

1 Antimicrobial and cytotoxic effects of the *Copaifera reticulata*
2 oleoresin and its main diterpene acids

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1

2 ABSTRACT

3 *Ethnopharmacological relevance:* The oleoresin of Brazilian *Copaifera reticulata* is a
4 traditional remedy used for the treatment of skin and urinary tract infections, respiratory
5 diseases, rheumatism, ulcer and tumours; thus, playing an important role in the primary health
6 care of the indigenous population.

7 *Aim:* As most previous pharmacological tests used the crude oleoresin and only a few studies
8 so far dealt with enriched fractions or pure chemically defined compounds, the aim of this study
9 was to systematically evaluate the antimicrobial and cytotoxic properties of the *Copaifera*
10 *reticulata* oleoresin and to assign traditional uses to specific secondary metabolites.

11 *Materials and methods:* The oleoresin, as well as its neutral and acidic fractions were tested for
12 their activity against six cancer cell lines, two clinically relevant bacterial strains, and two
13 dermatophytes. Both fractions were analysed by GC-MS and UHPLC-ELSD, respectively. The
14 antibacterial acidic phase was further fractionated by preparative chromatography to purify and
15 characterize the compounds responsible for the observed pharmacological effect.

16 *Results:* Whereas no cytotoxic activity was detected, the crude oleoresin and its acidic fraction
17 showed antibacterial activity against gram-positive bacteria *Enterococcus faecium* (IC₅₀ values
18 4.2 and 4.8 µg/mL, respectively) and methicillin-resistant *Staphylococcus aureus* (MRSA, IC₅₀
19 values 5.3 and 7.2 µg/mL, respectively). Purification of the acidic fraction of the *C. reticulata*
20 oleoresin yielded two dicarboxylic diterpene acids and the four main diterpene acids,
21 comprising three different diterpene scaffolds. Interestingly, the activity was not restricted to a
22 particular diterpene-type but rather depended on the compounds' lipophilicity, with the most
23 active constituent showing IC₅₀ values of 1.6 (*E. faecium*) and 2.5 µg/mL (MRSA),
24 respectively. Furthermore, *ent*-polyalthic acid, the major diterpenoid, was significantly active

1 against dermatophytes with IC₅₀ values of 6.8 µg/mL (*Trichophyton rubrum*) and 4.3 µg/mL
2 against (*T. mentagrophytes*).

3 *Conclusion:* The present study proved the antimicrobial effects of the *C. reticulata* oleoresin
4 and its diterpenoid constituents, confirming its wide use in folk medicine for the treatment of
5 skin and urinary tract infections. The inhibitory activity of copaiba diterpenoids against
6 dermatophytic fungi as well as the gram-positive bacteria *E. faecium* and MRSA is being
7 reported for the first time, providing potential lead structures for the treatment of these clinically
8 relevant bacterial strains.

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11 *Keywords:* *Copaifera reticulata*, copaiba oil, diterpenoid, polyalthic acid, kaurenoic acid,
12 kolavenic acid, natural product, MRSA, *Enterococcus faecium*, dermatophytes

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15 *Abbreviations:* IC₅₀, half maximal inhibitory concentration; MRSA, methicillin-resistant
16 *Staphylococcus aureus*; LC-MS, liquid chromatography coupled to mass spectrometry; SPE,
17 solid phase extraction; NMR, nuclear magnetic resonance; GC-MS, gas chromatography
18 coupled to mass spectrometry; MPLC, medium pressure liquid chromatography; NIST,
19 National Institute of Standards and Technology; UHPLC-ELSD, ultra-high performance liquid
20 chromatography coupled to evaporative light scattering detection; TSB, tryptic soy broth;
21 DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;
22 DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute
23 medium.

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1 **Introduction**

2 *Copaifera reticulata* Ducke (Fabaceae), is a tree growing in the tropical regions of Latin
3 America (Veiga Junior and Pinto, 2002; Costa et al., 2015). The oleoresins of *C. reticulata* and
4 a few other *Copaifera* species, which are obtained by perforation of the trunks during the rainy
5 season, are commonly referred to as copaiba oil and traditionally used as a panacea by the
6 indigenous population (Ohsaki et al., 1994; Veiga Junior and Pinto, 2002; Boaroto, 2014).
7 Typical medicinal applications include wound and scar healing as well as treatments of skin
8 and urinary tract infections, respiratory diseases, ulcers, rheumatism, herpes, tumours, and
9 tetanus disease. Thus, rendering the oleoresin an important natural remedy for people without
10 access to commercial drugs (Ohsaki et al., 1994; Veiga Junior and Pinto, 2002; Leandro et al.,
11 2012; Boaroto, 2014). Based on the comprehensive application in folk medicine, the oleoresin
12 has been subject of various scientific studies aiming to prove its activity in different *in vitro* and
13 *in vivo* models (Basile et al., 1988; Ohsaki et al., 1994; Veiga Junior et al., 2001; Lima et al.,
14 2003; Paiva et al., 2004; Carvalho et al., 2005; Gomes et al., 2007; Veiga Junior et al., 2007;
15 Gomes et al., 2008; Santos et al., 2008a; Santos et al., 2008b; Brito et al., 2010; Gomes et al.,
16 2010; Santos Jr., 2010; Neto et al., 2011; Almeida et al., 2012; Dias et al. 2014; Bardaji et al.,
17 2016).

18 Regarding the chemical composition, copaiba oleoresins have been reported to contain
19 sesquiterpenes and diterpenes. Sesquiterpenes represent the main components with contents
20 ranging from 58 to 98% by weight, depending on variable parameters such as species, growing
21 area, and harvest time (Lima et al., 2003; Izumi et al., 2012; Souza Barbosa et al., 2013; Bardaji
22 et al., 2016). In contrast to the diterpene pattern, the sesquiterpene pattern is stable and shows
23 only limited variation between different *Copaifera* species, with β -caryophyllene usually being
24 the major constituent (Leandro et al., 2012). However, for several *C. reticulata* samples of the
25 Pará region, β -bisabolene has been reported to be the main compound (Zoghbi et al., 2009).

1 Both compounds, as many other major sesquiterpenes (e.g. α -humulene or caryophyllene oxide)
2 are not restricted to the genus *Copaifera* and occur also in various other plant genera, such as
3 *Abies* (Legault et al., 2003), *Commiphora* (Yeo et al., 2016), *Eugenia* (Zheng et al., 1992),
4 *Heterotheca* (Kubo et al., 1996), *Taiwania* (He et al., 1997), and *Zanthoxylum* (Silva et al.,
5 2007). Therefore, previous pharmacological studies on pure compounds isolated from copaiba
6 oleoresins focused on the more particular diterpenes, e.g. *ent*-copalic acid, *ent*-agathic acid (**1**),
7 *ent*-kaurenoic acid (**4**), *ent*-polyalthic acid (**3**), and *ent*-hardwickiic acid (Leandro et al., 2012).
8 Tincusi et al. (2002) isolated thirteen diterpenoids from the oleoresin of *C. paupera*. The
9 authors found significant activity against gram-positive and gram-negative bacteria for *ent*-
10 copalic acid and *ent*-kaurenoic acid (**4**), with superior activity to cephalexin against *Bacillus*
11 *subtilis*. Cytotoxicity studies of the diterpene acids revealed no significant activity against colon
12 cancer cells (HT-29), lung cancer cells (A-549), and melanoma cells (MEL-28) with IC₅₀
13 values above 10 μ g/mL. Costa-Lotufo et al. (2002) reported moderate cytostatic activity for
14 *ent*-kaurenoic acid (**4**) isolated from *C. langsdorffii* oleoresin, inhibiting the cell growth of
15 leukemia cells (CEM) by 75%, breast cancer cells (MCF-7) and colon cancer cells (HCT-8) by
16 45% each, at a test concentration of 78 μ M. Cavalcanti et al. (2006) showed *ent*-kaurenoic (**4**)
17 acid to induce DNA-damage in Chinese hamster fibroblasts (V79 cells), but reported the
18 compound to possess poor therapeutic potential due to non-selective toxicity in a later study
19 (Cavalcanti et al., 2009). *Ent*-copalic acid was reported to be active against five different
20 *Streptococcus* species and *Lactobacillus casei* with MIC values ranging from 2.0 to 6.0 μ g/mL
21 (Souza et al., 2011). *Ent*-copalic acid was also active against amastigote forms of *Trypanosoma*
22 *cruzi* together with 3 β -hydroxycopalic acid and methyl copalate (Izumi et al., 2012). *Ent*-
23 polyalthic acid (**3**) was investigated against *Schistosoma mansoni* and found schistosomicidal
24 with LD₅₀ values from 33.4 to 41.7 μ g/mL and to affect the production and the development of
25 eggs (Borges et al., 2016).

1 Apart from the relatively few studies described above on pure compounds, the crude oleoresin
2 showed biological activity in a multitude of investigations (Leandro et al., 2012; Trindade et
3 al., 2018). Thus, the aim of the present study was to systematically evaluate the antimicrobial
4 and cytotoxic properties of the *Copaifera reticulata* oleoresin and its chemical constituents. The
5 cytotoxic effects were of special interest, because at the Veterinary Institute, Federal University
6 of Pará (Brazil), the oleoresin is currently used for the topical treatment of malignant melanoma
7 in horses.

8

9 **2. Materials and methods**

10 *2.1. Phytochemical study*

11 *2.1.1. Plant material and reagents*

12 Copaiba oleoresin was obtained through perforation of the trunk of a *C. reticulata* tree
13 growing in Rondon do Pará, Brazil, in August 2016. The plant species was determined by the
14 Brazilian Agricultural Research Corporation – Ministry of Agriculture, Livestock, and Food
15 Supply. Methanol, water (both of LC-MS grade), and other (analytical grade) solvents were
16 purchased from VWR International GmbH, Darmstadt, Germany. Solid phase extraction (SPE)
17 columns (Chromabond SB 3 mL/500 mg) were obtained from Macherey-Nagel GmbH & Co.
18 KG, Düren, Germany. Deuterated methanol (Lot P3021, 99.80%) for NMR spectroscopy was
19 obtained from Euriso-top GmbH, Saarbrücken, Germany, and conventional 5 mm sample tubes
20 were purchased from Rototec-Spintec GmbH, Griesheim, Germany. LC-MS grade formic acid
21 was obtained from Sigma Aldrich Co., St. Louis, MO, USA.

22

23 *2.1.2. General experimental procedures*

24 GC-MS measurements were done on a TraceTSQ Duo system (Trace 1310 GC coupled to
25 a Triple Stage Quadrupole Mass Spectrometer) equipped with a split/splitless (SSL) injector

1 and fitted with a TraceGOLD TG-SQC column (15 m x 0.25 mm i.d., film thickness 0.25 μ m).
2 UHPLC analyses were performed on a VWR-Hitachi Chromaster Ultra RS equipped with a
3 6170 binary pump, 6270 autosampler, 6310 column oven, 6430 DAD, and a Sederé 100
4 evaporative light scattering detector using a Phenomenex Luna Omega C18 column (100 \times 2.1
5 mm, 1.6 μ m particle size). LC-MS analyses were carried out on a Shimadzu Nexera 2 Liquid
6 chromatography connected to a LC-MS 8030 triple quadrupole mass spectrometer using
7 electrospray ionization in the negative mode. Preparative MPLC was accomplished with a
8 Buchi PrepChrom C-700 using a Buchi PrepChrom C18 column (250 \times 30.0 mm, 15 μ m
9 particle size). NMR spectra were recorded using a Bruker Avance III 300 NMR spectrometer
10 operating at 300 MHz for the proton channel and 75.5 MHz for the ^{13}C channel with a 5 mm
11 PABBO broad band probe with a z gradient unit.

12

13 *2.1.3. Solid phase extraction (SPE) and analytical chromatography*

14 For SPE, columns were equilibrated with two column volumes of *n*-hexane. 50.0 mg of
15 oleoresin were dissolved in 500 μ L dichloromethane, subjected to the SPE column and washed
16 with three column volumes of *n*-hexane to yield the neutral fraction. For GC-MS analysis, the
17 eluent was diluted to a volume of 10.0 mL with *n*-hexane (corresponding to 5.00 mg/mL
18 oleoresin), for bioassays, the eluent was evaporated to dryness. Further elution of the dried SPE
19 column with 5% formic acid in methanol yielded the acidic fraction, which was diluted to 10.0
20 mL with methanol for UHPLC-ELSD analysis or evaporated to dryness for testing. Yields of
21 the solid phase extraction were determined with 27.5 mg of neutral fraction (55%) and 22.5 mg
22 of acidic fraction (45%).

23 Conditions for GC-MS were: 70 $^{\circ}\text{C}$ to 220 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C}/\text{min}$, 220 $^{\circ}\text{C}$ for 15 min, 220 $^{\circ}\text{C}$ to 300
24 $^{\circ}\text{C}$ at 60 $^{\circ}\text{C}/\text{min}$, 300 $^{\circ}\text{C}$ for 2 min. Injector temperature was 250 $^{\circ}\text{C}$, carrier gas was helium
25 (1.2 mL/min), splitting ratio was 1:40 and detector temperature was 250 $^{\circ}\text{C}$. Identification of

1 compounds was done by comparison with NIST database. Conditions for UHPLC-ELSD
2 analysis are described in our previous study (Çiçek et al., 2018).

3

4 2.1.4. Isolation of diterpene acids

5 4.50 g oleoresin were dissolved in 300 mL diethyl ether and extracted five times with 50
6 mL of 5% potassium hydroxide aqueous solution. The combined aqueous layers were acidified
7 with 25% hydrochloric acid to pH 2 and extracted five times with 50 mL diethyl ether yielding
8 1.77 g of diterpene acids after evaporation of the solvent. 0.78 g of the acidic fraction were
9 chromatographed by preparative MPLC using isocratic elution with methanol-water (80:20 v/v)
10 for 60 minutes followed by methanol-water (85:15 v/v) for another 60 minutes. The resulting
11 192 fractions yielded *ent*-agathic acid (**1**, fractions 33-35, 4.8 mg), (13*E*)-*ent*-labd-8(17)-en-
12 15,18-dioic acid (**2**, fractions 41-47, 10.2 mg), *ent*-polyalthic acid (**3**, fractions 71-84, 124.4
13 mg) and *ent*-kaurenoic acid (**4**, fractions 120-132, 35.9 mg), kolavenic acid (**5**, fractions 149-
14 152, 6.7 mg), and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**6**, fractions 159-165, 4.9 mg) (Fig.
15 1). Structures of the isolated compounds were elucidated by comparison of their MS and NMR
16 spectra with the literature data (Lin et al., 1998; Salah et al., 2003; Xiang et al., 2004; Souza et
17 al., 2010;).

18

19 2.2. Biological activity testing

20 2.2.1. Antibacterial activity

21 The samples were tested against the ESKAPE panel of multidrug resistant bacterial human
22 pathogens, including the gram-positive bacteria *Enterococcus faecium* and methillicin-resistant
23 *Staphylococcus aureus* (MRSA), and the gram-negative bacteria *Klebsiella pneumoniae*,
24 *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli*. As inhibitory effects

1 were only observed for the Gram-positive strains, the respective bioassays are described in the
2 following.

3 *Staphylococcus aureus* DSM 18827 and *Enterococcus faecium* DSM 20477 were purchased
4 from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures,
5 Braunschweig, Germany. The cultivation of MRSA took place in TSB medium (1.2% tryptic
6 soy broth, 0.5% NaCl) and *E. faecium* was cultivated in M92 medium (3% trypticase soy broth,
7 0.3% yeast extract, pH 7.0-7.2). Overnight cultures of the test organisms were prepared and
8 diluted to an optical density (600 nm) of 0.01. To prepare the assay, the test samples (20 mg/mL
9 DMSO stock solution) were dissolved in medium and transferred into a 96-well microtiter plate
10 and 200 μ L of the cell suspension cultures were added to each well. The final assay
11 concentration of the substances was 100 μ g/mL. The inoculated microplates were incubated for
12 5 hours at 37 °C and 200 rpm (*E. faecium* without shaking). To detect the inhibitory effect of
13 the substances against MRSA 10 μ L of a resazurin solution (0.3 mg/mL phosphate-buffered
14 saline) was added to the microplates and incubated again for 5-30 min before the fluorescence
15 signal (560 nm/590 nm) was measured using the microplate reader (Tecan Infinite M200). For
16 *E. faecium* the pH indicator bromocresol purple was used to determine the acidification caused
17 by growing. The resulting values were compared with a positive control (chloramphenicol for
18 MRSA and ampicillin for *E. faecium*) and a negative control (no compound) on the same plate.
19 For IC₅₀ determination, a dilution series was prepared and the IC₅₀ value was calculated by
20 GraphPad Prism as the concentration that show 50% inhibition of viability on the basis of a
21 negative control (no compound).

22

23 2.2.2. Activity against dermatophytic fungi

24 *Trichophyton rubrum* I/95 and *T. mentagrophytes* 204/09 (patient isolates from Kiel
25 University Hospital) were cultivated for two weeks on Sabouraud agar (1% peptone, 2%

1 glucose, 1.5% agar-agar, pH 5.6). A suspension of 5×10^4 spores/mL Sabouraud medium was
2 prepared and added to each well of a microplate containing the test samples, which were
3 transferred as described before (bacterial assay). After incubation for 72 hours at 28 °C and 120
4 rpm the optical density at 600 nm was measured using the microplate reader. The resulting
5 values were compared with a positive (clotrimazol) and a negative control (no compound) on
6 the same plate. For IC₅₀ determination the test samples were diluted as described before
7 (bacterial assay) and calculated as the concentration that shows 50% inhibition of growing.

8

9 2.2.3. Cytotoxic activity

10 The sensitivity of liver cancer cell line HEP-G2 (DSMZ, Braunschweig, Germany),
11 colorectal adenocarcinoma cell line HT-29 (DSMZ, Braunschweig, Germany), malignant
12 melanoma cell line A-375 (CLS, Eppelheim, Germany), colon cancer cell line HCT-116
13 (DSMZ, Braunschweig, Germany), lung carcinoma cell line A-549 (CLS, Eppelheim,
14 Germany), human breast cancer line MDA-MB-231 (CLS, Eppelheim, Germany), and the non-
15 cancerous human keratinocyte line HaCaT (CLS, Eppelheim, Germany) to the compounds was
16 evaluated by monitoring the metabolic activity using the *CellTiterBlue* cell viability assay
17 (Promega, Mannheim, Germany). HEP-G2, HT-29 and HaCaT cells were cultivated in RPMI
18 medium, A549 and MDA-MB-231 cells in DMEM:Ham's F12 medium (1:1) supplemented
19 with 15 mM HEPES, and A-375 and HCT-116 cells were cultivated in DMEM medium
20 supplemented with 4.5 g/L D-glucose and 110 mg/L sodium pyruvate. All media were
21 supplemented with L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL
22 streptomycin. The cultures were maintained at 37 °C under a humidified atmosphere and 5%
23 CO₂. The cell lines were transferred every 3 or 4 days. For experimental procedure, cells were
24 seeded in 96-well plates at concentration of 10,000 cells per well in RPMI. After 24 hours
25 incubation, the medium was removed and 100 µL of the test sample were adjusted to a final

1 concentration of 100 $\mu\text{g/mL}$ by diluting with growth medium. Doxorubicin, as a standard
2 therapeutic drug was used as a positive control. Following compound addition, plates were
3 cultivated for 24 hours at 37 $^{\circ}\text{C}$. Afterwards, the assay was performed according to the
4 manufacturer's instructions and measured using the microplate reader at excitation with 560
5 nm and emission of 590 nm. For the determination of IC_{50} values, a dilution series of the
6 compounds was prepared and tested as described before. IC_{50} value was calculated as the
7 concentration that shows 50% inhibition of the viability.

8

9 *2.2.4. Statistical analysis*

10 Results were expressed as mean \pm SD. Statistical significance was determined by one-way
11 analysis of variance (ANOVA). Differences were considered significant if $p < 0.05$.

12

13 **3. Results**

14 *3.1. Preliminary tests*

15 Bioassays used in this study comprised two clinically relevant bacterial strains, two
16 dermatophyte strains, six different cancer cell lines, and one non-cancerous cell line. First tests
17 were accomplished using the oleoresin as well as its neutral and acidic fraction, which were
18 separated by anion exchange SPE. Cytotoxicity studies showed only moderate activity for the
19 oleoresin (IC_{50} values from 72.1 to 87.5 $\mu\text{g/mL}$), which was more pronounced in the neutral
20 fraction (IC_{50} values from 52.2 to 77.6 $\mu\text{g/mL}$) and less pronounced in the acidic fraction (IC_{50}
21 values from 84.7 to >100 $\mu\text{g/mL}$). In contrast, significant antibacterial results were observed
22 for the oleoresin and the acidic fraction, with IC_{50} values of 4.2 and 4.8 $\mu\text{g/mL}$ against *E.*
23 *faecium*, and 5.3 and 7.2 $\mu\text{g/mL}$ against MRSA, respectively, whereas the neutral fraction was
24 only moderately active (IC_{50} values from 52.5 to 87.1 $\mu\text{g/mL}$). Thus, our study concentrated on
25 the isolation of diterpene acids and the assessment of their biological activity. Additionally, the

1 neutral fraction was investigated for its array of sesquiterpenes by GC-MS, but was not further
2 separated due to its moderate antimicrobial and cytotoxic activity.

3

4 *3.2. Isolation of diterpene acids*

5 To separate the diterpene acids from the neutral constituents in a preparative scale, the
6 oleoresin was partitioned using potassium hydroxide and diethyl ether (Romero et al., 2009).
7 After UHPLC analysis of the partition showed a constituent pattern comparable to that of the
8 acidic fraction obtained by SPE, the acids were chromatographed using reversed phase MPLC.
9 Isocratic elution with methanol-water (80:20 v/v) yielded dicarboxylic acids *ent*-agathic acid
10 (**1**) and (13*E*)-*ent*-labd-8(17)-en-15,18-dioic acid (**2**) as well as *ent*-polyalthic acid (**3**), the
11 major diterpenoid of the *C. reticulata* oleoresin. Subsequent isocratic elution with methanol-
12 water (85:15 v/v) resulted in the isolation of *ent*-kaurenoic acid (**4**), kolavenic acid (**5**), and
13 (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**6**) (Fig. 1). With the exception of *ent*-copalic acid, the
14 isolated compounds comprised all relevant diterpene acids in the *C. reticulata* oleoresin.
15 Moreover, all three types of diterpene scaffolds known from copaiba oleoresins, labdane-type
16 (compounds **1**, **2**, **3**, and **6**), kaurane-type (**4**), and clerodane-type (**5**) diterpenes could be
17 investigated. All compounds were tested for their antimicrobial and cytotoxic potential, except
18 for compound **6**, which was not investigated on dermatophytes because of the minute amounts
19 of the compound isolated.

20

21 *3.3. Analysis of the neutral and acidic fractions*

22 The neutral fraction was analyzed by GC-MS for its composition and the content of the main
23 sesquiterpenes. Of the 35 present compounds, only eleven showed relative amounts of more
24 than 1%, with β -bisabolene (42.3%), caryophyllene oxide (18.7%), *cis*- α -bergamotene (11.7%),
25 and α -caryophyllene (10.0%) as the main constituents. UHPLC-ELSD analysis of the acidic

1 fractions gave the following relative amounts: *ent*-agathic acid (**1**, 0.92%), (13*E*)-*ent*-labd-
2 8(17)-en-15,18-dioic acid (**2**, 1.96%), *ent*-polyalthic acid (**3**, 51.6%), *ent*-kaurenoic acid (**4**,
3 21.6%), kolavenic acid (**5**, 8.52%), and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**6**, 10.8%). In
4 total, the neutral fraction accounted for 55% of the oleoresin and the acidic fraction for 45%.

5

6 3.4. Antimicrobial effects

7 As mentioned above, antibacterial assays resulted in significant effects for the crude
8 oleoresin, with IC₅₀ values of 4.2 µg/mL against *E. faecium* and 5.3 µg/mL against MRSA. The
9 antimicrobial activity was retained by the acidic fraction, with IC₅₀ values of 4.8 µg/mL against
10 *E. faecium* and 7.2 µg/mL against MRSA, while the neutral fraction showed poor activity with
11 IC₅₀ values of 52.5 and 75.9 µg/mL, respectively (Table 1). Regarding the pure diterpene acids,
12 except for the *ent*-agathic acid (**1**), which had no antibacterial activity, all other pure compounds
13 had significant activity against *E. faecium* and MRSA. Notably, the antibacterial activity of the
14 isolated diterpenoids increased with their lipophilicity, showing higher IC₅₀ values for
15 dicarboxylic acids **1** and **2** as well as for *ent*-polyalthic acid (**3**), while compounds **4** to **6** had
16 very low IC₅₀ values.

17 Due to the use of copaiba for the treatment of various skin infections in Brazilian traditional
18 medicine, we also assessed its inhibitory potential against two dermatophytic fungi from the
19 genus *Trichophyton* that cause athlete's foot, nail infection, ringworm and itches in head and
20 body skin. Here, the crude oleoresin had moderate antifungal activity (IC₅₀ value of 39.8 µg/mL
21 against *T. rubrum* and 15.1 µg/mL against *T. mentagrophytes*) and the acidic fraction was again
22 more active (IC₅₀ values 55.0 and 31.6 µg/mL, respectively) than the neutral fraction (IC₅₀
23 values 87.1 and 63.1 µg/mL, respectively). Among the five tested compounds, *ent*-polyalthic
24 acid (**3**) was the only metabolite that showed pronounced antifungal effects (IC₅₀ values 6.8
25 µg/mL against *T. rubrum* and 4.3 µg/mL against *T. mentagrophytes*, respectively), while the

1 other diterpenoids displayed moderate to low inhibitory potential (IC₅₀ values ranging from
2 15.5 to 91.2 µg/mL, Table 1) .

3

4 3.5. Cytotoxic effects

5 In the cytotoxicity tests, the oleoresin showed IC₅₀ values ranging from 72.1 µg/mL to 87.5
6 µg/mL against the six tested cancer cell lines. However, its cytotoxicity against the non-
7 cancerous human keratinocytes (HaCaT) cells was equipotent (IC₅₀ 86.5 µg/mL, Table 2). The
8 neutral fraction showed better activity against all cell lines than the crude oleoresin (IC₅₀ values
9 from 52.2 to 77.6 µg/mL), whereas the acidic fraction was only marginally active or inactive at
10 the highest concentration tested (100 µg/mL). Regarding the cytotoxicity of the pure
11 compounds, the dicarboxylic acids **1** and **2** were inactive at the highest concentration (100
12 µg/mL). The remaining compounds exerted moderate toxicity against cancer cell lines, which
13 was equal to their cytotoxicity on non-cancerous HaCaT cells, pointing out a non-specific
14 toxicity rather than a selective toxicity. *Ent*-kaurenoic acid (**4**) was most effective against A-
15 375, HepG2, HT-29, HCT-116, and MB-231 cell lines with IC₅₀ values from 50.2 to 65.2
16 µg/mL, whereas kolavenic acid (**5**) showed the highest activity against the A-549 cell line, with
17 an IC₅₀ value of 58.9 µg/mL. However, the compound showed the same toxicity towards HaCaT
18 cells, with an IC₅₀ value of 56.0 µg/mL.

19

20 4. Discussion

21 4.1. Cytotoxic effects

22 Several studies dealt with the cytotoxic and anticancer properties of copaiba oleoresin, its
23 fractions and pure compounds. Most notable activities were found for β-caryophyllene and the
24 diterpene kolavenol. β-Caryophyllene was active against HeLa, BT-20, B-16 and HTB cells,
25 with IC₅₀ values ranging from 3.86 to 4.86 µg/mL (Kubo et al., 1996). In two studies on the

1 cytotoxic potential of *ent*-kaurenoic acid (**4**), the compound was found active against CEM
2 leukemic cells as well as MCF-7 breast and HCT-8 colon cancer cells, but the activity was
3 found to be non-selective (Costa-Lotufo et al., 2002; Cavalcanti et al., 2009). *Ent*-kaurenoic
4 acid (**4**) and six other diterpene acids (including compounds **2** and **3**) were also part of a
5 cytotoxicity study on four different cancer cell lines, but none of the compounds showed
6 significant activity (Tincusi et al., 2002).

7 The weak activity of diterpene acids on human cancer cell lines was also evident in our own
8 study, where IC₅₀ values of all compounds exceeded 50 µg/mL. The neutral fraction did not
9 show superior results either, which was surprising with regards to the good effects observed for
10 the treatment of malignant horse melanoma at Pará University. Therefore, the neutral fraction
11 was analysed for its contents on sesquiterpenes by GC-MS, revealing that the oleoresin
12 investigated in our study is of the β-bisabolene-type (Zoghbi et al., 2009), in contrast to the
13 oleoresins of other *Copaifera* species, which were reported to contain up to 60% of β-
14 caryophyllene (Trindade et al., 2018).

15 β-Bisabolene was found to exhibit cytotoxic effects on breast cancer cell lines, with IC₅₀
16 values from 66.9 to 98.4 µg/mL (Yeo et al., 2015). These values were comparable to the IC₅₀
17 values observed in our own study and (together with the relatively small quantity of β-
18 caryophyllene) a possible reason for the low *in vitro* activity of our oleoresin. The fact that the
19 use of oleoresins for the treatment of horses is not restricted to *C. reticulata*, nor to a certain
20 chemotype, supports this theory. Because the horses at Pará University are treated topically,
21 where the responsible (lipophilic) compounds exhibit their effect after passive diffusion into
22 the tumour tissue, low solubility in culture media and thus reduced activity might be an
23 alternative explanation. This effect is confirmed by previous anticancer studies on copaiba
24 oleoresin, where the more pronounced effects were observed *in vivo*, such as activity against
25 Walker sarcoma in rats (Moraes et al. 1997), IMC carcinoma in mice (Ohsaki et al, 1994), Sp2/0

1 murine myeloma (Lima et al., 2003), or antineoplastic properties against Ehrlich tumours
2 (Gomes et al., 2008). These polarity issues will be more relevant for the sesquiterpenes than for
3 the more polar diterpene acids.

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5 4.2. Antimicrobial effects

6 Due to its wide use in folk medicine, copaiba oil was extensively studied for its antimicrobial
7 activity (Leandro et al., 2002). However, only few reports have been performed on the activity
8 of defined compounds isolated from the oleoresin. Tincusi et al. (2002) found significant
9 activities for *ent*-kaurenoic acid (**4**) and *ent*-copalic acid against *B. subtilis*, *S. aureus* and *S.*
10 *epidermidis*, with MIC values < 10 µg/mL for *S. aureus* and < 6 µg/mL for *B. subtilis* and *S.*
11 *epidermidis*, respectively. *Ent*-copalic acid was furthermore active against five different
12 *Streptococcus* species as well as *Lactobacillus casei*, with MIC values < 6 µg/mL and minimum
13 bactericidal concentrations below 15 µg/mL, respectively (Souza et al., 2011). Santos et al.
14 (2008b) investigated the effect of the oleoresin obtained from eight different *Copaifera* species
15 on their activity against *S. aureus*, MRSA, *S. epidermidis*, *B. subtilis* and *E. faecalis*. In the
16 same study, *C. reticulata* oleoresin obtained from two different regions was tested and showed
17 different results for different geographic regions of origin. The oleoresin obtained from Acre
18 (western Brazil) showed moderate activity against MRSA and good activity against the
19 remaining bacterial strains, while oleoresin collected in Pará (northern Brazil) was only
20 moderately active against *B. subtilis* and *E. faecalis*, and inactive against the other strains. In
21 the same study, antifungal properties were investigated, but *C. reticulata* oleoresin was neither
22 active against *T. rubrum* or *T. mentagrophytes*, nor against any of the other tested fungi or
23 yeasts.

24 Interestingly, in our own study good activity for the *C. reticulata* oleoresin was observed
25 against *T. mentagrophytes* and still moderate activity against *T. rubrum*. *Ent*-polyalthic acid

1 (3), the main diterpene acid that accounts around 23% of the oleoresin (Çiçek et al., 2018), was
2 found responsible for this effect as the compound showed IC₅₀ values of 4.3 (*T.*
3 *mentagrophytes*) and 6.8 µg/mL (*T. rubrum*). Bearing a heteroaromatic substructure *ent*-
4 polyalthic acid (3) significantly differs from the remaining diterpene acids in our study. This
5 structural difference and its much higher activity render the furylic moiety a possible key feature
6 for antifungal activity of diterpene acids, at least with regards to *Trichophyton* sp. In this sense,
7 investigations of *ent*-hardwickiic acid, another furanoditerpene acid known from copaiba, or
8 oleoresins containing this compound would be of interest. Regarding the use of copaiba for the
9 treatment of skin infections, the observed antifungal activity might provide a scientific
10 explanation for its beneficial effect.

11 Even more pronounced than the activity against dermatophytes, was the effect of *C.*
12 *reticulata* oleoresin against *E. faecium* and MRSA, with IC₅₀ values of 4.2 and 5.3 µg/mL,
13 respectively. *E. faecium* causes urinary tract infections, whereas MRSA mainly leads to skin
14 infections, but can also affect the respiratory system. Regarding the isolated diterpenoids, all
15 tested compounds except *ent*-agathic acid (1) were active. However, the antibacterial effects of
16 lipophilic compounds 4, 5 and 6 were more pronounced. Interestingly, these three compounds
17 represent all three types of copaiba diterpenes, kaurane-type, clerodane-type, and labdane-type
18 diterpenes, thus indicating that the antibacterial activity is not restricted to a certain scaffold.
19 As *E. faecium* and other *Enterococcus* species can cause urinary tract infections, another illness
20 that is traditionally treated with copaiba, our results give a possible scientific prove for its effect.
21 Similar to MRSA, *E. faecium* is prone to develop resistances against antibiotics. Therefore, the
22 results of our study reveal that the oleoresin and the pure diterpene components of copaiba are
23 useful in primary health care to treat bacterial and fungal infections. Several pure components
24 also have potential to serve as lead structures for the treatment of clinically relevant bacterial
25 strains, which is one of the major global challenges of the 21st century.

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5. Conclusion

In the present study, the biological activity of the *Copaifera reticulata* oleoresin was systematically analysed with regards to its neutral and acidic fractions and constituents. While reports on cytotoxic activities were not corroborated by our own *in vitro* experiments, we proved its antimicrobial activity and identified the compounds responsible for the observed pharmacological effects. We demonstrated that *ent*-polyalthic acid (**3**) was significantly active against both dermatophytic fungi, *Trichophyton rubrum* and *T. mentagrophytes*, which might be related to its furylic substructure. Furthermore, *ent*-kaurenoic acid (**4**), kolavenic acid (**5**), and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**6**) were found to be very active against *E. faecium* and MRSA. Differing antimicrobial effects observed for structurally different diterpene acids yield new details about the health-beneficial effects of copaiba. Thus, supporting the wide medical use of the chemically divers genus *Copaifera*. Giving a possible explanation for the ethnopharmacological use of the *C. reticulata* oleoresin for the treatment of skin and urinary tract infections, the present study moreover shows first reports for the activity of copaiba diterpenoids against *Trichophyton* dermatophytes and two clinically relevant bacterial strains.

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1 **Author contribution**

2 ALPB, AWS and SSC conducted the experiments, JDB collected the oleoresin, CP and DT
3 were supervising biological activity testing, CZ and DT revised the manuscript, SSC designed
4 the study and wrote the manuscript. All authors approved the final version of the manuscript.

5

6 **Conflict of interest**

7 None to declare.

8

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18 **Compound list:**

19 **1:** *ent*-agathic acid
20 **2:** (13E)-*ent*-labd-8(17)-en-15,18-dioic acid
21 **3:** *ent*-polyalthic acid
22 **4:** *ent*-kaurenoic acid
23 **5:** kolavenic acid
24 **6:** (13E)-*ent*-labda-7,13-dien-15-oic acid

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1 **Figure captions**

2

3 Figure 1: Chemical structures of isolated compounds: *ent*-agathic acid (**1**), (13*E*)-*ent*-labd-
4 8(17)-en-15,18-dioic acid (**2**), *ent*-polyalthic acid (**3**), *ent*-kaurenoic acid (**4**), kolavenic acid (**5**),
5 and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**6**).

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8 Figure 2: GC-MS chromatogram of the neutral copaiba fraction. Main sesquiterpenes were β -
9 caryophyllene (**A**), *cis*- α -bergamotene (**B**), β -bisabolene (**C**) and caryophyllene oxide (**D**).

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1 **Table 1**

2 Antimicrobial effects of the crude oleoresin, its neutral and acidic fractions, and compounds 1-
 3 **6**. The IC₅₀ values are in µg/mL., n.t. means not tested. Positive controls were ampicillin (*E.*
 4 *faecium*), chloramphenicol (MRSA) and clotrimazole (*T. rubrum* and *T. mentagrophytes*).

	<i>E. faecium</i>	MRSA	<i>T. rubrum</i>	<i>T. mentagrophytes</i>
Crude oleoresin	4.2 ± 0.1	5.3 ± 0.3	39.8 ± 1.8	15.1 ± 1.2
Neutral fraction	52.5 ± 2.4	75.9 ± 7.4	87.1 ± 1.0	63.1 ± 2.9
Acidic fraction	4.8 ± 0.1	7.2 ± 0.6	55.0 ± 3.9	31.6 ± 9.3
Compound 1	> 100	> 100	41.7 ± 0.5	30.2 ± 0.3
Compound 2	9.3 ± 0.3	10.7 ± 1.1	44.7 ± 3.0	38.0 ± 0.5
Compound 3	8.5 ± 0.4	8.9 ± 0.8	6.8 ± 0.1	4.3 ± 0.1
Compound 4	2.3 ± 0.2 ^{a,b}	3.4 ± 0.2 ^{a,b}	70.8 ± 1.6	15.5 ± 0.7
Compound 5	2.2 ± 0.1 ^{a,b}	3.0 ± 0.2 ^{a,b}	66.0 ± 0.8	91.2 ± 5.9
Compound 6	1.6 ± 0.1 ^{a,b,d}	2.5 ± 0.3 ^{a,b}	n.t.	n.t.
Positive control	0.4 ± 0.1 ^{a,b,c,d,e}	1.2 ± 0.1 ^{a,b,c,d,e}	0.1 ± 0.0	0.1 ± 0.0

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 6 Each value represents the mean ± SD. Significance was determined by one-way ANOVA for
 7 compounds **2-6** against *E. faecium* and MRSA.

8 ^a $p < 0.05$ as compared with compound **2**.

9 ^b $p < 0.05$ as compared with compound **3**.

10 ^c $p < 0.05$ as compared with compound **4**.

11 ^d $p < 0.05$ as compared with compound **5**.

12 ^e $p < 0.05$ as compared with compound **6**.

13

1 **Table 2**

2 Cytotoxic effects of the crude oleoresin, its neutral and acidic fraction, and compounds **1-6**. The IC₅₀ values are in µg/mL. Doxorubicin was used
 3 as positive control.

	A-375	HepG2	HT-29	HCT-116	A-549	MB-231	HaCaT
Crude oleoresin	80.2 ± 0.5	79.3 ± 0.3	83.8 ± 1.2	72.1 ± 0.6	87.5 ± 0.3	85.5 ± 0.1	86.5 ± 0.1
Neutral fraction	55.0 ± 0.8	59.2 ± 4.5	52.2 ± 2.5	53.3 ± 0.1	77.6 ± 2.2	54.3 ± 0.5	67.6 ± 7.8
Acidic fraction	85.3 ± 0.2	84.7 ± 0.9	92.9 ± 0.8	87.9 ± 0.3	> 100	91.4 ± 0.5	> 100
Compound 1	> 100	> 100	> 100	> 100	> 100	> 100	> 100
Compound 2	> 100	> 100	> 100	> 100	> 100	> 100	> 100
Compound 3	78.3 ± 1.3	83.2 ± 1.3	86.1 ± 1.4	66.2 ± 0.7	91.2 ± 0.5	96.4 ± 1.1	87.5 ± 0.8
Compound 4	55.7 ± 0.5	59.4 ± 0.2	61.8 ± 0.6	50.2 ± 4.1	79.6 ± 1.7	65.2 ± 0.3	69.0 ± 3.7
Compound 5	62.2 ± 0.7	68.9 ± 0.7	63.2 ± 0.5	62.4 ± 0.3	58.9 ± 0.5	72.8 ± 0.1	56.0 ± 0.3
Compound 6	> 50	> 50	> 50	> 50	> 50	> 50	> 50
Positive control	15.9 ± 2.2	14.9 ± 1.7	8.9 ± 0.5	10.6 ± 1.9	31.4 ± 4.4	15.2 ± 0.2	10.0 ± 4.5

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 5 Each value represents the mean ± SD.

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1 **References**

- 2 Almeida, M.R., Darin, J.D.C., Hernandes, L.C., Ramos, M.F.S., Antunes, L.M.G., Freitas, O.,
3 2012. Genotoxicity assessment of copaiba oil and its fractions in Swiss mice. *Genet. Mol.*
4 *Biol.* 35, 664–672. <https://doi.org/10.1590/S1415-47572012005000052>.
- 5 Bardají, D.K.R., da Silva, J.J.M., Bianchi, T.C., Eugênio, D.D., de Oliveira, P.F., Leandro, L.F.,
6 Rogez, H.L.G., Venezianni, R.C.S., Ambrosio, S.R., Tavares, D.C., Bastos, J.K., Martins,
7 C.H.G., 2016. *Copaifera reticulata* oleoresin: Chemical characterization and antibacterial
8 properties against oral pathogens. *Anaerobe* 40, 18–27.
9 <https://doi.org/10.1016/j.anaerobe.2016.04.017>.
- 10 Basile, A.C., Sertié, J.A.A., Freitas, P.C.D., Zanini, A.C., 1988. Anti-inflammatory activity of
11 oleoresin from Brazilian *Copaifera*. *J. Ethnopharmacol.* 22, 101–109.
12 [https://doi.org/10.1016/0378-8741\(88\)90235-8](https://doi.org/10.1016/0378-8741(88)90235-8).
- 13 Boaroto, J. A., 2014. Identification of β -caryophyllene sesquiterpene in copaiba oil using
14 Raman spectroscopy. Dissertation, Universidade Camilo Castelo Branco, São Paulo.
- 15 Borges, C.H.G., Cruz, M.G., Carneiro, L.J., da Silva, J.J.M., Bastos, J.K., Tavares, D.C., de
16 Oiveira, P.F., Rodrigues, V., Veneziani, R.C.S., Parreira, R.L.T., Caramori, G.F.,
17 Nagurniak, G.R., Magalhaes, L.G., Ambrosio, S.R., 2016. *Copaifera duckei* oleoresin and
18 its main nonvolatile terpenes: In vitro schistosomicidal properties. *Chem. Biodivers.* 13,
19 1348–1356. <https://doi.org/10.1002/cbdv.201600065>.
- 20 Brito, N.M.B., Brito, M.V.H., Carvalho, R.K.V., Matos, L.T.M.B., Lobato, R.C., Correa, S.C.,
21 Brito, R.B., 2010. The effect of copaiba balsam on Walker 256 carcinoma inoculated into
22 the vagina and uterine cervix of female rats. *Acta Bras. Cir.* 25, 176–180.
23 <https://doi.org/10.1590/S0102-86502010000200010>
- 24 Cavalcanti, B.C., Costa-Lotufo, L.V., Moraes, M.O., Burbano, R.R., Silveira, E.R., Cunha,
25 K.M.A., Rao, V.S.N., Moura, D.J., Rosa, R.M., Henriques, J.A.P., Pessoa, C., 2006.

1 Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in copaiba oil.
2 Food Chem. Toxicol. 44, 388–392. <https://doi.org/10.1016/j.fct.2005.08.011>.

3 Cavalcanti, B.C., Bezerra, D.P., Magalhaes, H.I.F., Moraes, M.O., Lima, M.A.S., Silveira, E.R.,
4 Camara, C.A.G., Rao, V.S., Pessoa, C., Costa-Lotufo, L.V., 2009. Kauren-19-oic acid
5 induces DNA damage followed by apoptosis in human leukemia cells. J. Appl. Toxicol.
6 29, 560–568. <https://doi.org/10.1002/jat.1439>.

7 Carvalho, J.C.T., Cascon, V., Possebon, L.S., Morimoto, M.S.S., Cardoso, L.G.V., Kaplan,
8 M.A.C., Gilbert, B., 2005. Topical antiinflammatory and analgesic activities of *Copaifera*
9 *duckei* Dwyer. Phytother. Res. 19, 946–950. <https://doi.org/10.1002/ptr.1762>.

10 Çiçek, S.S., Pfeifer Barbosa, A.L., Girreser, U., 2018. Quantification of diterpene acids in
11 copaiba oleoresin by UHPLC-ELSD and heteronuclear two-dimensional qNMR. J.
12 Pharm. Biomed. Anal. 160, 126–134. <https://doi.org/10.1016/j.jpba.2018.07.034>.

13 Costa, A.R.M., Freitas, L.A.P., Mendiola, J., Ibáñez, E., 2015. *Copaifera langsdorffii*
14 supercritical fluid extraction: Chemical and functional characterization by LC/MS and in
15 vitro assays. J. Supercrit. Fluids 100, 86–96.
16 <https://doi.org/10.1016/j.supflu.2015.02.028>.

17 Costa-Lotufo, L.V., Cunha, G.M.A., Farias, P.A.M., Viana, G.S.B., Cunha, K.M.A., Pessoa,
18 C., Moraes, M.O., Silveira, E.R., Gramosa, N.V., Rao, V.S.N., 2002. The cytotoxic and
19 embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii*
20 oleoresin. Toxicon 40, 1231–1234. [https://doi.org/10.1016/S0041-0101\(02\)00128-9](https://doi.org/10.1016/S0041-0101(02)00128-9).

21 Dias, D.S., Fontes, L.B.A., Crotti, A.E.M., Aarestrup, B.J.V., Aarestrup, F.M., Filho, A.A.S.,
22 Correa, J.O.A., 2014. Copaiba oil suppresses inflammatory cytokines in splenocytes of
23 C57Bl/6 mice induced with experimental autoimmune encephalomyelitis (EAE).
24 Molecules 19, 12814–12826. <https://doi.org/10.3390/molecules190812814>.

1 Gomes, N.D., Rezende, C.D., Fontes, S.P., Matheus, M.E., Fernandes, P.D., 2007.
2 Antinociceptive activity of Amazonian copaiba oils. *J. Ethnopharmacol.* 109, 486–492.
3 <https://doi.org/10.1016/j.jep.2006.08.018>

4 Gomes, N.D., Rezende, C.D., Fontes, S.P., Hovell, A.M.C., Landgraf, R.G., Matheus, M.E.,
5 Pinto, A.D., Fernandes, P.D., 2008. Antineoplastic activity of *Copaifera multijuga* oil and
6 fractions against ascitic and solid Ehrlich tumor. *J. Ethnopharmacol.* 119, 179–184.
7 <https://doi.org/10.1016/j.jep.2008.06.033>.

8 Gomes, N.D., Rezende, C.D., Fontes, S.P., Matheus, M.E., Pinto, A.D., Fernandes, P.D., 2010.
9 Characterization of the antinociceptive and anti-inflammatory activities of fractions
10 obtained from *Copaifera multijuga* Hayne. *J. Ethnopharmacol.* 128, 177–183.
11 <https://doi.org/10.1016/j.jep.2010.01.005>.

12 He, K., Zeng, L., Shi, G., Zhao, G.-X., Kozlowski, J.F., McLaughlin, J.L., 1997. Bioactive
13 Compounds from *Taiwania cryptomerioides*. *J. Nat. Prod.* 60, 38–40.
14 <https://doi.org/10.1021/np960513c>.

15 Izumi, E., Ueda-Nakamura, T., Veiga Junior, V.F., Pinto, A.C., Nakamura, C.V., 2012.
16 Terpenes from *Copaifera* demonstrated in vitro antiparasitic and synergistic activity. *J.*
17 *Med. Chem.* 55, 2994–3001. <https://doi.org/10.1021/jm201451h>.

18 Kubo, I., Chaudhuri, S.K., Kubo, Y., Sanchez, Y., Ogura, T., Saito, T., Ishikawa, H., Haraguchi,
19 H., 1996. Cytotoxic and antioxidative sesquiterpenoids from *Heterotheca inuloides*.
20 *Planta Med.* 62, 427–430. <https://doi.org/10.1055/s-2006-957932>.

21 Leandro, L.M., de Sousa Vargas, F., Barbosa, P.C.S., Neves, J.K.O., da Silva, J.A., da Veiga-
22 Junior, V. F., 2012. Chemistry and biological activities of terpenoids from copaiba
23 (*Copaifera* spp.) oleoresins. *Molecules* 17, 3866.
24 <https://doi.org/10.3390/molecules17043866>.

- 1 Legault, J., Dahl, W., Debiton, E., Pichette, A., Madelmont, J.-C., 2003. Antitumor Activity of
2 Balsam Fir Oil: Production of reactive oxygen species induced by α -humulene as possible
3 mechanism of action. *Planta Med.* 69, 402-407. <https://doi.org/10.1055/s-2003-39695>.
- 4 Lima, S.R.M., Veiga Junior, V.F., Christo, H.B., Pinto, A.C., Fernandes, P.D., 2003. In vivo
5 and in vitro studies on the anticancer activity of *Copaifera multijuga* Hayne and its
6 fractions. *Phytother. Res.* 17, 1048–1053. <https://doi.org/10.1002/ptr.1295>.
- 7 Lin, S.J., Short, R.E., Ford, S.P., Gringg, E.E., Rosazza, J.P.N., 1998. In vitro
8 biotransformations of isocupressic acid by cow rumen preparations: formation of agathic
9 and dihydroagathic acids. *J. Nat. Prod.* 61, 51–56. <https://doi.org/10.1021/np970372u>.
- 10 Neto, J.N., Lindoso, M.J.S., Coelho, L.F., Carvalho, R.A.F., Rodrigues, T.G.P.M., Araujo,
11 A.G.P., Girao, M.J.B.C., Schor, E., 2011. Changes in the volume and histology of
12 endometriosis foci in rats treated with copaiba oil (*Copaifera langsdorffii*). *Acta Bras. Cir.*
13 26, 20–24. <https://doi.org/10.1590/S0102-86502011000800005>.
- 14 Ohsaki, A., Yan, L.T., Ito, S., Edatsugi, H., Iwata, D., Komoda, Y., 1994. The isolation and in
15 vivo potent antitumor activity of clerodane diterpenoid from the oleoresin of Brazilian
16 medicinal plant *Copaifera langsdorffii* Desfon. *Bioorg. Med. Chem. Lett.* 4, 2889–2892.
17 [https://doi.org/10.1016/S0960-894X\(01\)80834-9](https://doi.org/10.1016/S0960-894X(01)80834-9).
- 18 Paiva, L.A.F., Gurgel, L.A., de Sousa, E.T., Silveria, E.R., Silva, R.M., Santos, F.A., Rao,
19 V.S.N., 2004. Protective effect of *Copaifera langsdorffii* oleo-resin against acetic acid-
20 induced colitis in rats. *J. Ethnopharmacol.* 93, 51–56.
21 <https://doi.org/10.1016/j.jep.2004.03.028>.
- 22 Romero, A.L., Baptistella, L.H.B., Imamura, P.M., 2009. Absolute configuration of some
23 dinorlabdanes from the copaiba oil. *J. Braz. Chem. Soc.* 20, 1036–1040.
24 <https://doi.org/10.1590/S0103-50532009000600006>.

- 1 Salah, M.A., Bedir, E., Toyang, N.J., Khan, I.A., Dewayne Harries, M., Wedge, D.E., 2003.
2 Antifungal clerodane diterpenes from *Macaranga monandra* (L) Muell. Et Arg.
3 (Euphorbiaceae). J. Agric. Food Chem. 51, 7607–7610.
4 <https://doi.org/10.1021/jf034682w>.
- 5 Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Veiga Junior, V.F., Pinto, A.C., Nakamura,
6 C.V., 2008a. Effect of Brazilian copaiba oils on *Leishmania amazonensis*. J.
7 Ethnopharmacol. 120, 204–208. <https://doi.org/10.1016/j.jep.2008.08.007>.
- 8 Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Veiga Junior, V.F., Pinto, A.C., Nakamura,
9 C.V., 2008b. Antimicrobial activity of Brazilian copaiba oils obtained from different
10 speies of the *Copaifera* genus. Mem. Inst. Oswaldo Cruz 103, 277–281.
11 <https://doi.org/10.1590/S0074-02762008005000015>.
- 12 Santos Junior, H.M., Oiveira, D.F., Carvalho, D.A., Pinto, J.M.A., Campos, V.A.C., Murao,
13 A.R.B., Pessoa, C., Moraes, M.O., Costa-Lotufo, L.V., 2010. Evaluation of native exotic
14 Brazilian plants for anticancer activity. J. Nat. Med. 64, 231–238.
15 <https://doi.org/10.1007/s11418-010-0390-0>.
- 16 Silva, S.L., Figueiredo, P.M.S., Yano, T., 2007. Chemotherapeutic potential of the volatile oils
17 from *Zanthoxylum rhoifolium* Lam leaves. Eur. J. Pharmacol. 576, 180–188.
18 <https://doi.org/10.1016/j.ejphar.2007.07.065>.
- 19 Souza, P.A., Rangel, L.P., Oigman, S.S., Elias, M.M., Ferreiera-Pereira, A., de Lucas, N.C.,
20 Leitao, G.G., 2010. Isolation of two bioactive diterpenic acids from *Copaifera glycyarpa*
21 oleoresin by high-speed counter-current chromatography. Phytochem. Anal. 21, 539–
22 543. <https://doi.org/10.1002/pca.1229>.
- 23 Souza, A.B., Martins, C.H.G., Souza, M.G.M., Furtado, N.A.J.C., Heleno, V.C.G., de Sousa,
24 J.P.B., Rocha, E.M.P., Bastos, J.K., Cunha, W.R., Veneziani, R.C.S., Ambrosio, S.R.,

1 2011. Antimicrobial activity of terpenoids from *Copaifera langsdorffii* Desf. against
2 cariogenic bacteria. *Phytother. Res.* 25, 215–220. <https://doi.org/10.1002/ptr.3244>.

3 Souza Barbosa, P.C., Moreira Wiedemann, L.S., da Silva Medeiros, R., Barbosa Sampaio, P.T.,
4 Vieira, G., da Veiga-Junior, V.F., 2013. Phytochemical fingerprints of copaiba oils
5 (*Copaifera multijuga* Hayne) determined by multivariate analysis. *Chem. Biodivers.*10,
6 1350–1360. <https://doi.org/10.1002/cbdv.201200356>.

7 Tincusi, B.M., Jimenez, I.A., Bazzocchi, I.L., Moujir, L.M., Mamani, Z.A., Barroso, J.P.
8 Ravelo, A.G., Hernandez, B.V., 2002. Antimicrobial terpenoids from the oleoresin of the
9 Peruvian medicinal plant *Copaifera paupera*. *Planta Med.* 68, 808–812.
10 <https://doi.org/10.1055/s-2002-34399>.

11 Trindade, R., Silva, J.K., Setzer, W.N., 2018. *Copaifera* of the neotropics: a review of the
12 phytochemistry and pharmacology. *Int. J. Mol. Sci.* 19, 1511.
13 <https://doi.org/10.3390/ijms19051511>.

14 Veiga Junior, V.F., Zunino, L., Calixto, J.B., Patitucci, M.L., Pinto, A.C., 2001. Phytochemical
15 and antioedematogenic studies of commercial copaiba oils available in Brazil. *Phytother.*
16 *Res.* 15, 476–480. <https://doi.org/10.1002/ptr.976>.

17 Veiga Junior, V.F., Pinto, A.C., 2002. O gênero *Copaifera* L.. *Quím. Nova* 25, 273–286.
18 <https://doi.org/10.1590/S0100-40422002000200016>.

19 Veiga Junior, V.F., Rosas, E.C., Carvalho, M.V., Henriques, M.G.M.O., Pinto, A.C., 2007.
20 Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera*
21 *cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne–
22 A comparative study. *J. Ethnopharmacol.* 112, 248–254.
23 <https://doi.org/10.1016/j.jep.2007.03.005>.

24 Yeo, S.K., Ali, A.Y., Hayward, O.A., Turnham, D., Jackson, T., Bowen, I.D., Clarkson, R.,
25 2016. Beta-bisabolene, a sesquiterpene from the essential oil extract of *Opoponax*

1 (*Commiphora guidottii*), exhibits cytotoxicity in breast cancer cell lines. *Phytother. Res.*
2 30, 418–425. <https://doi.org/10.1002/ptr.5543>.

3 Xiang, W., Li, R.T., Song, Q.S., Na, Z., Sun, H.D., 2004. Ent-clerodanoids from *Isodon*
4 *scoparius*. *Helv. Chim. Acta* 87, 2860–2865. <https://doi.org/10.1002/hlca.200490257>.

5 Zheng, G.-Q., Kenney, P.M., Lam, L.K.T., 1992. Sesquiterpenes from clove (*Eugenia*
6 *caryophyllata*) as potential anticarcinogenic agents. *J. Nat. Prod.* 55, 999–1003.
7 <https://doi.org/10.1021/np50085a029>.

8 Zoghbi, M.G.B., Andrade, E.H.A., Martins-da-Silva, R.C.V., Trigo, J.R., 2009. Chemical
9 variation in the volatiles of *Copaifera reticulata* Ducke (Leguminosae) growing wild in
10 the states of Pará and Amapá, Brazil. *J. Essent. Oil Res.* 21, 501–503.
11 <https://doi.org/10.1016/j.bse.2012.02.003>.