JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1179–1184
© 2004 The Authors
Received April 19, 2004
Accepted May 31, 2004
DOI 10.1211/0022357044058
ISSN 0022-3573

Evaluation of analgesic and anti-inflammatory activities of *Nectandra megapotamica* (Lauraceae) in mice and rats

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Funding: The authors are grateful to CAPES and CNPQ for scholarships, and FAPESP (Grant number 01/14219-7), Brazil, for financial support.

Abstract

The bioactivity-guided phytochemical investigation of the crude hydralcoholic extract of *Nectandra megapotamica* was carried out using the abdominal constriction test in mice, which led to the isolation of three active compounds: α-asarone (**1**), galgravin (**2**) and veraguensin (**3**). The crude extract (EBCA, 300 mg kg^{-1}) and isolated compounds **1**, **2**, and **3**, at different doses, were evaluated using the acetic acid-induced abdominal constriction test in mice, carrageenan-induced paw oedema in rats, and hot plate tests in rats. The EBCA showed a significant effect in the abdominal constriction and hot plate tests, but did not show activity in the rat paw oedema assay. All isolated compounds displayed activity in the abdominal constriction test, but only compound **1** was active in the hot plate test. Compounds **2** and **3** displayed activity in the anti-inflammatory assay. It was suggested that the analgesic effects obtained for EBCA could be due mainly to the presence of its major compound, α-asarone (**1**).

Introduction

The literature describing the indigenous medicine of central and South America contains references to the use of various plant species belonging to the genus *Nectandra* (Lauraceae) for the treatment of nervous disorders, fevers, snakebite, toothache and rheumatism (López et al 1995). The bark of *Nectandra megapotamica* (Spreng) Chodat et Hassler (Lauraceae) is used in folk medicine as an anti-rheumatic and to relieve pain (Da Silva Filho et al 2004). *N. megapotamica*, popularly known as 'canela-do-mato', is found in the southern and central parts of Brazil. Previous studies have shown that the hydralcoholic extract of *N. megapotamica* possesses significant antinociceptive effect (Novaes et al 1998). In addition, phytochemical investigation of this plant revealed the presence of alkaloids and tetrahydrofuran lignans (Da Silva Filho et al 2004). Tetrahydrofuran lignans have been recognized as important platelet-activating factor (PAF) receptor antagonists (Biftu et al 1986). Also, there are some lignans that display important anti-inflammatory and analgesic activities, such as the dibenzylbutyrolactone lignans from *Zanthoxylum naranjillo* (Bastos et al 2001; Souza et al 2004).

In this study, we have evaluated the analgesic and anti-inflammatory activities of *N. megapotamica* to corroborate the folkloric uses described for this plant. A bioactivity-guided study of the crude extract was carried out using the abdominal constriction test in mice. In addition, the crude extract and its isolated compounds were evaluated for antinociceptive and anti-inflammatory activities using the acetic acid-induced abdominal constriction test in mice, carrageenan-induced paw oedema in rats, and hot plate tests in rats.

Materials and Methods

Plant material

N. megapotamica (Lauraceae) was collected by A. A. da Silva Filho in August, 1999 in Ribeirão Preto, SP, Brazil. Ida de Vattimo kindly authenticated the plant and a

voucher specimen was deposited in the Herbarium of the Biology Department of the University of São Paulo, campus of Ribeirão Preto (SPFR 05655).

General procedures

Optical rotations were measured at λ 589 nm on a Shimidt-Haenseh polartronic HH8 polarimeter using a 1.0 cell. NMR spectra were recorded on a Brucker ARX 400 spectrometer. Vacuum-liquid chromatography (VLC) was carried out using Si gel 60H 100–200 mesh ASTM (Merck), in glass columns with 5–10 cm i.d. Flash chromatography was performed with Si gel 230–400 mesh (Merck) in a 450×25 mm glass column at $5\,mL\,min^{-1}$. Semi-preparative HPLC separation analyses were carried out on a Shimadzu SCL-10 AVP liquid chromatography system equipped with a SPD-M10AVP Shimadzu UV-diode array detector (the channel was set at 281 nm) and a Shimadzu column (ODS, 250×20 mm, 15 μm).

Extraction and isolation procedures

Air-dried, powdered barks (1500 g) were exhaustively extracted with EtOH:H_2O (9:1) at room temperature by maceration. The filtered extract was concentrated under vacuum to furnish 240 g crude extract (EBCA), which was dissolved in MeOH:H_2O (7:3), followed by sequential partition with hexane (CA1 16.0 g), $CHCl_3$ (CA2 12.0 g) and *n*-BuOH (CA3 94.0 g). The remaining H_2O fraction (CA4) was lyophilized. All the obtained fractions were screened using the abdominal constriction test in mice. This resulted in fractions CA1 and CA2 being selected for use in the experiments. The CA1 fraction (7.0 g) was chromatographed over silica gel under a VLC system, using hexane–ethyl acetate (EtOAc) mixtures in increasing proportions to afford four fractions. The resulting fraction I (hexane–EtOAc 9:1; 3.2 g) was crystallized (hexane–EtOAc 1:1) to afford 1.5 g of α-asarone (**1**). The CA2 fraction (12 g) was chromatographed over silica gel under a VLC system, using hexane–EtOAc mixtures in increasing proportions, furnishing six fractions. The resulting fractions III (hexane–EtOAc 1:1; 1.38 g) and IV (hexane–EtOAc 4:6; 0.51 g) from CA2 were submitted to flash column chromatography over silica gel, using hexane–EtOAc (9:1) as mobile phase, followed by semi-preparative HPLC (MeOH–H_2O 75:25). This gave the compounds galgravin (**2**, 60 mg) and veraguensin (**3**, 70 mg).

The chemical structures of all compounds were established by ^1H and ^{13}C NMR data analysis and by comparing the obtained data with those of authentic compounds. Purity of all isolated compounds were estimated to be higher than 95% by both HPLC analysis using different solvent systems (MeOH–MeCN–H_2O, 65:5:30; MeOH–H_2O 50 to 100% in 20 min) and ^{13}C NMR.

Animals

Animals used in this study were housed and cared for in accordance with the College of Medicine of the Ribeirão Preto of the University of São Paulo. The experiments were authorized by the Ethical Committee for Animal Care of the University of São Paulo (Process number 02.1.597.53.1), in accordance with the Federal Government legislation on animal care.

Male Swiss albino mice (20–25 g) were used for the abdominal constriction test, while male Wistar rats (150–180 g) were used for the hot plate and paw oedema tests. The animals were housed in groups of six in standard cages at room temperature ($25 \pm 3°C$) in 12-h dark/light control, with food and water freely available. Twelve hours before the experiments they were transferred to the laboratory, with water freely available only.

Abdominal constriction test

The abdominal constrictions were induced by intraperitoneal injection of 0.6% (v/v) acetic acid (0.1 mL/10 g; Merck) 30 min after drug administration, according to procedures described by Koster et al (1959). The number of abdominal constrictions was counted for 20 min, after the acetic acid injection. The data represent the average of the total number of constrictions observed. The EBCA was administered at 100, 300 or 500 $mg\,kg^{-1}$ (p.o.) and its CA1, CA2, CA3 and CA4 fractions were assayed at 150 $mg\,kg^{-1}$ (p.o.). These doses were based on studies by Novaes et al (1998). Compound **1** was administered at doses of 5, 10 or 20 $mg\,kg^{-1}$ (p.o.), while compounds **2** and **3** were administered at doses of 5, 10, 20 or 40 $mg\,kg^{-1}$ (p.o). Indometacin (10 $mg\,kg^{-1}$, Sigma Chemical Co.) was dissolved in vehicle and administered (p.o.) as the reference drug for comparison. Control animals received a similar volume of vehicle (5% Tween–saline solution).

Hot plate test

Experiments were performed using the method described by Woolfe & MacDonald (1944), which was slightly modified for rats. In these experiments, the hot plate (Ugo Basile, Model 7280) was maintained at $55 \pm 1°C$. Animals were placed into a glass cylinder and the time (s) between placement and shaking or licking of the paws or even jumping was recorded as the index of response latency. The reaction time was recorded for animals pretreated with compound **1** (10, 20 or 50 $mg\,kg^{-1}$, p.o.), compounds **2** and **3** (20 $mg\,kg^{-1}$, p.o.), or with morphine (4.0 $mg\,kg^{-1}$, s.c). The animals that remained on the apparatus for an average of 6 s were selected 24 h before the experiments on the basis of their reactivity in the model. A latency period (cut-off) of 30 s was defined as complete antinociception. The latency time was recorded 60 min after sample injection. The results were expressed as percent of maximum possible effect (% MPE) (Yaksh et al 1976). Control animals received a similar volume of vehicle (5% Tween-saline solution) used to dissolve the drugs.

Carrageenan-induced paw oedema in rats

The method of carrageenan-induced paw oedema in rats (Winter et al 1962) was used to evaluate the anti-inflammatory activity. The treatment was performed 30 min before

the injection of 0.1 mL carrageenan (100 μg) (kappa carrageenan type III, Sigma Chemical Co.) into the rat paw plantar surface. Foot volume was measured using a plethysmometer (Ugo Basile, Model 7140) at 1-h intervals after carrageenan injection, for 5 h, but the activity was acknowledged only for the third hour, in which the maximum oedema occurred. The treatments were undertaken orally using the following doses: 10 or 40 mg kg^{-1} compound **1**; 5, 10 or 20 mg kg^{-1} of compounds **2** and **3**; and 300 mg kg^{-1} EBCA. The results were obtained by measuring the volume difference between the right and the left paws in comparison with both the negative control group (treated with 5% Tween–saline solution) and the positive control group (treated with 10 mg kg^{-1} indometacin).

Statistical analysis

Data were analysed statistically by one-way analysis of variance followed by Tukey's multiple comparison test, with the level of significance set at $P < 0.05$ and $P < 0.01$.

Results

Phytochemical study

The bioactivity-guided phytochemical investigation using the abdominal constriction test for the EBCA furnished three active compounds, the major compound being the phenylpropanoid α-asarone (**1**), the two minor compounds being tetrahydrofuran lignans **2** (galgravin) and **3** (veraguensin) (Figure 1). The spectral data of all isolated compounds were in agreement with those reported by other investigators: α-asarone (**1**) (González et al 1996), galgravin (**2**) (Crossley & Djerassi 1962), and (+)-veraguensin (**3**) (Crossley & Djerassi 1962).

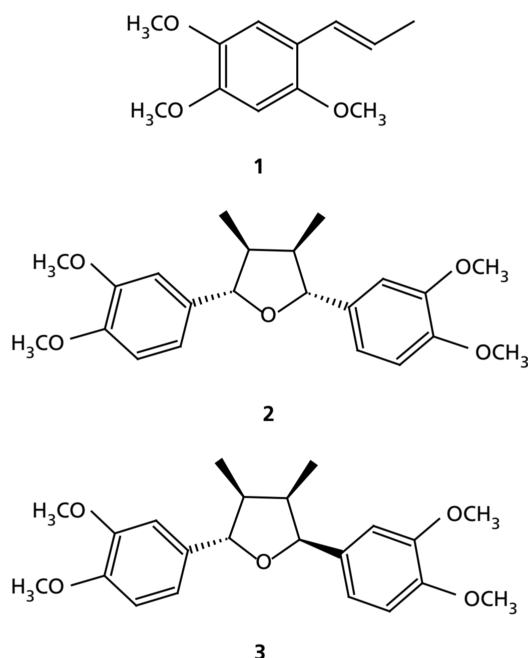

Figure 1 Compounds isolated from the crude hydralcoholic extract of *N. megapotamica*.

Abdominal constriction test

The oral administration of the bark crude extract (EBCA), at 300 or 500 mg kg^{-1}, produced a significant inhibition of the constrictions induced by intraperitoneal injection of acetic acid by 75.9% (5.17 ± 2.6) and 82.2% (3.83 ± 1.4), respectively, in comparison with the negative control group (21.5 ± 4.2), treated with vehicle (0.2 mL/animal, p.o.), as shown in Table 1. It was observed that the fractions CA1 and CA2 significantly reduced the number of constrictions by 75.2 and 53.5%, respectively. The fractions CA3 and CA4 did not reduce constrictions significantly compared with the negative control group. The positive control group, treated with indometacin, displayed 97.7% (0.5 ± 0.3) constriction inhibition (Table 1).

Compound **1** (20 mg kg^{-1}, p.o) significantly inhibited the constrictions by 70.6%. Similarly, compound **3** produced a dose–response inhibition of acetic acid-induced abdominal constriction, while compound **2** displayed activity, but it was not dose related. At 20 mg kg^{-1} each, compounds **2** and **3** inhibited the acetic acid-induced

Table 1 Effect of the tested drugs on acetic acid-induced abdominal constriction in mice

Drugs	Dose (mg kg^{-1}; p.o.)	Total number of abdominal constrictions[a]	% of inhibition
Control	–	21.50 ± 4.2	–
EBCA	100	12.00 ± 3.3	44.2
	300	5.17 ± 2.6**	75.9
	500	3.83 ± 1.4**	82.2
CA1	150	5.33 ± 2.5**	75.2
CA2	150	10.00 ± 2.3*	53.5
CA3	150	18.67 ± 2.6	13.2
CA4	150	17.83 ± 2.5	17.1
Compound **1**	5	20.12 ± 5.3	6.4
	10	16.67 ± 4.3	22.5
	20	6.33 ± 1.8**	70.6
Compound **2**	5	9.67 ± 3.3	55.0
	10	8.50 ± 1.4*	60.5
	20	8.50 ± 2.1*	60.5
	40	6.67 ± 0.9**	68.9
Compound **3**	5	9.50 ± 3.1	55.8
	10	8.00 ± 1.0*	62.8
	20	6.17 ± 1.8**	71.3
	40	1.00 ± 0.4**	95.3
Indometacin	10	0.5 ± 0.3**	97.7

[a]The numbers represent the mean ± s.e.m. (n = 6). *$P < 0.05$, **$P < 0.01$, analysis of variance followed by post-Tukey's test.

abdominal constriction by 60.5% and 71.3%, respectively.

Paw oedema

For the carrageenan-induced paw oedema test in rats, compound **2** at 10 and 20 mg kg^{-1} (p.o.) showed a significant anti-oedematous effect of 52.7 and 71.4%, respectively. Compound **3** at 20 mg kg^{-1} (p.o.) inhibited the oedema formation by 41.2% (Figure 2). However, compound **1** and EBCA were inactive in this assay at the tested doses (results are not shown).

Hot plate test

For the hot plate test, the oral administration of compound **1** at 20 and 50 mg kg^{-1} produced a significant analgesic effect of 29.0% and 46.7% MPE, respectively (Figure 3). The morphine-treated group displayed a quite significant analgesic effect of 100%. Lignans **2** and **3** at 20 mg kg^{-1} were inactive.

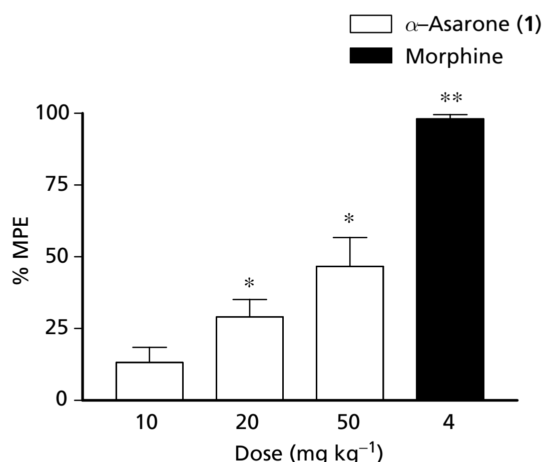

Figure 3 Analgesic effect (% MPE) of the oral administration of α-asarone (**1**) (10, 20 and 50 mg kg^{-1}) in the hot plate test in rats. The results were expressed as the mean percentage of maximal possible effect (% MPE) ± s.e.m. (n = 6). *$P < 0.05$ and **$P < 0.01$ were considered significant in comparison with negative control (analysis of variance followed by post-Tukey's test).

Discussion

The abdominal constriction test screening in mice was undertaken for hexane (CA1), chloroform (CA2), *n*-butanol (CA3) and water (CA4) fractions, which were obtained by liquid–liquid partition from EBCA, aiming to select the most active fractions to continue the phytochemical study. Thus, based on the results of the screening, it was possible to select the fractions CA1 and CA2 to continue the phytochemical study, which led to the isolation of compounds **1**, **2** and **3** (Figure 1).

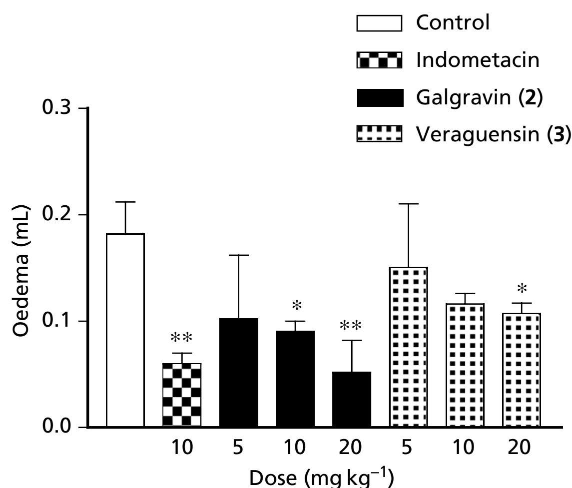

Figure 2 Effect of the oral administration of galgravin (**2**) and veraguensin (**3**) (5, 10 and 20 mg kg^{-1}) on rat paw oedema induced by carrageenan injection (100 μg/paw). The paw oedema volume was taken at 3 h after carrageenan injection. The results were expressed as the mean ± s.e.m. (n = 6). *$P < 0.05$ and **$P < 0.01$ were considered significant in comparison with negative control (analysis of variance followed by post-Tukey's test).

It is important to point out that the abdominal constriction test was chosen based on the mechanism of action of the acetic acid, which acts indirectly by releasing endogenous mediators, such as PGI_2 and PGE_2. These mediators are capable of stimulating both the peripheral receptors of pain and the neurons, which are sensitive to other drugs acting at the central nervous system, like opioids (Matshumoto et al 1998; Hiruma-Lima et al 2000). Therefore, the constrictions produced by acetic acid could be blocked by both opioids and non-steroidal anti-inflammatory drugs, thus allowing the use of this method for the analgesic and anti-inflammatory pharmacological screening. Thus, it could be used not only to select plant extracts but also to guide its phytochemical fractionation (Rates & Barros 1994). Nevertheless, this is not a specific model, since several non-analgesic compounds inhibit the constrictions induced by acetic acid, such as antihistaminic, mono-amino oxidase inhibitors (IMAO) and ansiolitics. Therefore, because of lack of specificity of this test, caution is required in interpreting the results until other tests have been performed (Sarkhail et al 2003; Rates & Barros 1994).

Regarding the rat paw oedema assay, the genesis of the oedema produced due to the action of carrageenan involves different phases, as related to prostaglandins and other mediators (Carvalho et al 1999). It is well known that the oedema formation induced by plantar injection of carrageenan reaches its highest volume at the third hour. The inhibitors of cyclo-oxygenase, such as indometacin, are able to inhibit the formation of the oedema and may be used as the standard drug for anti-inflammatory protocols (Di Rosa et al 1971).

Despite the negative effects of α-asarone (**1**) in the anti-inflammatory assay, both tetrahydrofuran lignans **2** and **3**

produced significant inhibition of the carrageenan-induced oedema formation. In addition to their anti-inflammatory activity, compounds **2** and **3** showed analgesic effects in the abdominal constriction test, but did not display activity in the hot plate test. This suggested that lignans **2** and **3** possessed peripheral analgesic activity, which might be accountable to alleviate the inflammatory pain and to reduce the oedema formation. Furthermore, these tetrahydrofuran lignans have been investigated for their ability to inhibit platelet-activating factor (PAF) (Biftu et al 1986). PAF, a powerful autacoid mediator of inflammation and allergy, may play an important role in different pathological conditions (Miguélez et al 1996). The administration of PAF-receptor antagonists could be effective for the treatment of many inflammatory diseases. Nevertheless, it should be taken into consideration that the mechanism involved in the genesis of both the carrageenan-induced oedema and the acetic acid-induced abdominal constriction may be related to the release of prostaglandins and kinins, among other mediators (Di Rosa et al 1971; Matshumoto et al 1998). Regarding the results of this study, the mechanism of action of lignans **2** and **3** might be related not only to the anti-PAF activity but also to the inhibition of other chemical mediators.

The hot plate test is considered partially selective for opioid-like compounds in several animal species, but other central nervous system-acting drugs, including sedatives and muscle relaxants might show activity in this test (Woolfe & MacDonald 1944; Hiruma-Lima et al 2000). Therefore, considering that α-asarone (**1**) is a sedative-like compound (Dandiya & Sharma 1962), the results obtained for **1** raised the possibility that the reduction in acetic acid-induced constrictions and the protection on the hot plate test were due to its sedative property, and not to its analgesic activity. Moreover, the results obtained for α-asarone (**1**) were in accordance with its sedative and tranquilizing activities reported by Menon & Dandiya (1967). According to Dandiya & Sharma (1962), α-asarone (**1**) was capable of increasing the latency time in the hot plate test, as observed in this study.

Overall, it is very important to point out that EBCA contained a large amount of α-asarone (**1**), but small amounts of the tetrahydrofuran lignans (**2** and **3**). This could explain why EBCA did not display anti-inflammatory activity in the rat paw oedema assay. Considering that EBCA showed a significant effect in the abdominal constriction and hot plate tests (Novaes et al 1998), it is suggested that the analgesic properties of EBCA could be due mainly to the presence of its major compound, α-asarone (**1**).

Conclusion

This study ratified the traditional indications of *N. megapotamica* and identified its major active compounds. However, α-asarone (**1**) is hepatotoxic and carcinogenic (López et al 1993), and so the use of *N. megapotamica* preparations should be discouraged until its safe use, as crude preparations, can be established.

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