| α-cadinene | 1538 | 0.18 |
| Germacrene B | 1561 | 1.89 |
| | | **96.16** |
| **Oxygenated sesquiterpenes** | --- | --- |
| **Unidentified compounds** | --- | **3.84** |
| **TOTAL** | --- | **100** |

Seventy-seven cows were tested using a strip cup and the CMT. Of those, 33 were healthy, one had clinical mastitis, and 43 had subclinical mastitis ranging from grades I to III. Twenty-four cows had grade III subclinical mastitis, and milk samples were collected from 36 teats for microbiological evaluation.

After cultivation, there was no yeast proliferation, and 60 bacteria were classified. Frequencies were 36.6% (22/60) of coagulase-negative *Staphylococcus* (CNS); 26.7% (16/60) of *S. aureus*; 6.7% (04/60) of *Corynebacterium* spp.; 5.0% (03/60) of *Streptococcus* of groups C, F, and G; 5.0% (03/60) of *Staphylococcus schleiferi schleiferi*; 3.3% (02/60) of *Escherichia coli*; 3.3% (02/60) of *Staphylococcus hyicus*; 3.3% (02/60) of *Staphylococcus schleiferi coagulans*; 3.3% (02/60) of *Leuconostoc* spp.; 1.7% (01/60) *of Staphylococcus intermedius*; 1.7% (01/60) of *Acinetobacter* spp.; 1.7% (01/60) of *Sphingomonas paucimobilis*; and 1.7% (01/60) of *Stomatococcus* spp.

The antimicrobial activity of the EO and the OR of *Copaifera* spp. was tested against CNS, coagulase-positive *Staphylococcus* (CPS: *S. aureus, S. schleiferi schleiferi, S. hyicus, S. schleiferi coagulans*, and *S. intermedius*), *Corynebacterium* spp., *Streptococcus* of groups C, F, and G, and *Escherichia coli*. The MIC test was not carried out for bacteria identified as *Leuconostoc* spp., *Acinetobacter* spp., *Sphingomonas paucimobilis* and *Stomatococcus* spp. because these are isolated etiological agents with low frequency in bovine mastitis.

For the CNS, the MIC of the EO of *Copaifera* spp. was ≤100 µg/mL for 9.1% (02/22) of the samples, 125 µg/mL for 9.1% (02/22), 1000 µg/mL for 22.7% (05/22), and >1000 µg/mL for the remaining 59.1% (13/22) (Table 3). Using the OR against the same bacteria, the MIC was ≤ 100 µg/mL for 40.9% (9/22), 1000 µg/mL for 31.8% (7/22), and > 1000 µg/mL for the remaining 27.3% (6/22) of the isolates (Table 3).

**Table 3.** Minimum Inhibitory Concentration of the essential oil (EO) and oleoresin (OR) of *Copaifera* spp. against coagulase-negative *Staphylococcus* (CNS) isolated from milk samples of cows diagnosed with grade III subclinical mastitis

| Microorganisms | MIC (µg/mL) | MIC (µg/mL) |
|---|---|---|
| CNS | *Copaifera* spp. EO | *Copaifera* spp. OR |
| (1) | >1000 | >1000 |
| (2) | >1000 | >1000 |
| (3) | >1000 | 1000 |
| (4) | >1000 | 62.5 |
| (5) | >1000 | 1000 |
| (6) | >1000 | >1000 |
| (7) | 1000 | 1000 |
| (8) | >1000 | 31.25 |
| (9) | >1000 | 15.62 |
| (10) | >1000 | 1000 |
| (11) | >1000 | >1000 |
| (12) | 1000 | 7.81 |
| (13) | >1000 | >1000 |
| (14) | 1000 | 7.81 |
| (15) | 1000 | 3.90 |
| (16) | 1000 | 7.81 |
| (17) | >1000 | >1000 |
| (18) | >1000 | 15.62 |
| (19) | 125 | 1000 |
| (20) | 62.5 | 1000 |
| (21) | 125 | 1000 |
| (22) | 15.62 | 31.25 |

The MIC values of the EO of *Copaifera* spp. for the isolates of *S. aureus* were 1000 µg/mL for 37.5% of the samples (6/16) and >1000 µg/mL for 62.5% (10/16). For *S. schleiferi schleiferi* (3/3), *S. hyicus* (2/2), *S. schleiferi coagulans* (2/2), and *S. intermedius* (1/1), the MIC was >1000 µg/mL (Table 4).

**Table 4.** Minimum Inhibitory Concentration of the essential oil (EO) and oleoresin (OR) of *Copaifera* spp. against coagulase-positive *Staphylococcus* (CPS) isolated from milk samples of cows diagnosed with grade III subclinical mastitis

| Microorganisms | MIC (µg/mL) | MIC (µg/mL) |
|---|---|---|
| CPS | *Copaifera* spp. EO | *Copaifera* spp. OR |
| ***S. aureus*** | | |
| (1) | 1000 | 31.25 |
| (2) | >1000 | 15.62 |
| (3) | >1000 | 15.62 |
| (4) | >1000 | 15.62 |
| (5) | 1000 | 7.81 |
| (6) | >1000 | 7.81 |
| (7) | >1000 | 31.25 |
| (8) | >1000 | 15.62 |
| (9) | >1000 | 125 |
| (10) | >1000 | 500 |
| (11) | 1000 | 7.81 |
| (12) | >1000 | 31.25 |
| (13) | 1000 | 7.81 |
| (14) | 1000 | 1.95 |
| (15) | 1000 | 31.25 |
| (16) | >1000 | 15.62 |
| ***S. schleiferi schleiferi*** | | |
| (1) | >1000 | 62.5 |
| (2) | >1000 | 62.5 |
| (3) | >1000 | 62.5 |
| ***S. hyicus*** | | |
| (1) | >1000 | 62.5 |
| (2) | >1000 | 31.25 |
| ***S. schleiferi coagulans*** | | |
| (1) | >1000 | 31.25 |
| (2) | >1000 | 250 |
| ***S. intermedius*** | | |
| (1) | >1000 | 31.25 |

The OR of *Copaifera* spp. had MIC ≤100 µg/mL against 87.5% (14/16) of the *S. aureus* isolates. For two isolates, *S. aureus* numbers (9) and (10), MIC values were 125 µg/mL and 500 µg/mL, respectively. MIC values against *S. schleiferi schleiferi* (3/3), *S. hyicus* (2/2), *S. schleiferi coagulans* (1/2), and *S. intermedius* (1/1) were ≤100 µg/mL, except for *S. schleiferi coagulans* number (2), where the MIC was 250 µg/mL, as seen in Table 4.

When the antimicrobial activity was tested against bacteria in genus *Corynebacterium*, the MIC value of the EO of *Copaifera* spp. was ≤100 µg/mL for 25% (1/4), but 1000 µg/mL for 50% (2/4), and >1000 µg/mL for 25% (1/4) of the remaining isolates. The MIC of the OR of *Copaifera* spp. was ≤100 µg/mL for 75% (3/4) of the same bacteria, and 125 µg/mL for 25% (1/4).

The EO of *Copaifera* spp. had antimicrobial activity against *Streptococcus* of groups C, F, and G, with MIC values of 1000 µg/mL for 33.3% (1/3) of the samples and >1000 µg/mL for 66.7% (2/3). Using the OR against the same bacteria resulted in a MIC≤100 µg/mL for 66.7% (2/3) and 1000 µg/mL for 33.3% (1/3) of the bacteria.

The MIC values for the EO of *Copaifera* spp. regarding the antimicrobial activity against *Escherichia coli* (2/2) were 1000 µg/mL and > 1000 µg/mL, while the OR had MIC > 1000 µg/mL against the same samples.

# Discussion

According to Brito et al.[26], bacteria in genus *Staphylococcus* are prominent in the etiology of intramammary infections of dairy cattle in Brazil. The same was observed in our results, where this genus predominated. The most common etiological agents were CNS and *S. aureus*, with frequencies of 36.6% and 26.7%, respectively.

The antimicrobial activity of the OR of *Copaifera* spp. against CPS was good in 87.5% (21/24) of the samples, in contrast to the EO, which was inactive in 75% (18/24) of the samples. Thus, CPS were more sensitive to the OR than the EO of *Copaifera* spp. These results corroborate the study of Santos et al.[16], who found that the OR of *Copaifera martii*, *Copaifera officinalis*, and *Copaifera reticulata* had good antibacterial activity (MIC ranging from 31.3 to 62.5 µg/mL) against Gram-positive bacteria (*S. aureus*, methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Enterococcus faecalis*), with lower bacterial viability after 3 hours.

The EO of *Copaifera* spp. had a good antimicrobial activity against 9.1% (02/22) of the CNS, but the OR had better antimicrobial effects against the same bacteria, with 40.9% (9/22). Therefore, CNS were also more sensitive to the OR than the EO of *Copaifera* spp. Current studies focused on CNS aim to reduce lineages resistant to antibiotics used in the treatment of mastitis. For example, Pereira et al.[27] demonstrated good antimicrobial activity of ethanol extracts of the bark of black jurema (*Mimosa tenuiflora* (Wild) Poiret- Leguminosae) and leaves of neem (*Azadirachta indica* A. Juss- Meliaceae) against CNS isolated from subclinical mastitis in buffaloes.

The EO of *Copaifera* spp. had good antimicrobial activity against 25% (1/4) of *Corynebacterium* spp., but the OR had a better antimicrobial effect, against 75% (3/4). No previous tests of antimicrobial activity of the EO and OR of *Copaifera* spp. against *Corynebacterium* spp. were found in the literature; however, we observed higher sensitivity to the OR than to the EO of *Copaifera* spp.

For *Streptococcus* of groups C, F, and G, the OR of *Copaifera* spp. also had a good antimicrobial activity against 66.7% (2/3) of the samples, unlike the EO of *Copaifera* spp., which was inactive against 66.7% (2/3). Thus, *Streptococcus* of groups C, F, and G had higher sensitivity to the OR than the EO of *Copaifera* spp. Other studies demonstrated that the OR of copaiba was active against bacteria in genus *Streptococcus*. For instance, Pieri et al.[28] showed in vitro antimicrobial activity of a solution of copaiba (*Copaifera officinalis*) against the oral microorganisms *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus pyogenes*, and *Enterococcus faecalis*. These studies suggest that the OR of copaiba is a potential alternative for orthodontic treatments.

In contrast to the other results, the antimicrobial activities of the EO and the OR of *Copaifera* spp.

against *Escherichia coli* were weak to inactive and inactive, respectively. Other studies found resistance of Gram-negative bacteria, including *E. coli and Pseudomonas aeruginosa*, to the OR of copaiba[16,29,30], corroborating our results.

Overall, the OR of *Copaifera* spp. had better antimicrobial activity (63.7%, 35/55) than the EO (5.5%, 3/55) against the 55 bacteria identified from milk samples of cows diagnosed with grade III subclinical mastitis ($p < 0.0001$). Pieri et al.[31] suggested that the copaiba OR has different components that act synergistically in many structures and metabolic paths of the bacterial cells, which may explain the results of our study.

According to Table 1, we identified sesquiterpenes, diterpenes, and the compounds with 22 atoms of carbon in the OR from *Copaifera* spp. samples. The sesquiterpens and diterpenes are formed by 15 and 20 carbon atoms, respectively[32]. While the compounds containing 22 atoms of carbon are suggestive as diterpenes esterified with ethyl or acetyl.

According to Veiga Júnior and Pinto[10], the 28 diterpenes described in OR from copaiba which were studied belong to cauran, labdan, and clerodan skeletons. Only two cauran have been described in scientific literature: *ent*-caura-16-eno-19-oic acid, which has also been found in the study samples, and *ent*-16-β-cauran-19-oic acid. By the ESI(-) FT-ICR MS method, used in this study, we could identify two more cauran in the OR from *Copaifera* spp.: (5β, 7α, 8α, 9β, 10α, 13α, 14R) -7,14-dihydroxy-15-oxokaur-16-en-19-oic (Table 1) and (1β, 5β, 9ξ, 10α, 11α, 13α, 14S) -1,11,14,20-tetrahydroxy-7,20-epoxikaur-16-en-15-ona (Table 1).

Veiga Júnior et al.[33] and Veiga Júnior and Pinto[10] argued that diterpene acids contribute for most of the therapeutic properties of the OR, and that antimicrobial activity can be explained by the presence of kaurenoic acid. Thus, the antimicrobial effect of the OR may be caused by diterpene acids, which can act synergistically with other components.

Antimicrobial activity of essential oils and their isolated constituents are related to chemical characteristics, functional and stereochemical groups of constituents, as well as with oxygenated compounds present in essential oils, particularly, those with fenolic structure (carvacrol, eugenol, timol), aliphatic terpenoids with ester grouping (geranyl acetate), alcohol (linalol) or aldheyde (cinamaldheyde), which are highlighted for their potential antimicrobial effect[34].

The EO, volatile fraction of OR from *Copaifera* spp., is constituted by oxygenated and hydrocarbon sesquiterpenes[10]. The EO from *Copaifera* spp. in this study presented hydrocarbons sesquiterpenes as the majority of compounds, with 96.16%. By taking into account the study by Henriques et al. [34], this fact would justify the weak and inactive EO antimicrobial activity.

The chemical compounds present in the EO in our study (Table 2) were similar to those found by other authors[12,15,17,35]. Both Veiga Júnior and Pinto[10] and Pieri et al. [36] attributed the anti-inflammatory, antibacterial, antifungal and anti-edema activities of the OR of copaiba to the sesquiterpene β-caryophyllene, which was the major component of the volatile fraction in the present study.

Although there are records in the literature of antimicrobial activity of β-caryophyllene, Souza et al.[37] demonstrated that the isolated caryophyllene compound had no antifungal activity, suggesting that certain phytochemicals only have an antimicrobial effect when they act synergistically with other components. Thus, the synergic functions of the various molecules found in the essential oils seem questionable compared to the effect of one or two compounds isolated from the oil[38].

Despite the different pharmacological activities recorded for the copaiba OR and the number of substances identified, little is known about the link between chemical structure and activity of each component[35]. For Tappin et al. [39], the OR of copaiba is an example where the full chemical profile, in addition to the characterization of the species present, is more important than isolated substances in determining putative pharmacological activities.

## Conclusions

In general, the OR of *Copaifera* spp. had good antimicrobial activity against the main pathogens that cause bovine mastitis, making this a promising raw plant material for the development of phytotherapeutic drugs to treat this disease. However, more studies are needed to better understand the connection between the chemical structure and the biological activities of its components.

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