Copaiba oleoresin activity against prevalent microorganisms in chronic wound infections

Authors
Viviane Vasconcelos Leite, Eline Lima Borges, Elisabeth Neumann, Karen Costa and Cristina Mariano Ruas

Background: Chronic wounds with excess bacteria present an increasing worldwide health problem. These wounds are difficult to heal, presenting prolonged inflammation and recurring microbial infections. Copaiba oleoresin (*Copaifera multijuga* Hayne) is a substance rich in sesquiterpenes and diterpenes, facilitating cicatrisation (the contraction of fibrous tissue formed at the site of a wound by fibroblasts, which decreases the wound size while distorting tissue). **Aim:** To analyse the antimicrobial activity (*in vitro*) from *C. multijuga* oleoresin against prevalent microorganisms in chronic wound infections. **Methods:** Characterisation of oils by gas chromatography coupled with mass spectrometry and antimicrobial sensitivity test by disk diffusion in agar and broth dilution. **Results:** Chromatographic analysis identified as major compounds: caryophyllene (60.89%), germachrene D (19.40%) and humulene (7.12%). The pure copaiba extract demonstrated activity against the pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Copaiba oleoresin, sunflower oil and mixtures in concentrations of 7%, 10%, and 12% presented no activity against *Escherichia coli* by means of broth dilution and small activity by agar diffusion. **Conclusion:** Copaiba oleoresin in concentrations utilised to treat cutaneous wounds presented no bactericide effects against prevalent microorganisms in wound infections.

Chronic wounds represent an increasing health problem in a wide range of nations (Di Giulio et al, 2018). Although analysing the prevalence and total incidence of chronic wounds presents difficulties, considering the diverse contexts and categories of these wounds, estimates suggest that 10% of the population will develop some kind of chronic wound during a lifetime, with a mortality rate of 2.5% related to such lesions (Chatterjee, 2012).

Studies show the prevalence of chronic wounds of different etiology types. A meta-analysis spanning 13 countries estimated a total prevalence of chronic wounds of 1.67 per 1,000 inhabitants (Martinengo et al, 2019). In a community from Barcelona, Spain, prevalence was 0.11% (Díaz-Herrera, 2021); and between 3.7% (O'Brien et al, 2016) and 5.0% (Skerritt and Moore, 2014) in Ireland. Prevalence was 7.8% in Germany's elderly care centres (Raeder et al, 2020) and 8.3% (Caron-Mazet et al, 2007) in France. In Brazil, studies show large data variation, with 8% prevalence in the Northeast region's older people (Vieira et al, 2017) and 0.164% among the general population of a city in the state of Minas Gerais (Borges et al, 2018). Considering such diverse contexts raises difficulty of analysing the total prevalence and incidence of chronic wounds.

Chronic wounds have a slow healing process with prolonged inflammation, and recurring

Author details can be found on the next page

microbial infections. Amongst the bacterial species found in these wounds are *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis* and *Klebsiella pneumoniae* (Di Giulio et al, 2018). Such wounds contribute to lower quality of life and bring discomfort to patients, while raising costs to healthcare systems (Di Giulio et al, 2018).

The most used forms of treatment against wound infections are topical and systemic antibiotic therapy (Negut et al, 2018). To mitigate risk of antibiotic resistant bacteria, healthcare professionals look for new treatment possibilities.

Historically in Brazil, myriad species of vegetables are utilised by the population as natural medicines (Masson et al, 2013; Penido et al, 2016). Among them, the copaiba (*Copaifera multijuga* Hayne) has been traditionally used for hundreds of years (Breitbach et al, 2013; Sousa, 1987). As Wikipedia states: "Copaiba is a stimulant oleoresin obtained from the trunk of several pinnate-leaved South American leguminous trees (genus *Copaifera*). The thick, transparent exudate varies in colour from light gold to dark brown, depending on the ratio of resin to essential oil (Wikipedia contributors, 2022)."

In 2011, 10% copaiba was included in the Brazilian *Pharmacopeia Form of Phytotherapics* (Brazil, 2011) as an anti-inflammatory and wound healer. In addition, *in vitro* and *in vivo* studies identified anti-inflammatory, antimicrobial, antiseptic and healing properties (da Trindade et al, 2018, Oliveira et al, 2020).

This study aimed to analyse antimicrobial activity of *C. multijuga* oleoresin's pure extract, and in concentrations of 7%, 10% and 12% mixed with sunflower oil. These concentrations include the recommended (10%) for wound treatment (Brazil, 2011), also values under (7%) and over (12%) this parameter.

Material and methods

C. multijuga Hayne's oleoresin (CM) was collected from the state of Amazonas (Brazil) by a team from the National Institute of Amazon Research — INPA (Manaus, Brazil), where the exsiccate is deposited. The use of this source guarantees the purity of the extract — which is also borne out by the results of the chromatography. The cold-pressed sunflower oil (SF) (Distriol®), utilised as a vehicle, was attested by Adolfo Lutz Institute (Sao Paulo/Brazil).

Preparation of samples

Antimicrobial activities from CM, from SF, and SF+CM 7%, 10% and 12% mixtures were tested against standard strains of *E. coli* (ATCC 25723) and *P. aeruginosa* (ATCC 25853) Gram-negative bacteria. Gram-positive bacteria used included: *E. faecalis* (ATCC 19433) and *S. aureus* (ATCC 29213), provided by the Microorganisms Ecology and Physiology Laboratory/ICB/UFMG, and *K. pneumoniae* (wild/multi-sensitive) given by the Microbiology Laboratory of the Clinical Hospital/UFMG.

The pathogens were activated in BHI agar (Acumedia®) and incubated for 24 hours in 37 ± 2ºC. Bacterial suspensions were prepared in a way as to correspond to the MacFarland standard of 0.5 (1 x 108 UFC/ml) (NCCLS, 2003).

Samples were prepared to obtain desired concentrations (7%, 10% and 12%), according to method utilised. All samples were vialed and stored in fresh, sunlight protected conditions.

Characterisation of oils

Volatile components of samples (CM, SF and SF+CM 10% were identified by gas chromatography, coupled with mass spectrometry (GC-MS) in QP2010 Ultra Shimadzu equipment, with an Agilent Technologies DB-5MS column.

Chromatographic conditions: starting temperature of 60°C, with heating of 10°C/minute, until 280°C in 35 minutes. Helium was utilised for gas drag. The temperature of the injector and detector was 280°C, and the ionisation source's was 200°C. Then 1 µl of diluted sample was injected in 1 ml of standard dichloromethane for analysis.

Compounds were identified by comparing the mass spectrums and retention indexes with the ones available in the equipment's library.

Antimicrobial activity tests

Disk diffusion

Agar disk diffusion test was done according to description found in Clinical and Laboratory Standards Institute (NCCLS, 2003). Positive control included antibiotics tetracycline (Laborclin®) (*E. coli, S. aureus, P. aeruginosa* and *E. faecalis*) or amikacin (*K. pneumoniae*), with 0.85% saline solution (SS) as negative control.

Standardised samples were spread in the Muller Hinton agar's surface, 6 mm sterilised paper filter disks were deposited and imbued with 10 µl of each sample. The plates were incubated at 37°C during 24–48 hours. The inhibition halos' diameter (in millimetres) formed around the disks were measured with a digital pachymeter, and results were expressed by the average, followed with standard deviation.

Viviane Vasconcelos Leite *is Nurse, Doctor of Medication and Pharmaceutical Assistance, Pharmacy College, Federal University of Minas Gerais, Belo Horizonte, and Nurse Osvaldo Rezende Franco Public Regional Hospital of Betim, Betim, Minas Gerais, Brazil; Eline Lima Borges is Nurse, Professor of Basic Nursing Department, Nursing School, Federal University of Minas Gerais, Belo Horizonte, Brazil; Elisabeth Neumann is Pharmacist, Professor Post-graduate Program in Microbiology, Microbiology Department, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Brazil; Karen Costa is Food Engineer, Doctoral student in Post-graduate Program in Microbiology, Microbiology Department, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Brazil; Cristina Mariano Ruas is Pharmacist, Professor of Social Pharmacy Department and in the Post-graduate Program in Medication and Pharmaceutical Assistance, Pharmacy College, Federal University of Minas Gerais, Belo Horizonte Brazil.*

Table 1: Main compounds identified in preparations of copaiba oleoresin, sunflower oil and sunflower oil mixture with 10% copaiba oleoresin.

Compounds	Preparations			
		CM	SF	SF+CM 10%
	Rt(min)	area%	area%	area%
β-elemene	9.97	1.26	–	–
Copaene	10.55	2.14	–	–
α-cubebene	10.56	–	–	5.98
Caryophyllene	11.18	60.89	–	55.66
α-bergamotene	11.28	–	–	6.11
Humulene	11.63	7.12	–	6.18
α-amorphene	11.84	1.25	–	–
Germacrene D	11.95	19.40	–	–
Germacrene B	12.12	1.64	–	–
β-mirceno	12.21	–	–	1.85
δ-cadinene	12.36	2.20	–	–
α-farnezene	13.21	–	–	1.48
τ-cadinol	13.88	1.06	–	–
diisopropilglicol	17.12	–	–	3.73
3-methyl-cyclooctene	18.78	–	–	5.57
1-undecanol	18.83	–	–	9.60
Palmitic acid	24.00	–	8.8	–
Stearic acid	30.40	–	3.6	–
Oleic acid	30.70	–	26.5	–
Vaccenic acid	30.80		2.2	
Linoleic acid	32.40	–	47.8	–
Linolelaidic acid	32.90	–	1.3	–
Linolenic acid	34.50	–	3.9	–

Abbreviations: – = absent; Rt = retention time; CM = *Copaifera multijuga* oleoresin; SF = sunflower oil; SF+CM 10% = sunflower oil mixture with 10% *C. multijuga* oleoresin.

Broth dilution

In plates from 96 wells were added 100 ul of BHI broth (double concentration) + Tween80 1%, then 100 ul of the preparations were added, as to obtain concentrations of 7%, 10% and 12% SF+CM, with 2% bacterial inoculum, followed by incubation at 37°C for 24 hours. To verify bactericide activity, after incubation, a sample of each well was striated in BHI agar, and plates were incubated according to microbial growth conditions (NCCLS, 2012). Colorimetric method evaluated Inhibition of bacterial growth, as described by Araújo and Longo (2016). After 24 hours of incubation, 20 µl of 0.01% sodium resazurin solution (R) was added to each well, followed by incubation at 37°C for 1 hour. Blue colouration demonstrates bacterial inactivity,

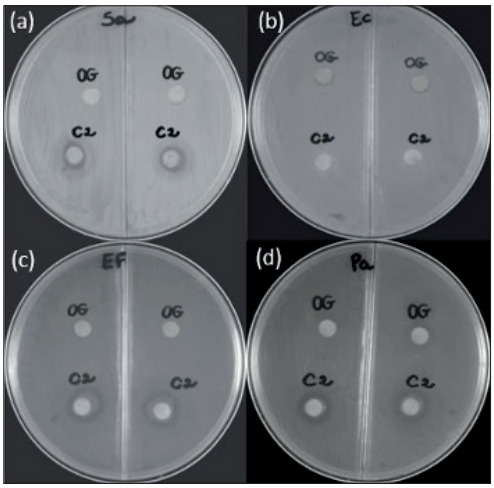

Figure 1. Image of test plates of antimicrobial activity from pure C. multijuga extract against the S. aureus (a), E. coli (b), E. faecalis (c) and P. aeruginosa (d) pathogens. Abbreviations: OG = pure sunflower oil; C2 = ORCM; Ec = E. coli; Ef = E. faecalis; Pa = P. aeruginosa; Sa = S. aureus.

and red, bacterial activity. Growth control used BHI broth + Tween80 + bacterial inoculum; while negative control utilised BHI broth + Tween80, and positive control of growth inhibition employed BHI broth + Tween80 + bacterial inoculum + tetracycline (Laborclin®) 30 µg/ml. Both tests were done in triplicate.

The author performed the broth dilution test and compared the results with the disk-diffusion test. In some cases, a MIC or MBC would be considered, however, in this instance it was not considered necessary because we start from a known concentration, already used as a healing agent.

If the research had used essential oils above 5%, the author would have needed to consider that they contain toxic terpenes as this research was with 'in natura' vegetable oils, widely used in Brazil.

Results

CM's chromatographic analysis identified three major compounds, mostly sesquiterpenes caryophyllene (60.89%), germachrene D (19.40%) and humulene (7.12%). In SF, the main compounds were the fatty acids: linoleic acid (47.80%), oleic acid (26.50%) and palmitic acid (8.80%). In the mixed sample SF+CM 10% the identified compounds were caryophyllene (55.66%) which remained the majority, -bergamotene (6.11%), humulene (6.18%) and -cubebene (5.98%) *[Table 1]*. Germacrene-D, a compound present in CM (19.40%), was not found in the mixture. Likewise, compounds

Table 2: Antimicrobial activity of pure copaiba extract, sunflower oil, and mixtures of sunflower oil and *C. multijuga* oleoresin against *S. aureus, E. coli, E. faecalis, P. aeruginosa* and *K. pneumoniae bacteria*.

Tested compounds	Analysed pathogens				
	S. aureus	E. coli	E. faecalis	P. aeruginosa	K. pneumoniae
	Average (SD[1])	Average (SD)	Average (SD)	Average (SD)	Average (SD)
CM	8.740 (0.212)	6.825 (0.262)	13.04 (0.191)	12.34 (0.622)	n/a
SF	n/a	n/a	n/a	n/a	n/a
SF+CM 7%	n/a	6.980 (0,156)	n/a	n/a	n/a
SF+CM 10%	n/a	6.705 (0.120)	n/a	n/a	n/a
SF+CM 12%	n/a	7.010 (0.170)	n/a	n/a	n/a
PC	27.060 (0.425)	23.745 (0.417)	25.210 (0.368)	26.535 (0.785)	22.570 (0.156)
NC	n/a	n/a	n/a	n/a	n/a

Results express arithmetic average (mm) from inhibition halo diameter around the disks in three trials. Abbreviations: CM = pure *C. multijuga* extract; SF = pure sunflower oil; SF+CM 7% = sunflower oil + 7% *C. multijuga* oleoresin; SF+CM 10% = sunflower oil + 10% *C. multijuga* oleoresin; SF+CM 12% = sunflower oil + 12% *C. multijuga* oleoresin; PC = positive control; NC = negative control; SD = standard deviation; n/a = not apparent.

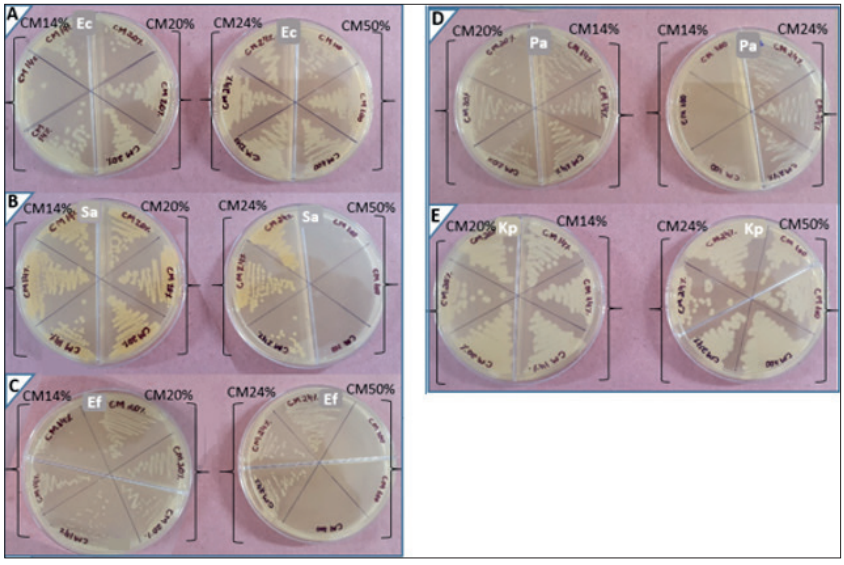

Figure 2. Image of the plates with cultures where the substances acted upon: sunflower oil + copaíba oleoresin 7%, 10%, 12% and 50% against microorganisms (A) E. coli, (B) S. aureus, (C) E. faecalis, (D) P. aeruginosa, (E) K. pneumoniae. Abbreviations: Ec = E. coli; Sa = S. aureus; Ef = E. faecalis; Pa = P. aeruginosa; Kp = K. pneumoniae; CM50%: C. multijuga pure extract; SF = pure sunflower oil; CM7% = sunflower oil + 7% C. multijuga oleoresin; CM10% = sunflower oil + 10% C.multijuga oleoresin; CM12% = sunflower oil + 12% C. multijuga oleoresin.

present in the mixture (SF+CM10%) were not observed in the pure samples. This is probably because the intermolecular interactions between the compounds present in the mixture do not favour volatilisation, and consequently are not detectable in the GC-MS analysis.

In disk diffusion test, pure extract of *C. multijuga* demonstrated microbial activity against *S. aureus, E. coli, E. faecalis* and *P. aeruginosa* *[Figure 1]*, presenting inhibition halo averages of 8.74 mm, 6.82 mm, 13.04 mm and 12.34 mm, respectively *[Table 2]*. *K. pneumoniae* did not present an apparent inhibition halo. Unsurprisingly, SF presented no antimicrobial activity. Mixtures of SF+CM (7%, 10% and 12%) presented some antimicrobial activity exclusively against *E. coli*, presenting inhibition halo averages of 6.98 mm for SF+CM 7% sample; 6.70 mm for SF+CM 10%; and 7.01 mm for SF+CM 12% *[Table 2]*.

In the study's second stage, bactericidal activity of *C. multijuga* was verified by broth dilution technique. The wells containing samples with CM 50% concentrations remained blue coloured, indicating activity against *S. aureus, E. faecalis* and *P. aeruginosa*. This did not happen with samples of CM 7%, 10% and 12%, indicating no bacterial growth inhibition.

Inhibition of growth of all microorganisms happened by means of tetracycline antibiotic (PC). No inhibitory action was observed by the addition of Tween80. Tween80 (polysorbate 80) is a surfactant and emulsifier, which was added to solubilise the oils with the culture medium; therefore, a control was performed to see if it interfered with the inhibition of bacterial growth. Growth control was effective for all microorganisms, and all reagents were tested.

After the incubation period, seeded plates were inspected for evaluation of the samples' antibacterial effect *[Figure 2]*. Lack of growth in plates B, C and D were confirmed, corresponding respectively to CM 50% samples inoculated with *S. aureus, E. faecalis* and *P. aeruginosa*.

Discussion

Two classes of substances mainly form the copaiba: sesquiterpenes (C15H24) and diterpenes (C20H32). The quantity of these compounds varies according to environment, climate, sample gathering techniques, plant characteristics, and soil aspects, amongst other factors (Veiga Junior and Pinto, 2002; Morelli et al, 2015). Results from chromatographic analysis of *C. multijuga* revealed cryophyllene as the main constituent; strengthening data found in literature (Veiga Junior and Pinto, 2002; Souza Barbosa et al, 2013; Morelli et al, 2015) and confirming sample authenticity.

β-caryophyllene is one of the main constituents of *C. multijuga*, presenting anti-inflammatory action, as well as antimicrobial

(Ziech et al, 2013; Bonan et al, 2015), antioxidant and analgesic properties (Leandro et al, 2012; Fidyt et al, 2016). D-germachrene presents antibacterial action due to lipophilic properties, which can facilitate penetration of antimicrobial agents inside cells, intensifying antibacterial activity (Cowan, 1999; Murari et al, 2008). Also identified were sesquiterpenes humulene and copaene, both of which have anti-inflammatory (Turkez et al, 2014) and anti-oxidising properties (Legault and Pichette, 2007). Other compounds in lower percentages were found in oleoresin, but they do not have properties related to wound healing described in the scientific literature.

Copaiba oleoresin constituents present in lesser quantities can affect antibacterial activities, as is the case with humulene's antimicrobial effect (Pichette et al, 2006), attributed to an interaction with bacteria cell's structures present in the cellular membrane, raising permeability and attaching itself to bacteria's vitally important constituents (Singh et al, 2002). It can be observed in analysis results that germacrene-D, δ-β-elemeno, α-β-copaeno, δ-cadinene, α-muurolene, germacrene-B, and τ-cadinol appear in the pure OR's composition. However, when observed in the preparation at SF+CM10%, these compounds disappear. The presence of these compounds in higher percentages in pure copaiba oleoresin can explain the antibacterial action against microorganisms *S. aureus, E. faecalis* and *P. aeruginosa*. Lowering the concentration of copaiba oleoresin, this activity disappears.

Considering the disk diffusion test, CM inhibited growth of pathogens *S. aureus, E. coli, E. faecalis* and *P. aeruginosa*, corroborating previous research data (Mendonça and Onofre, 2009; Pereira et al, 2018). Inhibition halos in disks with pure extracts were higher for *E. faecalis* and *P. aeruginosa*, demonstrating that copaiba's activity can be more effective against these microorganisms. Samples of 7%, 10% and 12% CM presented halo measures that befits lower antimicrobial activity against *E. coli* when compared to tetracycline.

Comparing previous results to the broth dilution test, inhibition of microbiological activity by pure copaiba extract was confirmed against *S. aureus, E. faecalis* and *P. aeruginosa*. No inhibition of *K. pneumoniae* happened in both tests, in any tested CM concentrations. Contrary to what was observed in the disk diffusion test, no inhibition of *E. coli* in liquid medium tests happened.

This finding is relevant, as wound healing in many chronic wounds does not proceed in normal stages, due to recurring bacterial infection (Frykberg and Banks, 2015). These results signal that utilisation of *C. multijuga* in chronic wound treatment could be a cost-effective option to mitigate infection occurrence, especially in still developing countries.

Still, it is important to know the compound's purity and concentrations of major and minor components for a successful wound treatment. In this study, purity of copaiba oil was guaranteed by origin of the extract (provided by INPA), and confirmed by chromatography, demonstrating the presence of the main chemical signalers in *C. multijuga*'s oleoresin.

Conclusion

The *C. multijuga* pure extract causes antimicrobial activity against prevalent bacteria in chronic wound infections, such as *S. aureus, E. faecalis* and *P. aeruginosa*. In concentrations of 7%, 10% and 12%, activity against *E. coli* was only observed in disk diffusion testing.

Copaiba oleoresin in concentrations utilised to treat cutaneous lesions presented no bactericide effects against microorganisms prevalent in wound infections. In the case of using higher concentrations as a therapeutic option, it is necessary to consider the cytotoxic action. WINT

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