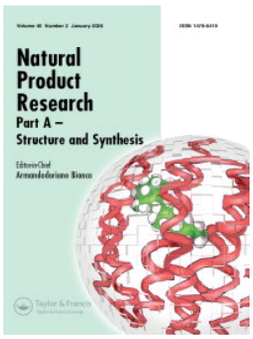

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Preclinical safety and antimicrobial evaluation of *copaifera epunctata* amshoff oleo-resin from the Amazon region

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ABSTRACT

Copaiba oleo-resin is widely used in Amazon folk medicine, but information about its toxicity is limited. We study the possible toxic effects of the oleo-resin of *Copaifera epunctata* Amshoff. Chemical analysis by GC-MS, acute oral toxicity test, acute dermal toxicity test, and acute eye irritation/corrosion test were conducted using Copaiba oleo-resin from the Amazon region. Chromatographic analysis revealed the presence of seven sesquiterpenes and five diterpenes, with emphasis on β-caryophyllene (39.7%) and β-bisabolene (7.1%). No signs of toxicity were observed in any of the tests, except for the repeated-dose dermal toxicity test, which induced changes in behaviour and in the hematological parameters of animals treated with 1000 mg/ml. There were no changes in body weight gain, macroscopic analysis, organ weight, feed and water intake or biochemical parameters. In the ocular irritation test, there were no signs of lysis, haemorrhage or coagulation. Copaiba oleo-resin has a low risk of toxicity.

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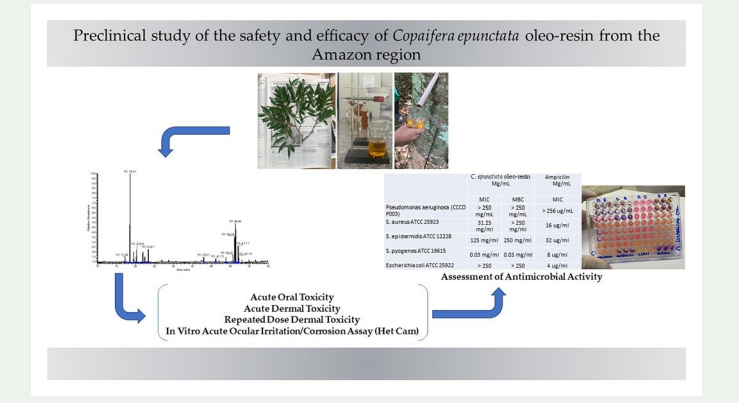

1. Introduction

Medicinal plants are frequently used by man because of the therapeutic potential of their biomolecules. The use of these plant products first started empirically, with knowledge about their effects on the treatment and cure of diseases accumulating over time until being recognised by modern medicine (Subramanian et al. 2018).

People in the Amazon region traditionally use these natural products for therapeutic purposes. This area is recognised worldwide for its great biodiversity and potential therapeutic arsenal available in its flora. The copaiba oleo-resin is a traditional herbal product found the Amazon region, which has several therapeutic actions, such as antibacterial, anti-inflammatory, anti-leishmaniasis, anti-proliferative, and anti-trypanosoma effects (Trindade et al. 2018; Medeiros et al. 2018).

Species of the genus *Copaifera* are among the medicinal plants most extensively used by traditional populations of the Amazon region due to the therapeutic properties of the oleo-resin obtained by perforation of the trunk and used to treat wounds, infections, and other diseases (Medeiros et al. 2018).

The diversity of medicinal plants in the Amazon region facilitates access by the population, but the lack of knowledge about their toxic effects has been a health risk (Nunes and Maciel 2017). In this context, in-depth scientific studies are important in order to understand the possible toxicity of these plants. Indeed, although medicinal plants have therapeutic actions, their possible toxicological effects should not be ruled out. Therefore, care should be taken to determine the correct identification of the plant and the possible contamination, overdose, and allergic and toxic reactions to its products even though they have been used for thousands of years (Subramanian et al. 2018; Pedroso et al. 2021).

Thus, we aimed to identify the possible toxic effects of *Copaifera epunctata* (also know as *C. reticulata* Ducke) oleo-resin in order to guarantee its safe use in traditional medicine, in addition to providing data for the production and development of an herbal product.

2. Results and discussion

The chemical constituents of the preparations were identified by comparing their retention indexes (RI) with the literature (Adams 2007), by co-injecting standards

(β-caryophyllene and α-humulene) and by comparing the fragmentation profiles obtained with a spectrometer. The percentage of each constituent is reported as a relative concentration value (%). The major chemical constituents identified in the COR were seven sesquiterpenes (73.28%) and five diterpenes (26.72%), as shown in the Table S1 and Figure S1.

Acute oral toxicity analysis of Wistar rats showed no changes in behaviour, nor any signs of intoxication such as tremors, convulsions, salivation, diarrhoea, lethargy, sleep, coma, pain, and suffering. The weight of the animals was also evaluated, showing no loss of body mass that could be considered as a sign of toxicity. On the contrary, the animals showed weight gain when compared to the control group (Table S2). These results classify the product as category 5, i.e. a product with a high level of biological safety according to the criteria of the experimental protocol used.

During the 14 days of the acute dermal toxicity experiment, the two animal groups showed no signs of intoxication or of weight loss and no physical changes, for example, injuries or changes in the skin, eyes and mucous. Also, no identified changes in respiratory frequency, circulatory or central nervous system or changes in behavioural patterns. Changes in weight of individually treated animals were calculated and compared to control animals, with no evidence of weight loss per animal or changes in average weight between groups (Table S3).

On the 14th day, evaluation according to the Draize criteria (Draize et al. 1944) yielded a score of "zero" (0), with no formation of edoema, bedsores or erythema on the animals' skin. After euthanasia and autopsy, macroscopic analysis of the animals' external and internal organs revealed no signs of toxicological reactions in the treated animals compared to the control group.

In the 21-day repeated-dose dermal toxicity assay, the intoxication results observed were similar to those of the acute dermal toxicity assay. The weight of the animals gradually increased, and feed consumption continued to rise during the study (Table S4). However, animals of both sexes treated with 1,000 mg/kg COR showed agitation, restlessness, piloerection, and diarrhoea, whereas animals treated with 1/4 and 1/8 of the maximum dose allowed by the protocol did not show any changes.

Regarding biochemical and hematological parameters, the animals did not show significant changes that could be characterised as toxic signs when compared to the male and female control groups (Table S5 and Table S6). However, analysis of leukocyte parameters revealed that male animals treated with 1,000 mg/kg COR showed changes in the number of leukocytes (Table S7). In the treated animals, the organs (kidneys, liver and testicles) were dissected and weighed immediately after autopsy to avoid drying out, and, no were identified changes in relative weight, when compared to organs of the control group (Table S8).

The treatment with COR at a dose of 1,000 mg/kg did not induce signs of lysis, haemorrhage or coagulation using the Het cam test. The positive control (0.1 N NaOH) showed a score of 17.60, confirmed the toxicity, and the negative control (0.9% saline with 1% Tween) showed no signs of toxicity (Figure S2).

Table S9 shows the MIC and MBC values of COR against a panel of bacteria frequently associated with skin discontinuity infections. At the concentrations of the COR tested, promising MIC results were obtained against Gram-positive strains (*S. aureus, S. epidermidis and S. pyogenes*), whereas no activity was detected against

Gram-negative bacterial strains (*P. aeruginosa* and *E. coli*) even at the highest concentration used. The results of the MBC obtained showed bactericidal action of the COR against strains of *S. epidermidis* and *S. pyogenes*. Against *S. aureus*, MBC values were above 250 mg/ml.

Several plants are known to have a therapeutic potential. Oil-resins from the *Copaifera epunctata* species are used by traditional people in the Amazon region. Several studies have demonstrated some of its pharmacological properties, such as antibacterial, anti-inflammatory and antineoplastic actions. However, in addition to these benefits, it is necessary to consider possible harmful effects. Therefore, safety tests on these natural products are necessary to elucidate the possible toxic effects of medicinal plants (Trindade et al. 2018; Gaston et al. 2020).

In the present study, we chemically characterised and investigated the possible toxic effects of *C. epunctata* oleo-resin (COR), in addition to its antimicrobial action. The major constituents present in COR are sesquiterpenes, including β-caryophyllene (42.61%), β-bisabolene (7.58%), α-humulene (6.29%), β-selinene (5.73%), trans-α-bergamotene (5.26%), α-selinene (3.21%) and β-elemene (2.60%). Santos et al. (2020) identified the following sesquiterpene components of COR: β-bisabolene, trans-α-bergamotene, and β-caryophyllene, while Bardají et al. (2016) identified β-bisabolene, trans-α-bergamotene, and β-selinene, in agreement with some of the compounds detected in the present study.

Previous research (Lima et al. 2020) reported a chemical composition for COR similar to that observed in the present study, but with quantitative differences, which can be justified due to environmental factors, characteristics inherent to the plant itself, in addition to the method adopted for the oil extraction process. The main COR compounds described in the literature are β-caryophyllene, with antibacterial and anti-inflammatory activity (Yoo and Jwa 2019; Parisotto-Peterle et al. 2020), and β-bisabolene, with anti-inflammatory activity (Almeida Júnior et al. 2021).

The chemical constituents present in the oleoresin can exert biological activity in isolation, however, it is believed that a synergistic or additive effect occurs between them (Arruda et al. 2019). Therefore, these oils continue to have the same pharmacological relevance, despite the differences in composition and concentration.

In the present study, we did not identify toxicity of the oleoresin up to a dose of 2,000 mg/kg when used for 14 days. This result is in agreement with previous reports using the oleoresin of *Copaifera ssp.* (Almeida Júnior et al. 2021; Silva et al. 2021). Therefore, *C. epunctata* oleoresin was classified as category 5, since the acute oral lethal dose was estimated to be greater than 2,000 mg/kg according to the OECD Guide 423 (OECD 2002), indicating a safety margin for pharmacological use as an alternative source. Furthermore, we did not identify dermal toxicity or changes in the animals' internal organs after macroscopic analysis. There was also no change in the animals' feed intake or body weight during the treatment period. This information is essential to ensure the safety of medicinal plants and the development of phytotherapeutic medicines.

In the present study, the evaluation of repeated-dose dermal toxicity revealed that the animals that received the oleoresin consumed food and water regularly and also showed a gradual increase in body weight. Regarding behaviour, animals treated with the maximum dose authorised by the protocol (1,000 mg/kg) showed agitation,

restlessness, piloerection and diarrhoea. Thus, our results are in accordance with previous research (Lima et al. 2020) in animals treated only with topical *C. epunctata* oleo-resin in natura, which showed behavioural signs of skin irritability after application for 5 consecutive days.

Regarding hematological parameters, we observed changes in the number of leukocytes only in the group of male animals treated with 1,000 mg/kg COR. An increase in leukocytes indicates an inflammatory process promoted by macrophages when faced with a stimulus (Freitas et al. 2019). Therefore, this change may be related to possible signs of intoxication in the animals of this group.

The ocular membrane irritation test for identifying the danger of chemical products is important as an alternative approach to toxicity in different areas of research using the *in vitro* model (Lanzerstorfer et al. 2021). Our study demonstrated that *C. epunctata* oleoresin does not cause eye irritation at the dose tested and is not find in the literature studies on the ocular safety of *C. epunctata* oleoresin or on its adverse effects when in contact with the ocular region. In the study of antimicrobial activity, we found that COR was effective only against Gram-positive bacteria, with emphasis on its potential against *S. pyogenes*, a bacterium with effects ranging from common bacterial pharyngitis to more serious diseases such as scarlet fever, a clinical syndrome characterised by the appearance of skin rashes (Ferretti et al. 2016). Although the variation of chemical constituents present in the COR influences the antimicrobial action (Tincusi et al. 2002; Santos et al. 2020), diterpenes have been associated with this biological action described for this oil-resin (Bardají et al. 2016). Tincusi et al. (Tincusi et al. 2002) isolated thirteen diterpenoids from the oleo-resin of *C. paupera* and attributed significant antimicrobial activity to these compounds.

We demonstrated that COR contains compounds of natural origin, such as β-caryophyllene (42 0.61%) and kaurenoic acid (10.5%), that are promising for the development of new antimicrobial agents, as demonstrated by Moo et al. (2020). These authors reported that β-caryophyllene caused alterations of the bacterial membrane, what may be involved with the antimicrobial mechanism of this chemical constituent. Therefore, it is possible to infer that the antimicrobial potential of COR, rich in β-caryophyllene and kaurenoic acid, is promising for the development of efficient antimicrobial agents for the treatment of infections caused by clinically relevant microorganisms.

3. Experimental

See Supplementary Material.

4. Conclusions

The present results demonstrated that oleoresin from *C. epunctata* (also know as *C. reticulata*) is highly safe. Most of its compounds belong to the group of sesquiterpenes, mainly β-caryophyllene, and some diterpenes. The oleoresin showed low toxicity in preclinical tests of acute oral, acute dermal and repeated-dose toxicity, and of

ocular irritation. We also proved the antimicrobial effects of COR against Gram-positive bacteria, but not against Gram-negative ones, which partly accounts for its wide use in popular medicine, mainly for the treatment of skin infections, thus representing a viable alternative for the treatment of infections caused by clinically relevant Gram-positive bacterial strains.

Finally, although the oleo-resin of *C. epunctata* is widely used in folk medicine, there are no literature studies evaluating all the preclinical safety tests required by the Brazilian regulatory agency for the development of herbal medicines based on *Copaifera epunctata*. Thus, the present study contributes to the knowledge of this practice as a therapeutic alternative.

Authors' contributions

CRediT: **José Sousa de Almeida Junior**: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft; **Daniela Vieira de Castro Macambira**: Data curation, Formal analysis, Investigation, Methodology, Resources; **Aline de Morais Gomes**: Data curation, Formal analysis, Investigation, Methodology, Resources, Software; **Francisco Flávio Vieira de Assis**: Formal analysis, Investigation, Methodology, Validation; **Sandra Layse Ferreira Sarrazin**: Investigation, Methodology, Resources, Supervision, Writing – review & editing; **Valdir Florencio Veiga Junior**: Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing; **Elaine Cristina Pacheco de Oliveira**: Data curation, Methodology, Resources, Validation, Visualization; **Tânia Mara Pires Moraes**: Formal analysis, Project administration, Resources, Supervision, Visualization; **Antonio Humberto Hamad Minervino**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing; **Waldiney Pires Moraes**: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing; **Lauro Euclides Soares Barata**: Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Disclosure statement

The authors declare no conflict of interest.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Federal University of Western Pará (protocol code 0320220198, approved in May 2022).

Informed consent statement

Not applicable.

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Data availability statement

The raw data from this work are available upon request to the corresponding author.

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