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Phytochemical screening and biological activities of *Copaifera langsdorffii* Desf. (Fabaceae) organic extracts

Caio Cézar Oliveira de Lucena*, Camila Joyce Alves da Silva,
Raudiney Frankilin Vasconcelos Mendes, Kêsia Xisto da Fonseca Ribeiro de Sena,
Teresinha Gonçalves da Silva and Rafael Matos Ximenes

Departamento de Antibióticos, Universidade Federal de Pernambuco, 50740-525, Recife, Brazil.

This study explores the therapeutic potential of *Copaifera langsdorffii* Desf. (Fabaceae), commonly known as Copaíba, a legume renowned for its oleoresin extraction. The organic extracts from Copaíba's leaves, stem bark, and wood, using n-hexane, ethyl acetate and methanol were investigated for their antimicrobial, antioxidant and anti-inflammatory activities. *In vitro* assessments included determining minimum inhibitory concentration (MIC) against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, DPPH• radical scavenging and quantifying inflammatory mediators (NO, TNF-α, IL-6, IL-10) produced by LPS-stimulated murine peritoneal macrophages (MPM). Phytochemical analysis using thin-layer chromatography (TLC) unveiled various secondary metabolites in the extracts, with particularly active ones exhibiting high levels of flavonoids, terpenes and coumarins. The findings emphasize the importance of conserving Copaíba species and leveraging its traditional medicinal use across diverse ailments. Notably, this research paves the way for exploring alternative plant parts without compromising the integrity of oleoresin extraction. The identified bioactive compounds in Copaíba suggest a promising correlation with its observed therapeutic effects, highlighting its potential as a valuable natural resource in the treatment of various diseases.

Key words: Phytochemical screening, biological activities, *Copaifera langsdorffii*, extracts.

INTRODUCTION

Natural resources have always been utilized by humans for various purposes, with plants standing out for their medicinal properties applied in the treatment of various illnesses. The Brazilian flora is widely known for its vast biodiversity, and studies on the therapeutic properties of natural products, as well as the bioactive molecules found in these plants, have been growing each year, piquing the interest of the pharmaceutical industry for the development of new drugs (Calixto, 2019; Castro, 2021; Valli et al., 2018).

Legumes (Fabaceae) are considered the third largest family of angiosperm plants, with a wide spectrum of distribution, biodiversity, and uses by humans. Legumes are also described as plants rich in phytochemicals with

*Corresponding author. E-mail: caio.colucena@ufpe.br. Tel: +55 81 2126 8866.

high therapeutic potential, as flavonoids, alkaloids, carotenoids, saponins, lectins, and gallic acid. Among the various types of molecules, phenolic compounds are by far the most abundant secondary metabolites found in these plants (Motta et al., 2019; Tor-Roca et al., 2020). These phytochemicals exhibit a wide structural diversity and are strongly associated with a range of biological activities, such as the reduction of various types of cancer, infections, inflammation, diabetes, oxidative stress, among others, making them important targets for the development of new products in various sectors of interest (de Vargas et al., 2015; Caleja et al., 2017; Kokoska et al., 2019; Aly et al., 2019; Macêdo et al., 2020; Albuquerque et al., 2021; Salehi et al., 2021; Usman et al., 2022).

The specie *Copaifera langsdorffii* Desf. is commonly known as "pau d'óleo" or "pau d'óia" due to the oleoresin obtained from perforating its stem. This natural product is also widely used because of its well-known medicinal properties, such as antimicrobial and anti-inflammatory effects (Teixeira et al., 2017; Diefenbach et al., 2018; Oliveira et al., 2020). The literature is abundant regarding the activities and characterization of Copaíba oil. However, there are few studies on the properties of organic extracts from different parts of this plant, presenting a promising research strategy.

Among the ethnopharmacological activities of secondary metabolites found in Copaíba species described in the literature, inflammation stands out. This is a complex process that occurs when there are infections or tissue injuries triggered by pathogens or irritants. Immune cells, blood vessels, and various mediators are involved in this process (Ahmed et al., 2017; Truong et al., 2019). Acute inflammation is a physiological response of the body resulting from an exogenous stimulus, often caused by pathogenic microorganisms, which leads to the activation of the immune system. Consequently, there is an increase in vascular permeability caused by blood vessel dilation, influencing leukocyte migration and the release of inflammatory mediators (Huber-Lang et al., 2018; Ludewig et al., 2019).

Several mediators are involved in the inflammatory process, including vasoactive amines, arachidonic acid metabolites, platelet-activating factor (PAF), tumor necrosis factor (TNF), chemokines, and cytokines, including interleukins (IL) IL-1β, IL-6, and IL-18. All these components constitute a complex physiological response of the body aimed at neutralizing, inactivating, and eliminating the agent that initiated the inflammatory process, ultimately leading to tissue repair and the suppression of the inflammatory response (Etienne et al., 2021).

Considering the aforementioned, the present study aims to investigate the organic extracts of *C. langsdorffii* regarding their potential anti-inflammatory, antioxidant, antimicrobial, and cytotoxic properties, as well as to identify the main compounds responsible for the biological activities found

MATERIALS AND METHODS

Plant materials and extractions

The aerial parts (leaves, stem bark, and wood) of *Copaifera langsdorffii* Desf. (Fabaceae) were collected in December 2019, in the municipality of Exú – PE (7°21'15.9"S 39°53'20.3"W). A voucher specimen was deposited in the Dárdano de Andrade Lima Herbarium of the pernambuco agronomic institute (IPA), and identified by A. Bocage, under number IPA 93794. The leaves, stem bark, and wood of *C. langsdorffii* were individually separated and dried in an oven at 45ºC.

After drying, each part was ground in a knife mill and sieved to separate non-ground particles. The powdered material (20 g from each plant part) was used for metabolite extraction through cyclic maceration, using a Soxhlet apparatus with approximately 500 mL of solvent (n-hexane, ethyl acetate, and methanol) over a period of 6 h. Subsequently, the solvent was removed by rotary evaporation, and the yield of the concentrated material was determined.

Total phenolic, flavonoid, and proanthocyanidin contents

The quantity of phenolic compounds present in the *C. langsdorffii* extracts was determined following the method described by Li et al. (2008). Aliquots (20 µL) of the extracts at 1 mg/mL, dissolved in DMSO, were dispensed into a microplate, and subsequently, 100 µL of the Folin-Ciocalteu reagent was added. After three minutes of reaction, 80 µL of 7.5% sodium carbonate was added, and after two hours, the absorbances were measured at 735 nm using a spectrophotometer. A standard curve with different concentrations of gallic acid was prepared following the same procedures. The results were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g of extract).

The flavonoid content of the extracts was determined as quercetin equivalent per gram of extract (Pękal and Pyrzynska, 2014). Initially, a quercetin standard curve was prepared for data calibration. Aliquots of 100 µL from the extracts (1 mg/mL) were mixed with 50 µL of 2% aluminum chloride and 50 µL of 1 M sodium acetate and vigorously shaken to initiate the reaction. After 10 minutes, the samples were placed in a 96-well plate and read at 425 nm using a spectrophotometer.

The amount of proanthocyanidins was also estimated, using a catechin standard curve as a calibration control. In a 96-well plate, 40 µL of each sample was added and subsequently mixed with 100 µL of 1% vanillin and 100 µL of 20% sulfuric acid, both diluted in methanol. The samples were incubated for 15 min at 30ºC. After the incubation time, the samples were read at 500 nm in a spectrophotometer. The results were expressed based on the equivalence in milligrams of catechin per gram of extract (mg EC/g of extract) (Sun et al., 1998).

Antioxidant activity

The free radical scavenging capacity of the extracts was evaluated using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) method (Blois, 1958). In a 96-well microplate, 40 µL of extracts at different concentrations were distributed in triplicate, followed by the addition of 250 µL of the DPPH• solution (1mM). After 30 minutes of reaction, the plates were quantified by spectrophotometry at 517 nm. The scavenging of free radicals was determined by the formula: %RSA = (Abs control – Abs sample) x 100 / Abs control, where the

Table 1. Secondary metabolites classes, standards, and elution systems in the phytochemical analysis of organic extracts from *C. langsdorffii* through thin-layer chromatography.

Secondary metabolites classes	Standards	Elution systems	Chromogenic agent	References
Flavonoids, cinnamic derivatives and phenylpropane glycosides	Quercetin, rutin e chlorogenic acid	AcOEt-HCOOH-AcOH-H_2O (100:11:11:27 v/v)	NEU reagent	(Wagner and Bladt, 1996; Brasseur and Angenot, 1986)
Triterpenes and steroids	β-Sitosterol	Toluene-AcOEt (90:10 v/v)	Lieberman and Burchard reagent	(Harborne, 1998)
Diterpenes	Copalic acid	Hexane-AcOEt (90:10 v/v)	Anisaldehyde-sulfuric reagent	(Harborne, 1998)
Mono and sesquiterpenes	Thymol	Toluene-AcOEt(97:3 v/v)	Anisaldehyde-sulfuric reagente	(Harborne, 1998)
Coumarins and Quinones	Coumarin e lapachol	$CHCl_3$-MeOH (98:2 v/v)	KOH	(Wagner and Bladt, 1996)
Saponines	Saponines	AcOEt-HCOOH-AcOH-H_2O(100:11:11:27 v/v)	Lieberman & Burchard; Vanilina sulfúrica	(Harborne, 1998)
Alkaloids	Pilocarpine	AcOEt-HCOOH-AcOH-H_2O(100:11:11:27 v/v)	Dragendorff reagent	(Wagner and Bladt, 1996)
Condensed proanthocyanidins and leucoanthocyanidins	Catechin	AcOEt-HCOOH-AcOH-H_2O(100:11:11:27 v/v)	Hydrochloric vanillin	(Roberts et al., 1957)
Hydrolysable tannins	Gallic acid	AcOEt-Toluene-HCOOH (10:3:1 v/v)	Iron alum 1%	(Stiasny, 1912)

control refers to the absorbance of DPPH• without any sample. The results were expressed as the value of the median effective concentration (EC_{50}), which corresponds to the concentration of the antioxidant required to reduce 50% of the initial amount of free radicals.

Phytochemistry profile

The phytochemical profile of each extract was analyzed by thin-layer chromatography (TLC); using silica gel 60_{F254} plates (Merck, Germany). Various organic solvents were used as the elution system, specifically prepared according to the class of metabolites to be investigated, as shown in Table 1 (Richardson and Harborne, 1990).

Antimicrobial activity

The antimicrobial activity of the extracts was evaluated by susceptibility test to determine the Minimum Inhibitory Concentration (MIC). Strains of *Staphylococcus aureus* (UFPEDA 02), *Pseudomonas aeruginosa* (UFPEDA 416), and *Candida albicans* (UFPEDA 1007) from the microorganism collection of the Department of Antibiotics – UFPE (UFPEDA) were used. Cells were suspended in 0.9% saline solution, and the suspension was adjusted in a spectrophotometer at 625 nm, corresponding to a final concentration of 1.5 x 10^4 CFU/mL for bacteria and 1.5 x 10^5 CFU/mL for *C. albicans* according to the McFarland

scale.

To determine the MIC, a microdilution of the extracts was performed in a 96-well plate, following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). Subsequently, 10 μL of the standardized microbial suspension in liquid Mueller-Hinton (MH) medium was added. Appropriate wells were also reserved for sterility and growth control. The plates were incubated with the extracts for 24 h at 37°C. After this time, microbial growth was indicated by the addition of 20 μL of a 0.01% aqueous solution of resazurin (Sigma-Aldrich), with further incubation at 37ºC for 2. The MIC was defined as the lowest concentration at which there was no reduction in dye oxidation, indirectly indicating no microbial activity.

Animals

Female Swiss mice, aged 4 to 6 weeks and weighing between 25 and 30 grams, were used. They were kept under controlled lighting conditions (12-h light/dark cycle), temperature (22 ± 2ºC), and humidity (45-65%), with free access to water and food. All experimental procedures were conducted following standard protocols and were submitted to the Ethics Committee for Animal Use at the Federal University of Pernambuco. control refers to the absorbance of DPPH• without any sample. The results were expressed as the value of the median effective concentration (EC_{50}), which corresponds to the

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Isolation and culture of murine peritoneal macrophages (MPM)

Initially, macrophage proliferation was stimulated by 1 mL of sterile 3.8% (w/v) sodium thioglycolate solution injected into the peritoneal cavity of the animals. After 72 h of the injection, a peritoneal lavage was performed using 10 mL of buffered phosphate saline solution (PBS) to obtain the cell suspension. After centrifugation, the cells were resuspended in DMEM medium with 10% FBS, and the cell concentration was determined by Neubauer chamber counting (Rios et al, 2017).

Cytotoxicity evaluation using sulforhodamine B

MPM were added to 96-well plates and kept in an incubator at 37ºC for cell adherence. On the following day, the extracts, at different concentrations, dissolved in DMSO were added to the wells in triplicate to determine the IC_{50}. After 24 h of incubation, the supernatant was discarded, and the cells were fixed with 100 μL of 10% trichloroacetic acid for 1 h at 4ºC. Afterward, the plates were washed and incubated with a 0.4% solution of sulforhodamine B (SRB) for 30 min in an incubator. Following incubation, the wells were washed with 1% acetic acid, and the dye was solubilized with 200 μL of 10 mM Tris Base buffer under agitation, allowing measurement with a spectrophotometer at 570 nm (Skehan et al, 1990).

Quantification of nitric oxide and cytokines production by LPS-stimulated MPM

To evaluate inflammatory markers, MPM was stimulated by LPS (5μg/mL) for one hour. After the stimulation, cells were treated with all the extracts at a concentration of 25 μg/mL for 24 h, and after this period, the supernatants from each well were collected for further quantification. The concentration of NO was assessed indirectly by the accumulation of sodium nitrite in the culture medium, using the Griess reagent (Tsikas, 2007). In a 96-well plate, the collected supernatants and the Griess reagent were mixed (1:1). After a 10-min reaction, the plates were quantified using a spectrophotometer at 540 nm. A standard curve of sodium nitrite was previously established to determine the average nitrite concentration of the analyzed samples. The concentration of inflammation-associated cytokines (IL-6, IL-10, and TNF-α) was also quantified in the sample supernatants following the manufacturer's instructions.

Statistical analysis

At least three independent replicates of all experiments were conducted to ensure the independence and correlation of the results. The results were expressed as mean ± standard error of the mean (SEM), evaluated through analysis of variance (ANOVA), followed by the Dunnett post-test. GraphPad Prism software (v. 8.0) was used, and values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Extractions and phytochemical screening

Different parts of the *C. langsdorffii*, such as leaves, stem bark, and wood, were obtained to investigate the distribution profile of bioactive compounds and elucidate the parts with the highest pharmacological potential.

It was observed that the methanolic extracts (extract number 3, 6, and 9) had higher yields depending on the plant parts used, with leaf extraction (3) being the most efficient, yielding approximately 18%, followed by stem bark (6) at 13.5%, and wood (9) at 2%. This difference in yield can be attributed to the polarity differences of the solvents used, which play a crucial role in the solubility of phytochemical compounds determined by their structural differences (Felhi et al., 2017). The polarity and type of solvent, extraction time and temperature, as well as the physical and chemical characteristics of the plant material, are among the main factors influencing the extractions (Salih et al., 2021).

The choice of solvent used for plant extraction depends on the part of plant and nature of bioactive compounds to be extracted. Hexane, ethyl acetate, and methanol are most used for the extraction of nonpolar and polar compounds, such as polyphenols, flavonoids, and tannins. However, compared to different types of solvents, methanol stands out for its efficiency in extracting phenolic compounds (Abubakar and Haque 2020; Thien et al., 2020; Fagbemi et al., 2022). Thus, it is possible to suggest that the higher yields of methanolic extractions from different parts of *C. langsdorffii* may be related to the greater presence of polyphenols, as some studies demonstrate the presence of these compounds in different Copaíba species (Batista et al., 2016; Arruda et al., 2019).

After extracts processing of obtaining, the chemical diversity of each of the nine samples obtained was assessed. The presence of total phenolic compounds, flavonoids, and proanthocyanidins were quantified. Furthermore, DPPH• radical reduction capacity was investigated. All data are presented in Table 2.

It was observed that the extracts with the highest amount of total phenolic compounds were 2, 5, and 8, corresponding to leaves, stem bark, and wood extracted

Table 2. Levels of total phenolic compounds, flavonoids, proanthocyanidins in organic extracts of *Copaifera langsdorffii*, and their capacity of DPPH• radical reduction.

Plant part	Solvent	Yield (%)	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)	Proanthocyanidin content (CE/g)	DPPH· EC$_{50}$ (µg/mL)
	n-hexane	7.47	98.6	519.75	42.11	>1000
Leaves	ethyl acetate	11.25	564.04	865.3	8.2	160
	methanol	18.03	253.44	269.75	ND	299.3
	n-hexane	1.41	151.92	491.97	ND	>1000
Stem bark	ethyl acetate	1.52	839.8	151.14	95.44	68.09
	methanol	13.49	549.95	26.7	126	40.87
	n-hexane	0.15	111.47	251.4	9.8	>1000
Wood	ethyl acetate	1.48	476.32	ND	6	275.4
	methanol	2.10	311.62	ND	1.55	267.8

ND: not detected.

with ethyl acetate, respectively. Extract 5 stood out for the highest presence of these compounds, with approximately 840 mg GAE/g of extract.

Phenolic compounds are one of the main products of the secondary metabolism of plants, and are involved in various activities, such as cytotoxicity in cancer cells, antioxidant, anti-inflammatory and antimicrobial effects (Bouziane et al., 2018; El Yadini et al., 2023; Soltani et al., 2023). Although studies with similar extracts of *C. langsdorffii* are scarce in the literature, vegetables of the same family (Fabaceae) have a similar profile when compared. The work of de Oliveira Rodrigues et al. (2020) demonstrates that the ethyl acetate fraction of *Bauhinia ungulata* L. (Fabaceae) extract has high concentrations of phenolic compounds. Kamal et al. (2022) demonstrated that methanolic extract of leaves and bark of *Bauhinia variegata* L. (Fabaceae) showed highest phenolic and flavonoids contents, with antibacterial, antidiabetic, and anticancer activity. The methanolic extraction of *Senna racemosa* (Mill.) H.S. Irwin and Barneby

(Fabaceae) stem bark also showed phenolic compounds, such as some anthraquinones, which demonstrated antiparasitic activity (Caamal-Fuentes et al., 2016). *Sophora flavescens* Ailton (Fabaceae), another legume, has some flavonoids, such as quercetin, rutin and kushenol C, in extracts of the aerial parts. These phenols play antioxidant and anti-inflammatory responses by regulating the expression of important proteins involved in these processes, such as kinases and cytokines (Oh et al., 2023).

Others phenolic profiles were also investigated in organic extracts of *C. langsdorffii*, mainly flavonoids and proanthocyanidins. Condensed tannins, also called proanthocyanidins, are polymeric compounds whose structure is formed by the condensation of two or more flavonoid nuclei (Jonker and Yu, 2017). The use of drugs containing proanthocyanidins, as well as all polyphenols, also have biological activities widely described in the literature, such as anti-inflammatory, antioxidant and antiapoptotic (Singh et al., 2017; Han et al., 2019; Zeng et al., 2020).

Due to this wide therapeutic spectrum, this is a class of special interest to pharmacology.

Analyzing the extracts used, it was observed a high content of flavonoids in extracts 1, 2 and 4. The presence of these metabolites, in smaller quantities, in extracts 3, 5 and 7 was also noted. Extracts 6, 8 and 9 showed small or no significant expression of flavonoids. Motta et al. (2019) describe that flavonoids are the main phenolic compounds found in the leaves of *C. langsdorffii*, and the amount of the compound may vary according to the geographical location in which the material is collected.

It was also identified a prominent presence of proanthocyanidins in samples 5 and 6. The literature demonstrates the presence of these compounds in ethanolic extracts from the bark of some legumes, such as *Acacia mearnsii* De Wild (Fabaceae) and *Dalbergia monetaria* L. f. (Fabaceae). Proanthocyanidins, like many phenolic compounds, are also associated with pharmacological activities such as antioxidant, antidiabetic, anticancer and antimicrobial (Xiong

Table 3. Phytochemical screening of organic extracts from *Copaifera langsdorffii* Desf. (Fabaceae).

Secondary metabolite classes	*Copaifera langsdorffii*								
	1	2	3	4	5	6	7	8	9
Flavonoids	-	+++[1]	+++[1]	tr[2]	+[2]	++[2]	-	-	-
Cinnamic derivaties	-	-	-	-	-	-	-	-	-
Saponines	-	-	tr	tr	+	++	-	+	+
Alkaloids	-	-	-	-	-	-	-	-	-
Triterpenes and steroids	+	+	-	+++	+++	+	++	++	+
Diterpenos	+++	++	-	+++	++	+	++	+	tr
Monoterpenes and Sesquiterpenes	+++	++	-	+++	++	+	++	tr	tr
Coumarins	+	++	-	+++	+++	+	++	+++	+
Quinones	-	-	-	-	-	-	-	-	-
Proanthocyanidins	-	-	-	-	+[3]	+[4]	-	tr	-
Hydrolysable tannins	-	-	-	-	-	-	-	-	-

[1] flavonoid heterosides 3'-OH and 4'-OH; [2] flavonoid heterosides 3'-OH; [3] oligomeric proanthocyanidins; [4] polimeric proanthocyanidins. (+++) strong; (++) moderate; (+) weak; (-) absent; (tr) traces.

et al., 2017; Ogawa and Yazaki, 2018; Chen et al., 2018; de Moura et al., 2020).

Oxidative stress is caused by the imbalance between the inevitable production of free radicals and the presence of antioxidant agents that eliminate these species in the body. Thus, the accumulation of reactive oxygen species (ROS) is one of the main factors that trigger the onset of various degenerative diseases, such as inflammation, cardiovascular disorders, mutagenesis, and cancer (Senoner and Dichtl, 2019; Jelic et al., 2021; Forman and Zhang, 2021).

Vegetables are a rich source of compounds with antioxidant properties, and the Fabaceae family is highlighted for presenting metabolites associated with such properties (Amarowicz and Pegg, 2019). By scavenging the DPPH• radical, the oxygen free radical modulating capacity of the organic extracts obtained from the collected parts of *C. langsdorffii* was investigated.

As previously demonstrated in Table 2, the extracts that showed the most prominent antioxidant activity were 2, 5, and 6, which exhibited relatively equivalent EC_{50} values. This activity may be associated with the presence of total phenols, which were also found in greater quantities in this extract. The literature correlates the antioxidant capacity of polyphenols with their chemical structures and aromaticity (Feng et al., 2017; de Lima Cherubim et al., 2020; Shen et al., 2022). According to Ghizoni et al. (2017), Copaíba oil has a systemic, anti-inflammatory, and antioxidant action in animals with arthritis. In addition to the oil, the fruits of *C. langsdorffii* also showed a high content of polyphenols with antioxidant capacity (Batista *et al.* 2016). However, studies on the antioxidant properties of the organic extracts from *C. langsdorffii* are still scarce, which raises the interest in this species, given its wide ethnopharmacological effects.

The chromatographic profile of the organic extracts of *C. langsdorffii* revealed the presence of several secondary metabolites, as shown in Table 3. Among the compounds analyzed, flavonoids, terpenes, steroids, and coumarins were found in greater quantities in most of the extracts, while saponins and proanthocyanidins are found only in extracts 5 and 6. Literature data demonstrate that oleoresin and some organic extracts of Copaíba contain different secondary metabolites, such as terpenes and flavonoids, often associated with various biological activities, which reinforces the use of this plant in traditional medicine. The antitumor and anti-inflammatory potential of oleoresin from different Copaíba species has been associated with the presence of some diterpenes and sesquiterpenes, which are considered chemical markers of the genus under study (Carneiro et al., 2020; Mangabeira da Silva et al., 2020).

Antimicrobial activity

The treatment of infectious diseases caused by microorganisms continues to be a significant challenge today, due to the increasing cases of infections caused by several antibiotic-resistant bacteria. Therefore, the search for antimicrobial compounds in natural sources, such as plant extracts, still proves to be an important alternative in current medicine (Tsamo et al., 2021). Thus, Copaíba extracts were investigated for their antimicrobial capacity, using strains of *S. aureus*, *P. aeruginosa*, and *C. albicans*.

One of the main pathways which different molecules induce antibacterial activity is related to the permeability of the bacterial cell membrane, which is an essential characteristic for a drug to exert the desired effect, whether it is bactericidal or bacteriostatic. Gram-negative bacteria, such as *P. aeruginosa*, are intrinsically less permeable to drugs due to the presence of an outer

Table 4. Antimicrobial susceptibility test from *Copaifera langsdorffii* organic extracts against *S. aureus*, *P. aeruginosa* and *C. albicans*. Data are shown as minimal inhibitory concentrations (MIC) in µg/mL.

Sample	S. aureus	P. aeruginosa	C. albicans
1	>1024	>1024	512
2	32	>1024	512
3	>1024	>1024	1024
4	>1024	>1024	1024
5	1024	>1024	128
6	1024	>1024	64
7	>1024	>1024	>1024
8	>1024	>1024	1024
9	>1024	>1024	>1024

membrane in their cell wall, which prevents the diffusion and, consequently, the action of compounds in the cell's cytoplasm. In contrast, Gram-positive bacteria are more sensitive and susceptible to the effects of toxic compounds (Arzanlou et al., 2017; Morrison and Zembower 2020). These data support those in the present study, as shown in Table 4.

No extract was able to reduce the metabolism of *P. aeruginosa* at low concentrations, suggesting that structural alterations may have occurred, and the action of the extracts may be inhibited by bacterial enzymes, such as phenol hydroxylases. The literature reports that some bacteria, such as the species in question, are capable of biodegrading phenolic compound under favorable conditions, which can hinder the antibacterial action of these compounds (Jõesaar et al., 2017; Wang et al., 2018; Mahgoub et al., 2023). Nevertheless, by evaluating the extracts against *S. aureus* and *C. albicans*, a reduction in the activity of these microorganisms was observed in some treatments.

Extracts from leaves and stem bark showed better activity compared to wood. Extract 2 showed the lowest bacteriostatic concentration against *S. aureus*, with a MIC of 32 µg/mL. Regarding the yeast *C. albicans*, extract 6 showed a MIC of 64 µg/mL, while extract 5 was 128 µg/mL. The remaining extracts did not show significant inhibition or had very high inhibitory concentrations.

Several studies correlate phenolic compounds with antimicrobial activity. Extracts from the leaves and stem bark of the legume *Amburana cearensis* (Allemão) A.C.Sm. (Fabaceae), using polar solvents, showed antibacterial effects against several bacterial species, including *S. aureus*, as assessed in the present study (Silveira et al., 2022). Isolated flavonoids from different types of *Glycyrrhiza glabra* L. (Fabaceae) showed different biological activities, including antibacterial (Wang et al., 2020). In a review on the biological activities of several extracts from *Mimosa tenuiflora* Willd (Fabaceae), authors have found several antimicrobial activities *in vitro*, with flavonoid and tannin compounds probably the mainly responsible for this activity (Ferreira

and Evangelista 2021). Compounds extracted from *Rhynchosia minima* (L.) DC. (Fabaceae) showed antifungal activity against *C. albicans* due to the presence of different flavonoids found in the various parts of this plant (Adewole et al., 2022). Other legumes also present antimicrobial activity against the same microorganisms evaluated in the present study, associating such activities with the presence of different polyphenols (Obistioiu et al., 2021).

The chemical structure of phenolic compounds, such as the position of different radicals in the phenolic skeleton, can modulate antimicrobial activity (Araya-Cloutier et al., 2017). They can interact with extracellular proteins of the bacterial cell wall, causing disruption of cellular peptidoglycan and altering membrane permeability. Furthermore, its hydroxyl groups can also contribute to its activity through hydrogen bonding with enzymatic active sites, thereby altering bacterial metabolism and its liposolubility (Amini et al., 2021). Based on the previously presented assays, phenolic compounds are widely distributed in the parts of *C. langsdorffii*, and their antibacterial activity may be associated with the presence of these metabolites.

In vitro anti-inflammatory activity

The presence of Copaíba in traditional medicine is widely associated with the treatment of various health complications, such as urinary, respiratory, cutaneous, and inflammatory disorders. Ethnopharmacological studies support most of these uses (Arruda et al., 2019). Therefore, it is essential that toxicity studies be carried out to ensure the maximum safety in the use of various preparations with this plant.

The organic extracts of *C. langsdorffii* were evaluated for their cytotoxicity in murine peritoneal macrophages (MPM) and using the colorimetric assay of sulforhodamine B (SRB). The experimental tests did not show significant cytotoxicity in MPM by extracts, where the majority showed IC_{50} values close to or greater than 100 µg/mL,
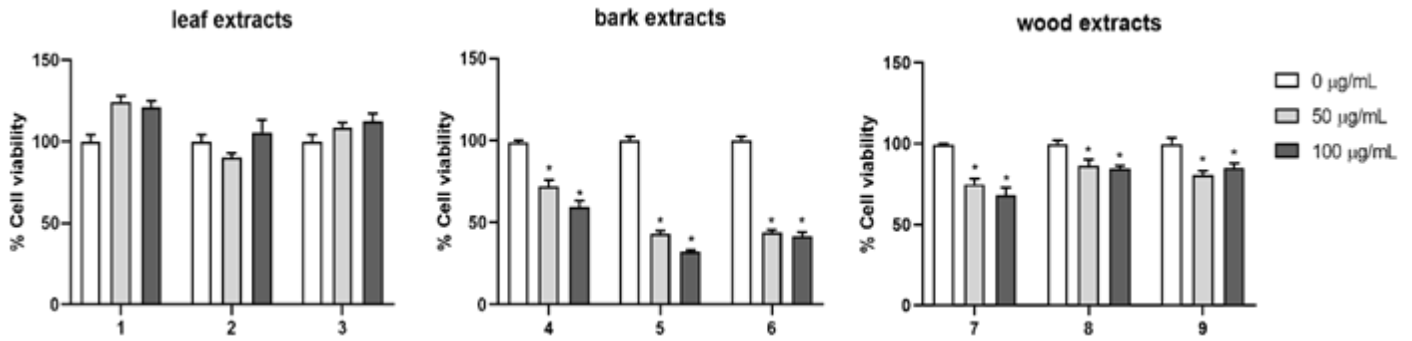
Figure 1. Cytotoxicity activity of *Copaifera langsdorffii* organic extracts against murine peritoneal macrophages by sulforhodamine B quantification after 24 h of incubation. *$p < 0.05$. Significance was determined with two-way ANOVA followed by Dunnett's post-test when compared to the control group.

as observed in Figure 1.

Only extracts 5 and 6 showed IC_{50} values of 33.64 ± 1.06 µg/mL and 38.97 ± 1.1 µg/mL, respectively. These extracts, as highlighted above, obtained interesting results, especially in terms of their phenolic content and antioxidant potential. The data obtained after quantification of total proteins by SRB may suggest that some of the secondary metabolites present in these extracts are inducing an immunomodulatory response, reducing the amount of proteins synthesized by macrophages and resulting in a decrease in their inflammatory activity.

Macrophages are key cells in the innate inflammatory response, where the release of chemical mediators by these cells is essential for triggering inflammation. Evaluating the cytotoxic effects of different compounds on macrophages can provide insights into their modulation of the inflammatory process (da Luz et al., 2020). Although plant extracts with anti-inflammatory potential are not cytotoxic to healthy cells in the body, according to Marques-Santos et al. (2023), it is possible that the same extract presents different responses to macrophages, potentially affecting their viability. Other studies, such as Buyinza et al. (2021) correlates the presence of phenolic compounds, such as isoflavones, to the cytotoxicity of *Millettia dura* (Dunn) (Fabaceae) extract on different cell lines. The ethanol extract of *Senna septemtrionalis* (Viv.) H.S. Irwin and Barneby (Fabaceae) displays an in vitro and in vivo anti-inflammatory action, reducing levels of cytokines by LPS-stimulated macrophages (Arana-Argáez et al., 2020).

Inflammation is one of the primary and essential protective responses to infections, irritations, injuries, or physiological disorders. Macrophages are the main cells involved in this process, acquiring specific functions and phenotypes in response to various external activators. The main mediators of this response include cytokines and chemokines released at the site of the inflammatory reaction, serving as targets for anti-inflammatory or pro-inflammatory molecules (Kuprash and Nedospasov,

2016).One of these mediators is nitric oxide (NO), an important molecule in the defense system, acting as a vasodilator and cytotoxic agent in infectious processes (Bailey et al., 2019). Among various intracellular mediators related to inflammation, cytokines TNF-α, IL-6, and IL-10 stand out because they are involved in various immune system-related pathologies, as viral and bacterial infections, cancer, among others (Roe, 2021).

Macrophages can be classified at least in two phenotypes when related to the inflammatory process. The M1 phenotype has pro-inflammatory characteristics, which release mediators that promote this process, while the M2 phenotype has an anti-inflammatory effect (Orecchioni et al., 2019). When stimulated with pro-inflammatory factors like LPS, macrophages can adopt the M1 phenotype. Thus, the present results suggest that the extracts that showed cytotoxicity against M1 macrophages may contribute to reduction of the inflammatory response mediated by such cells.

Due to the anti-inflammatory potential of some of the organic extracts of *C. langsdorffii*, the expression profile of some inflammatory markers produced by LPS-stimulated murine peritoneal macrophages treated with Copaíba organic extracts was evaluated, as presented in Figure 2.

After treatment with Copaíba organic extracts, several of them significantly reduced the nitrite levels and consequently the NO production by LPS-activated macrophages. Extracts 1 and 4 showed a similar reduction in the percentage of NO. Similarly, all acetate extracts (2, 5, and 8) also reduced nitrite levels, with extract 2 causing the most prominent effect, reducing about 82% of nitrite. No methanolic extract (3, 6, and 9) was efficient in reducing nitrite levels, as observed in Figure 2. These results suggest that compounds extracted with nonpolar solvents were more effective when compared to polar or moderately polar ones.

The literature reports that flavonoids play an important regulatory role in inflammation and oxidative stress, acting on different signaling pathways, including the
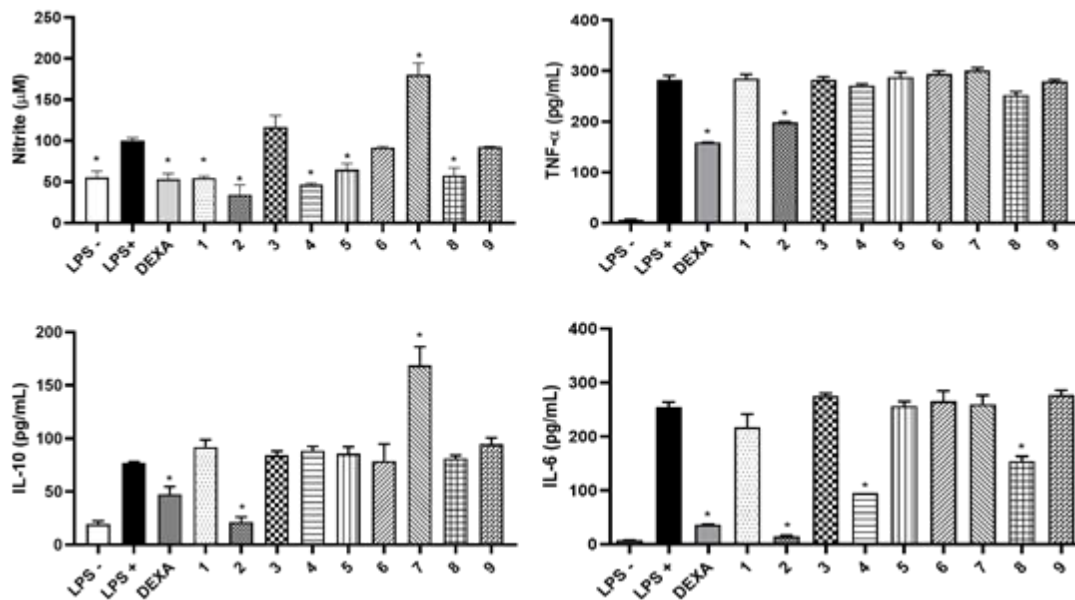
Figure 2. Immunomodulatory effect of C. langsdorffii organics extracts on murine peritoneal macrophages after 24 h of treatment. LPS -: control group; LPS +: cells treated with LPS; DEXA: Dexamethasone. *p < 0.05. Significance was determined with one-way ANOVA followed by Dunnett's post-test when compared to the control group.

inhibition of reactive oxygen species production. This inhibition occurs through the binding of flavonoids to plasma membrane receptors on certain cells, suppressing various intracellular signaling cascades and pro-inflammatory mediators (Li et al., 2020). Quercetin, a known flavonoid, has shown potential anti-inflammatory effects by suppressing the expression of the superoxide dismutase enzyme, in addition to significantly reducing the levels of pro-inflammatory cytokines such as NF-kB, IL-6, and TNF-α (Li et al., 2021)

In view of the above, the literature supports the findings, suggesting that flavonoids found in the Copaíba extracts may be inactivating NO expression pathways by peritoneal macrophages. Furthermore, it expands the spectrum for the compounds to act on the levels of other mediators associated with inflammation. The *in vitro* action of these metabolites related to inflammation depends on the modulation of different inflammatory mediators, such as enzymes and cytokines (Al-Khayri et al., 2022).

As shown in Figure 2, extract 2 significantly reduced the levels of TNF-α produced by macrophages. This data, together with the reduction in NO levels, suggests its anti-inflammatory potential through changes in the profile of these evaluated mediators. Furthermore, the same extract also significantly reduced interleukin-6 (IL-6) levels, another pro-inflammatory marker. Similar to extract 2, extracts 4 and 8 also showed a significant reduction in this cytokine. Based on these results, it is possible to observe that there is a broad spectrum of metabolites distributed throughout different parts of

Copaíba, both in the leaves, bark, and wood, which can play an anti-inflammatory role by suppressing these mediators.

Inflammation is a highly regulated process that operates through a balance in the release of a series of pro-inflammatory and anti-inflammatory mediators and mechanisms. If there is an imbalance between these factors, it can result in the development of a wide range of inflammatory diseases. It is in this context that the phenotypic plasticity of inflammatory cells, such as macrophages, plays a crucial role in mediation. The expression of the M1 phenotype, or pro-inflammatory phenotype, characterizes macrophages as producers of inflammation-inducing factors, such as the cytokines IL-6, IL-1, and TNF-α. On the other hand, the M2 phenotype is associated with immunomodulatory and anti-inflammatory factors, such as the release of the cytokines IL-10 and TGF-β (Shapouri-Moghaddam et al., 2018; Ross et al., 2021).

Quercetin, a well-known flavonoid obtained from extracts of various legumes, has shown to be an important suppressor of the inflammatory response in LPS-stimulated macrophages (Lu et al., 2018). Luteolin ,another flavonoid from natural origin, is correlated to reduce pro-inflammatory and increase anti-inflammatory mediators. Given the data obtained in the present work, such as the presence of flavonoids in the evaluated extracts and their involvement in the inflammatory response, it was investigated the influence of the studied extracts on the expression of interleukin 10 (IL-10).

It was observed that, among all the studied organic

extracts, only extract 7 increased the expression of IL-10, suggesting a correlation between this extract and the suppression of the inflammatory response. Conversely, this extract did not interfere with the expression of the pro-inflammatory cytokines evaluated. For instance, the expression of TNF-α can be inhibited by the high production of IL-10. This observation may be correlated with the body's natural defense processes, resulting in the balance of the Th1/Th2 cytokine response (Wen et al., 2022).

On the other hand, extract 2, which also reduced the expression of TNF-α and IL-6, exhibited a decrease in the levels of IL-10. This finding is consistent with the results of Saiki et al. (2018), who demonstrated that compounds like quercetin, a well-known plant-derived flavonoid with anti-inflammatory properties, reduced both IL-6 and IL-10 levels in LPS-stimulated macrophages. According to the literature, quercetin not only diminishes the production of various reactive oxygen species but also plays a role in balancing the M1/M2 macrophage profile. It reduces the expression of markers associated with the M1 profile (pro-inflammatory) while increasing the levels of M2 markers (anti-inflammatory), such as IL-6 and IL-10, respectively. Consequently, it is suggested that quercetin may serve as a compound with high potential for the treatment of inflammation-related diseases (Tang et al., 2019; Chen et al., 2020; Tsai et al., 2021).

Given that various studies demonstrate the presence of phenolic compounds in legume extracts with significant biological potential, particularly in terms of anti-inflammatory properties, further research is imperative to isolate, purify, and elucidate the main components present in the organic extracts of *C. langsdorffii.* In the present study, different responses were observed among the various evaluated extracts, enabling the correlation of their biological activities with the presence of phenolic compounds, especially flavonoids (Arulselvan et al., 2016; Zhu et al., 2018; Al-Khayri et al., 2022; Juárez-Chairez et al., 2022). Based on the available ethnobotanical information regarding the use of the species under study, it is widely recognized that Copaíba oil holds significant importance in traditional medicine. This oil is notably rich in metabolites such as sesquiterpenes, which are associated with various biological activities, as previously described. Consequently, the utilization of Copaíba oil is becoming increasingly prevalent in traditional medicine, leading to a rise in demand for its extraction. However, this heightened demand adversely affects the conservation of these tree species. Therefore, the development of new alternative methods for obtaining bioactive compounds from Copaíba is becoming more imperative to mitigate the risks to the population of these trees. Unsustainable extraction practices constitute one of the primary causes of the extinction of species with pharmacological potential, posing a growing threat to their conservation

(Costa et al., 2021; Santos et al., 2022; Almeida-Bezerra et al., 2022).

Given the data obtained in the present study, it is evident that various bioactive compounds can be derived from different parts of Copaíba, such as the leaves and bark. These plant structures are relatively perennial, and their extraction causes less damage to the species compared to traditional oil extraction methods. Obtaining compounds with biological activities similar to those found in the oil obviates the need to drill the tree trunk for extraction, thus offering alternatives for the ethnopharmacological use of Copaíba and mitigating the risk of its extinction.

Building upon the data collected thus far, the intention is to proceed with the isolation, identification, and evaluation of pharmacologically active secondary metabolites in the species *C. langsdorffii.* This endeavor includes exploring additional biological activities of the organic extracts used, such as anticancer, antiparasitic, and antivenom activities, which will provide valuable insights into the chemical composition and confirm the bioactive potential of this species.

It is also noteworthy to highlight the novelty of this study, as there is a significant gap in the literature regarding the chemical composition, biological activity, and mechanisms of action of some of the extracts presented here.

Conclusion

In conclusion, the organic extracts of *C. langsdorffii* demonstrated the ability to reduce the activity of *S. aureus and C. albicans*, with extracts 1, 2, 5, and 6 exhibiting lower inhibitory concentrations. These extracts also displayed antioxidant capacity by effectively reducing the amount of the DPPH• radical, with EC50 values below 300 µg/mL. Additionally, some extracts demonstrated cytotoxic effects against murine peritoneal macrophages, diminishing their viability and suggesting an action through the inflammatory pathway.

Upon evaluating the anti-inflammatory potential of Copaíba extracts, a notable reduction in inflammatory markers was observed, including decreased nitrite levels (a precursor of nitric oxide) and pro-inflammatory cytokines such as TNF-α and IL-6. Furthermore, most extracts maintained high levels of the IL-10 anti-inflammatory cytokine. Thus, the anti-inflammatory potential of certain extracts was confirmed, with this activity being associated with the presence of various secondary metabolites, such as flavonoids, terpenes, and coumarins, identified through chromatography.

These findings underscore the ethnopharmacological potential of this species, not only in the form of oleoresin but also in organic extracts derived from different parts of the plant, particularly the leaves and bark, for the treatment of infections and inflammations. They provide

valuable insights for the safe utilization of Copaíba in traditional and popular medicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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