JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 1405–1410

Received September 21, 2005
Accepted July 24, 2006
DOI 10.1211/jpp.58.11.0015
ISSN 0022-3573

The inhibition of paw oedema formation caused by the oil of *Copaifera multijuga* Hayne and its fractions

Valdir F. Veiga Junior, Luciano Zunino, Maria Lúcia Patitucci, Angelo C. Pinto and João B. Calixto

Abstract

Two oils exuded from a *Copaifera multijuga* Hayne tree (Leguminosae-Caesalpinoideae), collected from the same plant, but in different periods of the year, and the hexanic, dichloromethanic and methanolic fractions of one of these oils were analysed by high-resolution gas chromatography (HRGC) and HRGC coupled with mass spectrometry (HRGC-MS). In addition, the in-vivo preliminary anti-oedematogenic actions of the oil and some fractions of it were assessed against carrageenan- and bradykinin-induced oedema formation in the rat paw. Twenty-seven sesquiterpenes and six diterpenes were identified, β-caryophyllene, α-copaene and copalic acid being the main components. The dichloromethanic and methanolic fractions obtained from *C. multijuga* oil given by the intraperitoneal route caused a significant inhibition of paw oedema caused by carrageenan with inhibition of 49 ± 13% and 64 ± 9 %, respectively. Likewise, dexamethasone (the positive control drug) also greatly inhibited carrageenan-induced paw oedema formation (60 ± 4% at 2 h). The hexanic fraction also significantly inhibited (50 ± 6%) the paw oedema formation caused by bradykinin. These results suggest the presence of still non-identified active terpene compounds in the oil of *C. multijuga* that exhibit anti-oedematogenic properties. Of note, the yield of these compounds and the pharmacological actions of the oil, exhibited great seasonal variations, a relevant aspect that should be carefully observed for the correct medicinal use of this plant by the population.

Departamento de Química, Instituto de Ciências Exatas, Universidade Federal do Amazonas, 69077-000, Manaus, AM, Brazil

Valdir F. Veiga Junior

Instituto de Química, Centro de Tecnologia, Universidade Federal do Rio de Janeiro, 21945-970, Rio de Janeiro, RJ, Brazil

Valdir F. Veiga Junior, Maria Lúcia Patitucci, Angelo C. Pinto

Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900-Florianópolis, SC, Brazil

Luciano Zunino, João B. Calixto

Correspondence: V. F. Veiga Junior, Departamento de Química, Instituto de Ciências Exatas, Universidade Federal do Amazonas, 69077-000, Manaus, AM, Brazil. E-mail: valdirveiga@ufam.edu.br

Acknowledgements and funding: The authors thank CAPES and CNP8 for financial support and Dr Simon Garden for his useful suggestions.

Introduction

The plants belonging to the genus *Copaifera* L. (Leguminosae-Caesalpinoideae) grow abundantly in South American countries. *Copaifera multijuga* Hayne is very common in the Amazon region, especially in the central and western Amazonia, at Manaus neighbourhood. The copaiba oil is exuded from the trunk of the trees of some of the *Copaifera* species and has been demonstrated to be mainly constituted of sesquiterpenes and diterpenes (Delle-Monache et al 1969, 1970, 1971; Veiga Junior et al 1995, 1997; Braga et al 1998; Cascon & Gilbert 2000; Lima et al 2003). In Brazil, folk medicine widely prescribes the copaiba oil, either systemically or topically, for the management of several pathological states, such as inflammation, bronchitis, cough, cancer and as antiseptic for the urinary system (Veiga Junior & Pinto 2002). However, only a few pharmacological studies have been so far carried out with the oil or the constituents isolated from *C. multijuga* (Basile et al 1988; Fernandes et al 1992; Osaki et al 1994; Veiga Junior et al 2001). In a previous study, we have shown that the resin, as well as the diterpene and sesquiterpene fractions, obtained from *C. multijuga* given orally to mice exhibited anti-tumoral activity (Lima et al 2003).

In this study, we have further examined the chemical composition and also the in-vivo effects of the crude oil and the hexanic, dichloromethanic and methanolic fractions isolated from the oil of *C. multijuga* against carrageenan- and bradykinin-induced oedema formation in the rat paw.

Materials and Methods

Plant material

The copaiba oil was collected from the *Copaifera multijuga* Hayne tree in the Ducke Reserve of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil. Two samples were obtained from the same tree in different seasons: summer (August),

the sample named *C. multijuga* 1, and winter (February), *C. multijuga* 2.

Fractionating procedures

The sample of *C. multijuga* 1 was fractionated using silica gel column chromatography as previously reported (Pinto et al 1997). Briefly, silica gel was impregnated with a 5% aqueous solution of KOH and the column was eluted with hexane, dichloromethane and methanol. The methanol fraction, containing the potassium salts from the diterpenic carboxylic acids, was acidified with hydrochloric acid and concentrated under reduced pressure to obtain the diterpene carboxylic acids fraction, called methanolic fraction. The process afforded three fractions: hexanic (F1), dichloromethanic (F2) and methanolic (F3).

Gas chromatography analyses

Oils and fractions were methylated with diazomethane and analysed by infrared spectroscopy and high-resolution gas chromatography-mass spectrometry (HRGC-MS). The constitution of each oil and fraction is presented in Table 1.

Table 1 Chemical composition of *Copaifera multijuga* Hayne oils

Compound	Retention indices			*C. multijuga* 1				*C. multijuga* 2
	A1	A2	Polar	Oil (%)	F1	F2	F3	Oil (%)
α-Elemene	1344	—	—	1.0	1.7	—	—	—
α-Cubebene	1352	1378	1431	0.4	0.6	—	—	0.3
α-Copaene	1382	1383	1457	5.0	8.1	—	—	2.5
Calarene	1397	—	—	5.3	8.6	—	—	1.1
β-Caryophyllene	1426	1413	1559	29.6	48.0	—	—	58.4
α-Bergamotene	1436	—	—	4.4	7.2	—	—	2.6
β-Sesquiphellandrene	1442	—	—	0.2	0.3	—	—	0.1
Humulene	1457	—	1620	5.7	9.2	—	—	8.4
γ-Amorfene	1478	—	—	2.3	3.7	—	—	1.9
D-Germacrene	1483	1464	1645	1.5	2.4	—	—	2.5
Allo-aromadendrene	1490	—	1595	0.6	1.0	—	—	—
B-Germacrene	1499	1495	—	0.7	1.1	—	—	1.0
NI	1503	—	—	0.7	1.1	—	—	0.3
γ-Cadinene	1515	122	1704	1.3	2.0	—	—	0.6
δ-Cadinene	1524	1528	1715	1.9	3.1	—	—	1.7
α-Cadinene	1531	—	1730	0.7	1.1	—	—	0.2
β-Vetivene	1542	—	—	0.5	0.8	—	—	0.1
Total of hexanic fraction				61.6	100	—	—	82.8
α-Caryophyllenol	1554	—	—	5.8	—	16.8	—	0.8
Ledol	1565	—	—	—	—	—	—	0.2
Caryophyllene oxide	1582	—	—	13.0	—	37.4	—	0.5
Guaiol	1595	—	—	—	—	—	—	0.2
NI	1607	—	—	1.4	—	4.1	—	—
Cedrol	1616	—	—	3.6	—	10.3	—	0.4
T-Cadinol	1625	—	—	1.2	—	3.4	—	—
Cadalene	1637	1667	—	0.9	—	2.5	—	0.4
NI	1643	—	—	1.0	—	2.8	—	0.7
α-Cadinol	1649	—	—	2.2	—	6.6	—	0.4
β-Bisabolol	1666	—	—	0.7	—	2.1	—	0.1
Epi-α-bisabolol	1690	—	—	3.0	—	8.7	—	—
14-Hydroxy-α-humulene	1712	—	—	1.0	—	2.9	—	—
NI	1759	—	—	0.9	—	2.5	—	—
Total of dichloromethanic fraction				34.6	—	100	—	3.6
Methyl eperuate				—	—	—	—	0.4
Methyl copalate				1.9	—	—	50.2	6.3
Dimethyl pinifolate				—	—	—	—	0.2
NI				—	—	—	—	1.3
Dimethyl agathate				0.4	—	—	9.8	2.1
Methyl 3β-hydroxy-copalate				—	—	—	—	0.6
Methyl 3β-acetoxy-copalate				1.5	—	—	41.0	3.4
Total of methanolic fraction				3.8	—	—	100	14.3
Total on crude copaiba oil				100	61.6	34.6	3.8	100

A1, SE54; A2, DB-5; Polar, DB-Wax; Oil, crude copaiba oil; F1, hexane fraction; F2, dichloromethane fraction; F3, methanol fraction; NI, not identified.

High resolution gas chromatography (HRGC) analysis was carried out with a Hewlett-Packard (HP) model 5890 instrument equipped with a flame ionization detector (FID). Three columns were used: SE-54 glass column (15 m × 0.20 mm i.d., film thickness 0.20 μm); DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm); DBWax fused silica column (15 m × 0.25 mm i.d., film thickness 0.25 μm). Samples were injected using the split mode (1:20) with the injector temperature 270°C and the detector at 300°C, with a flow rate of 2 mL min^{-1} H_2.

HRGC-MS analysis was performed using a Hewlett-Packard model 5880 (quadrupole analyser), operated in the electron impact mode (70 eV) coupled to a Hewlett-Packard model 5897A mass spectrometer. High pure hydrogen was used as carrier gas at a linear velocity of 2 mL min^{-1}. The oven temperature was submitted to differentiated programs, to each column: SE-54, 120–160°C at 2°C min^{-1} and (no hold) to 260°C at 10°C min^{-1}; DB-5, 65–300°C at 4°C min^{-1}; DB-Wax, 50–150°C at 2°C min^{-1} and (no hold) to 250°C at 15°C min^{-1}, followed by a 15-min o final isothermal.

Retention indices (RI) were calculated using co-chromatographed standard linear alkanes. Identifications were done by comparison with MS literature data, computer matching with the Wiley 275 Library and by comparison of their RI values with those of pure standards and confirmed with the aid of RI from published sources, whenever necessary.

Biological assays

Experiments were conducted using non-fasted male Wistar rats, 140–200 g, housed at 22 ± 2°C with a 12-h light–dark cycle (lights on at 0600 h). The studies reported in this manuscript were carried out in accordance with the current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals and approved by the Ethics Committee of our university (process No. 262/CEUA and 23080.035334/2003–16/UFSC). In experiments with bradykinin, rats were pre-treated with the angiotensin converting enzyme inhibitor captopril (5 mg kg^{-1}, s.c.) 1 h before the experiment to prevent kinin degradation (Corrêa & Calixto 1993). Under ether anaesthesia, the rats received 0.1 mL intraplantar injections in one hindpaw of phosphate-buffered saline (PBS; composition mmol L^{-1}: NaCl 137; KCl 2.7 and phosphate buffer 10) containing carrageenan (300 μg/paw) or bradykinin (3 nmol/paw). The contralateral paw received 0.1 mL PBS and was used as control. Oedema was measured by use of a plethysmometer (Ugo Basile) at several time points (0.5, 1, 2 and 4 h for carrageenan and 10, 20, 30, 60 and 120 min for bradykinin) following the injection of carrageenan or bradykinin, as described previously (Campos & Calixto 1995). Oedema is expressed in μL as the difference between the test and control paws.

The crude oils, their fractions, or dexamethasone (used as positive control drug) were dissolved in absolute ethanol (100%) and then suspended in saline and were administered by the intraperitoneal route 30 min before the injection of carrageenan or bradykinin. The control rats received the same volume of saline containing ethanol. As positive control, rats were treated with the steroidal anti-inflammatory drug dexamethasone (0.5 mg kg^{-1}, s.c.) 4 h before carrageenan administration. The final concentration of ethanol was less than 0.5%, which had no effect against bradykinin- or carrageenan-induced oedema formation.

The following drugs were used: bradykinin, dexamethasone acetate, captopril, carrageenan grade IV (all from Sigma Chemical Company, St Louis, MO). The stock solutions for bradykinin were prepared in PBS (1–10 mM) in siliconized plastic tubes, maintained at −18°C, and diluted to the desired concentration just before use. Carrageenan was prepared daily in 0.9% w/v NaCl.

Statistical analysis

The results are presented as the mean ± s.e.m. Statistical comparison of the data was performed by means of analysis of variance followed by Dunnett's test or by the unpaired Student's *t*-test, when indicated and differences with $P < 0.05$ or less were considered significant.

Results

The main constituents of the *Copaifera multijuga* 1 copaiba oil and its fractions are presented in Table 1. From this Table it can be seen that samples of the oil collected from the same tree, but at different periods of the year, *C. multijuga* 2, showed qualitative and quantitative differences regarding its constituents.

The intraperitoneal injection of different fractions from *C. multijuga*, namely dichloromethane and methanol fractions (30 mg kg^{-1}, 30 min earlier), significantly attenuated the paw oedema formation elicited by the intraplantar injection of carrageenan. The dichloromethanic and methanolic fractions from *C. multijuga* 1 oil and *C. multijuga* 2 oil were the most active, with inhibition of paw oedema of 49 ± 13 (0.5 h), 64 ± 9 (0.5 h) and 33 ± 7% (2 h), respectively (Figure 1B, D, E). Likewise, dexamethasone (used as positive control), also significantly inhibited carrageenan-induced oedema formation, with an inhibition of 60 ± 4% (2 h) (Figure 1F). The hexanic fraction and the sample obtained from *C. multijuga* 1 showed few or no anti-oedematogenic properties (Figure 1, A, C).

When assessed in the paw oedema caused by intraplantar injection of bradykinin, the hexanic fraction and the sample of total oil (*C. multijuga* 1) also significantly inhibited the paw oedema formation with an inhibition of 50 ± 6% (at 30 min) and 31 ± 6% (at 10 min). On the other hand, the dichloromethanic and methanolic fractions from *C. multijuga* 1 oil and *C. multijuga* 2 oil exhibited few, or even no, inhibition on bradykinin-induced paw oedema formation (Figure 2A, B, C, D, E).

Discussion

Results of this study greatly extend the existing phytochemical analysis on this plant and show that the hexanic fraction from *Copaifera multijuga* Hayne assessed by HRGC-MS revealed the presence of eighteen sesquiterpene hydrocarbons. To perform the identification of the peaks, three chromatographic systems were used: two apolar and one polar

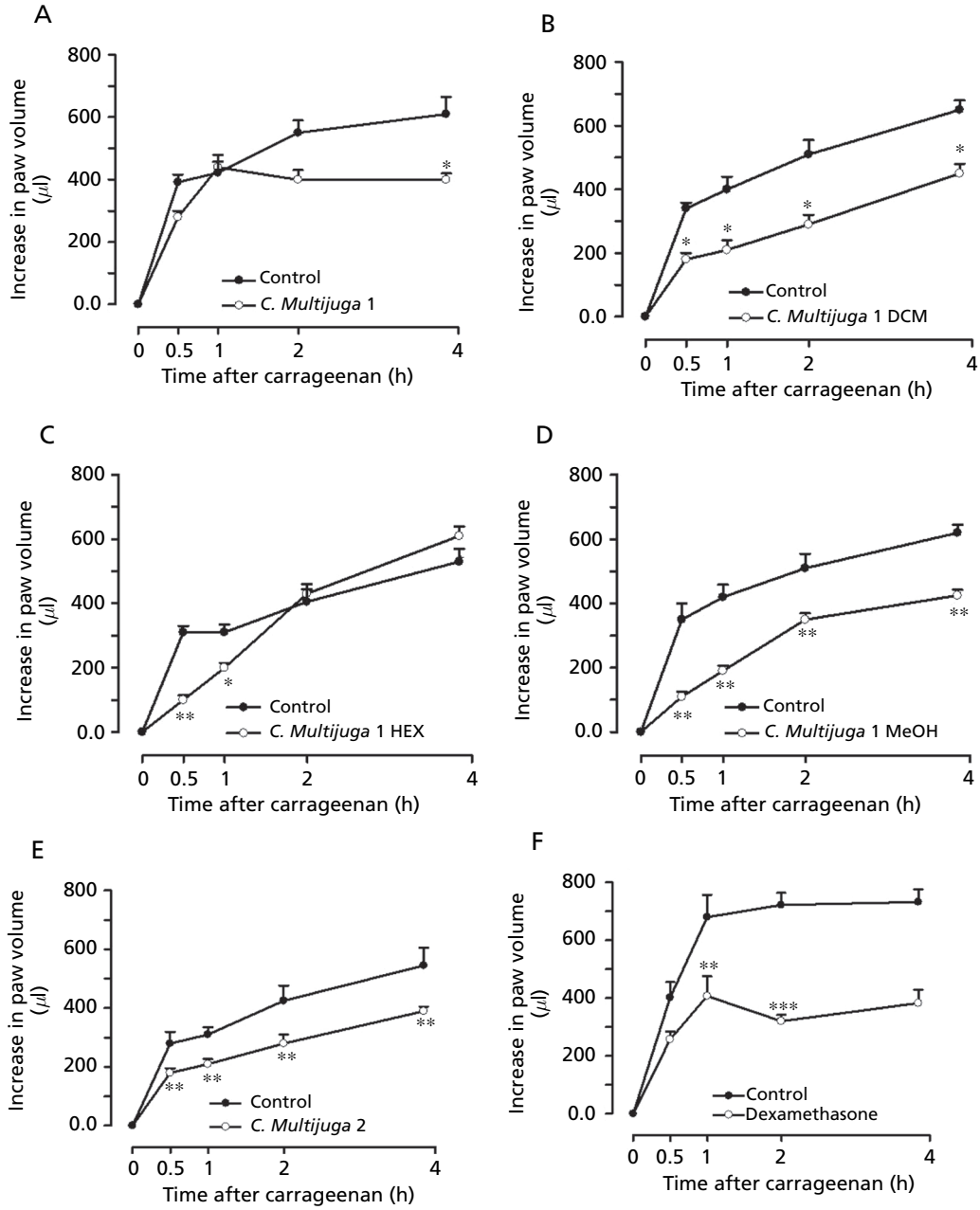

Figure 1 Influence of different oils and fractions obtained from *Copaiba multijuga* or dexamethasone on carrageenan-induced rat paw oedema. Each point represents the mean of 5 rats and the vertical bars the s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs control group (analysis of variance). Different rats have been used in the several control groups.

(DB-WAX). The two apolar retention indices are furnished in Table 1, named A1 (obtained with an SE-54 column) and A2 (obtained with a DB-5 column). A mixture of β-caryophyllene, α-humulene and caryophyllene oxide was prepared with pure standards and it was used to correlate the relative indices obtained from copaiba oils with those from literature in SE-54 column. The sesquiterpenes were identified using the three retention relative indices obtained (correlated with literature), fragmentation patterns and comparison with the mass spectrometry library Wiley 275. The hexanic fraction comprises ~60% of the total area in the FID chromatogram of the copaiba oil and the main component of this fraction is β-caryophyllene.

The sesquiterpenic alcohols and ketones present in the dichloromethanic fraction were characterised in a similar manner to the sesquiterpenic hydrocarbons in the hexanic fraction. Interestingly, the sample of oil collected during the summer (*Copaifera multijuga* 1) showed lower concentrations of β-caryophyllene (29.6%), but in contrast exhibited higher concentrations of more oxygenated caryophyllene species, such as α-caryophyllenol and caryophyllene oxide (5.8% and 13.0%, respectively), when compared with the

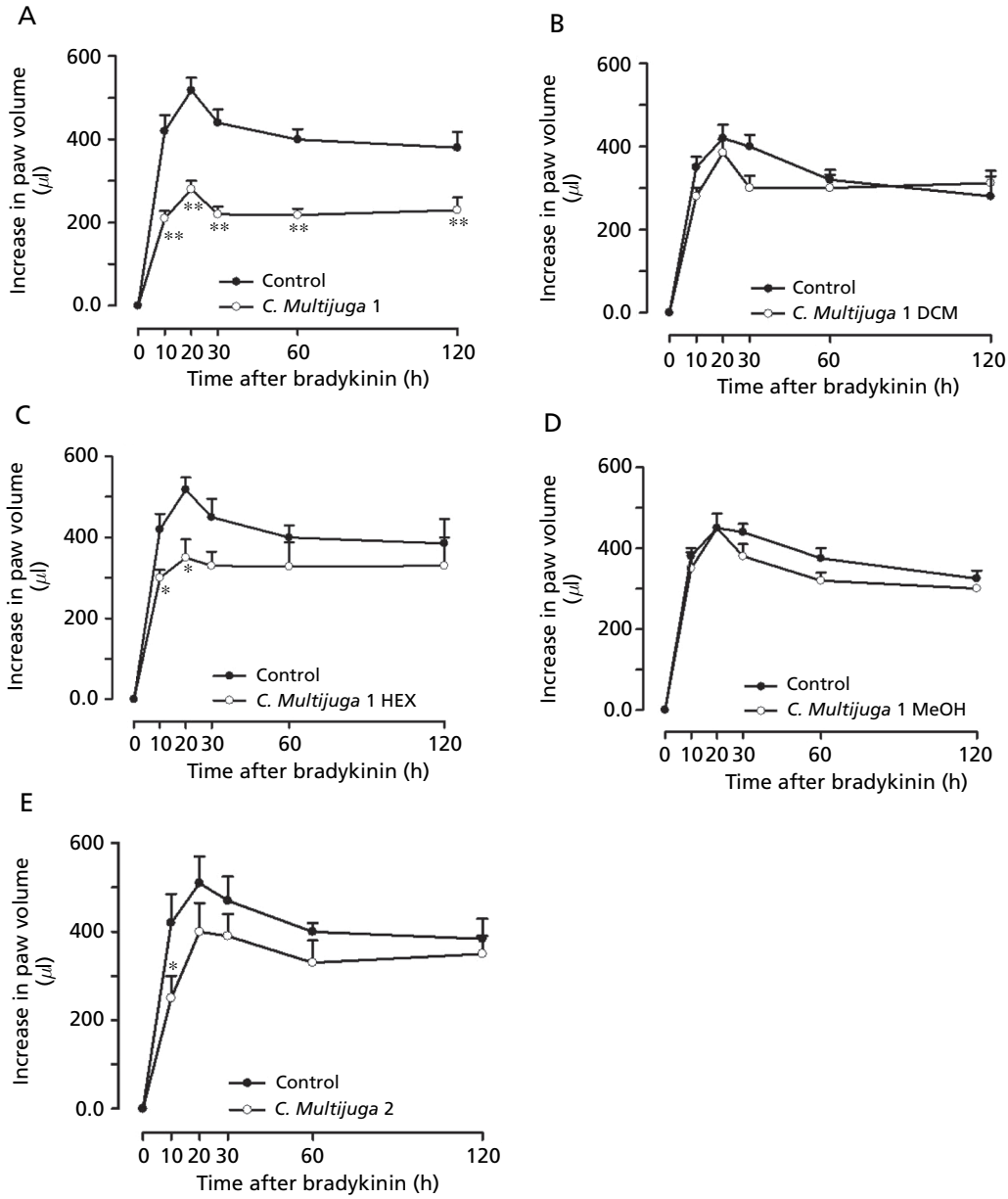

Figure 2 Influence of different oils and fractions obtained from *Copaiba multijuga* on bradykinin-induced rat paw oedema. Each point represents the mean of 5 rats and the vertical bars the s.e.m. $*P<0.05$, $**P<0.01$, vs control group (analysis of variance). Different rats have been used in the several control groups.

sample of oil collected during the winter (*Copaifera multijuga* 2). The later oil contained 58.4% of β-caryophyllene, 0.8% of α-caryophyllenol and 0.5% of caryophyllene oxide. The great variations of caryophyllene yield in the same three as a function of the time of the oil collection have been previously described in other plant species (Müller-Riebau et al 1997). These findings strongly suggest that the time of the oil collection should be carefully observed to maintain the same composition and pharmacological activity of *C. multijuga* oil.

The methanol fraction was then submitted to chromatography in a silica gel column to give eperuic, copalic, 3β-hydroxy-copalic, 3β-acetoxy-copalic, pinifolic and agathic acids, which were identified by their ^{1}H and ^{13}C NMR data, and confirmed by using bi-dimensional experiments and comparison of their spectroscopic properties with those reported in literature. HRGC-MS was used to perform the qualitative and quantitative analysis of the carboxylic acids of this fraction that, after reaction with diazomethane, was detected and reported as their respective methyl esters (Table 1). Copalic acid is the main component of this fraction, as previously reported from this oil (Lima et al 2003).

A search of the literature revealed that very few pharmacological studies have been carried out with *C. multijuga* oil or with its main constituents (see Introduction). The sesquiterpene β-caryophyllene is the most studied, in light of its reported anti-inflammatory activity (Martin et al 1993). The high yield of β-caryophyllene in the *C. multijuga* 1 oil in the hexane fraction suggests that, at least in part, the anti-oedematogenic activity observed in this study against bradykinin-induced

paw oedema appears to be associated with its presence in this specie. Of interest, the same hexanic fraction of *C. multijuga* 1 oil failed to significantly interfere with carrageenan-mediated paw oedema formation. It its well demonstrated that besides bradykinin, many other inflammatory mediators, such as serotonin, histamine, nitric oxide and prostaglandin E_2, among others, are involved in carrageenan-induced paw oedema (Di Rosa et al 1971). The presence of the diterpenes eperuic, copalic, 3β-hydroxy-copalic, 3β-acetoxy-copalic, pinifolic and agathic acids in the methanolic fraction and of the sesquiterpenic alcohols and ketones in the diclorometh ane fractions of *C. multijuga* oil might be the main reason for the marked inhibition of carrageenan-mediated paw oedema formation. On the other hand, as β-caryophyllene is the main sesquiterpene present in the *C. multijuga* oils, it is likely to be mainly responsible for the inhibition of bradykinin-induced oedema formation. Since the yields of these compounds greatly vary in function of the period of the oil collection, it is suggested that the marked differences in the pharmacological in-vivo activity observed for the two samples of this oil could be due to its great seasonal fluctuations. However, the definitive proof of this hypothesis deserves further experiments using the purified sesquiterpene β-caryophyllene.

Conclusions

These results confirm and largely extend existing data by showing that *C. multijuga* oil, one of the most important and widely used copaiba species in Brazilian traditional medicine, is comprised of a mixture of about 27 sesquiterpenes, besides 6 diterpenic acids, namely eperuic, 3β-hydroxy-copalic, 3β-acetoxy-copalic, pinifolic, copalic and agathic acid. Preliminary in-vivo pharmacological studies revealed that the oil and some of its fractions inhibited carrageenan- and bradykinin-mediated paw oedema formation. The phytochemical studies also revealed a great seasonal variation of the *C. multijuga* constituents according to the period of the year of the oil collection. Such great fluctuations also reflect substantial differences in the pharmacological activity.

These results are in line with the use of *C. multijuga* Hayne oil in the folk medicine for the management of inflammatory diseases, mainly for treating rheumatism. Since several compounds have been identified in these oils, our results do not presently permit us to define which of them is (are) responsible for the inhibition of carrageenan- and bradykinin-induced oedema in the rat paw, but suggest that β-caryophyllene may have a central role in bradykinin-induced paw oedema, while a mixture of sesquiterpenes present mainly in the dichloromethanic and hexanic fractions seem to be responsible for the inhibition of carrageenan-mediated paw oedema. Additional experiments using purified compounds from *C. multijuga* Hayne are required to clarify this point.

Finally, our results show a probable synergistic effect among the copaiba oil fractions, contributing to the main copaiba oil activity, and alert that chemical variation of the composition of natural medicinal products can change its pharmacological properties.

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