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Antinociceptive Effect in Mice of a Hydroalcoholic Extract of *Neurolaena lobata* (L.) R. Br. and its Organic Fractions

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Abstract

An infusion of the aerial parts of *Neurolaena lobata* (L.) R. Br. (Compositae–Asteraceae) is used in Caribbean folk medicine to treat several kinds of pain. In this investigation we studied the acute oral toxicity of the hydroalcoholic extract of the plant and the antinociceptive effect of the extract and of its hexane- and chloroform-partitioned fractions, given orally, in nociception and inflammatory models in mice.

No signs of toxicity were observed for oral doses up to 5000 mg kg^{-1} in mice. Morphine hydrochloride (10 mg kg^{-1}), dipyrone sodium (200 mg kg^{-1}), the hydroalcoholic extract (1000 mg kg^{-1}), and its chloroform- and hexane-partitioned fractions (100 mg kg^{-1}) significantly inhibited acetic acid-induced abdominal constriction in mice (100, 95, 47, 62 and 60% inhibition, respectively when compared with the negative control). In the hot-plate test in mice, morphine hydrochloride, the chloroform- and hexane-partitioned fractions, but not the hydroalcoholic extract, resulted in a significant latency increase in all observation times. In the acetic acid-induced abdominal constriction in mice, pretreatment of the animals with naloxone significantly reversed the analgesic effect of morphine, but not that of the hydroalcoholic extract or of its hexane- and chloroform-partitioned fractions. Finally, administration of the hexane- and chloroform-partitioned fractions (100 mg kg^{-1}) had a significant anti-oedematogenic effect on carrageenan-induced oedema in mice.

These data show that the hydroalcoholic extract of *N. lobata* and, in particular, its partitioned fractions have significant analgesic properties when assessed through these pain models. Their antinociceptive effect might be the result of interference with the inflammatory process.

The plant family Compositae consists of approximately 920 genera and more than 19 000 species widely distributed in tropical and subtropical countries (Joly 1977). *Neurolaena lobata* (L.) R. Br., previously incorrectly named *Pluchea symphytifolia* (Miller) Gillis, is a herbaceous plant of the Compositae-Asteraceae family (Khan & Jarvis 1989) which is widespread in Central America and has also been found in north-western South America, including the north of Brazil (Pasrreiter 1995). The Guatemala Caribbeans use *N. lobata* as a remedy for several diseases, including malaria (François et al 1995), stomach pains, diabetes, skin diseases (Pasrreiter 1995) and other kinds of pain (Germosén-Robineau 1995). This plant is also used by some ethnic groups in the Antilles for cancer treatment (François et al 1995; Pasrreiter 1995). People use extremely bitter-tasting decoctions of the leaves, and other preparations. The doses and the frequency of administration differ among ethnic groups (François et al 1995).

Chemical substances isolated from *N. lobata* include a thymol derivative (Bohlmann et al 1979), twelve flavonoids including one new sulphate, several cuathemone derivatives, germacranolides, terpenoids such as α-amirine, and one obscure alkaloid (Germosén-Robineau 1995). Eleven sesquiterpene lactones, among them two named neurolenin-A and neurolenin-B (Manchand & Blount 1978) have also been isolated from this species.

Considering its use in Central American folk medicine and the large number of chemical substances isolated from *N. lobata*, it seemed

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appropriate to evaluate the activity of this species. In this study we have investigated the acute toxicity of the hydroalcoholic extract of *N. lobata* and the antinociceptive effect of the extract and its hexane- and chloroform-partitioned fractions against acetic acid-induced abdominal constriction and the hot-plate analgesic test. Because of the use of this species for cancer treatment (François et al 1995; Pasrreiter 1995), we also investigated the possible involvement in its antinociceptive effect of opioid receptors. Finally, the anti-oedematogenic effect of the hexane- and chloroform-partitioned fractions was investigated using carrageenan-induced paw oedema in mice.

Materials and Methods

Drugs

Dipyrone sodium (Hoechst Marion Roussel, Brazil) and the *N. labata* extract and its hexane- and chloroform-partitioned fractions were dissolved in 12% Tween 80 (Synth, Brazil) solution. Acetic acid, morphine hydrochloride, naloxone hydrochloride, carrageenan and indomethacin (Sigma, MO, USA) were dissolved in 0·9% NaCl (saline) or 5% $NaHCO_3$ solution. All reagents were of a high grade of purity. Substances, reagents and extract or fractions were prepared immediately before use.

Animals

Experiments were performed, during the morning, on Swiss albino mice, 30 ± 5 g, from the Central Animal House of the Universidade Estadual de Campinas (Cemib/Unicamp). They were fed a certified Nuvilab CR-a (Nuvital) diet, had free access to tap water and were kept in the animal house under standard conditions of 12-h light–dark, humidity (55%) and temperature (25 ± 1°C). Animals were fasted before the assays because the standard drugs or extract and fractions of *N. lobata* were always administered orally (p.o.), except for indomethacin, morphine and naloxone. All experiments were performed according to current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann 1983).

Preparation of Neurolaena lobata *extract and fractions*

The leaves of *N. lobata* were collected by a botany group from Enda-Caribe, Santo Domingo, Dominican Republic, coordinated by Dr Lionel Germonsén-Robineau. The dried leaves were minced and extracted with ethanol–water, 9 : 1, in a Soxhlet apparatus. The resulting extract was evaporated to dryness under vacuum at 50°C and the residue was successively submitted to hexane and chloroform partition to obtain appropriate partitioned fractions. The solvent was then removed from the resulting fractions by evaporation.

Acute toxicity and LD50

Studies of the acute toxicity of the hydroalcoholic extract were performed on mice. Increasing doses of *N. lobata* hydroalcoholic extract were administered to groups of 10 animals for each dose level after a 12-h fast. The animals were observed for 14 days, when the number of survivors was recorded. The acute toxicologic effect was estimated by the method described by Souza Brito (1995) and was expressed as LD50 (the dose resulting in the death of half the mice) according to Litchfield & Wilcoxon (1949).

Abdominal constriction response caused by intraperitoneal injection of acetic acid

The response to intraperitoneal injection of a 0.6% acetic acid solution; contraction of the abdominal muscle and stretching of the hind limbs; was induced according to procedures described by Koster et al (1959). Animals were pretreated with the hydroalcoholic extract ($1000\,mg\,kg^{-1}$), or its hexane- or chloroform-partitioned fractions ($100\,mg\,kg^{-1}$) and negative-control animals received a similar volume of 12% Tween 80 ($10\,mL\,kg^{-1}$). Positive-control mice received dipyrone ($200\,mg\,kg^{-1}$, p.o.) and subcutaneous morphine ($10\,mg\,kg^{-1}$). The drugs were administered 30 min before injection of 0·6% acetic acid. After challenge, pairs of mice were placed in separate transparent boxes and the number of abdominal constrictions over a period of 6–21 min were counted. Antinociceptive activity was expressed as the reduction of the number of abdominal constrictions. The number of abdominal constrictions and stretchings was recorded and percentage protection was calculated by use of the formula:

$$[(\text{control mean} - \text{treated mean})/\text{control mean}] \times 100$$

Hot-plate test

The hot-plate test was used to measure latencies according to the method described by Eddy & Leimback (1953), with minor modifications. In these experiments the hot-plate apparatus (Ugo Basile, Model-DS 37) was maintained at 56 ± 1°C. Animals were placed in a 24-cm diameter glass cylinder on the heated surface and the time between placement and shaking or licking of the paws or jumping was recorded as latency. Latency was recorded for control mice (treated with vehicle) and

for animals pretreated with morphine (10 mg kg^{-1}, s.c.) used as positive-control or pretreated with the hydroalcoholic extract (1000 mg kg^{-1}), or with its hexane- or chloroform-partitioned fractions (100 mg kg^{-1}). All substances were administered 30 min before the beginning of the experiment. Animals were selected 24 h previously on the basis of their reactivity in the test. Only animals showing a reaction within the 3·9–6·9 s range were selected. Negative-control animals received a similar volume of 12% Tween 80 (10 mL kg^{-1}, p.o.). All animals were observed before (0 min) and 30, 60 and 90 min after drug administration. A latency period of 30 s was defined as complete analgesia.

Analysis of the mechanism of analgesic action of Neurolaena lobata *hydroalcoholic extract and its chloroform- and hexane-partitioned fractions*

The possible participation of the opioid system in the antinociceptive effect of the hydroalcoholic extract of *N. lobata* and its chloroform- and hexane-partitioned fractions was investigated. To analyse this mechanism we also used the acetic acid-induced abdominal constriction model in mice, with some modifications. Animals were pretreated with intraperitoneal naloxone (5 mg kg^{-1}) 15 min before oral administration of the hydroalcoholic extract (1000 mg kg^{-1}) or its chloroform- or hexane-partitioned fractions (100 mg kg^{-1}), or subcutaneous administration of morphine (10 mg kg^{-1}). Control animals received a similar volume of 12% Tween 80 (10 mL kg^{-1}) orally.

Carrageenan-induced paw oedema in mice

The method utilized was similar to that described by Henriques et al (1987) who used groups of male mice. Pretreatment with indomethacin (20 mg kg^{-1}, s.c.; used as positive control) or with its hexane- or chloroform-partitioned fractions (100 mg kg^{-1}, p.o.) was 30 min before injection of 300 μg carrageenan (1% suspension in normal saline). The paws were weighed 3 h after carrageenan injection. The increase in weight caused by the irritant was found by subtracting the weight of the untreated left paw from that of the treated right paw.

Statistical analysis

Results are expressed as means ± s.d. or means ± s.e.m.; statistical significance between results from different groups was assessed by analysis of variance followed by Dunnett's pairwise test. P values < 0.05 were considered indicative of significance.

Results and Discussion

In this study relatively large doses of the plant extract (1000 mg kg^{-1} p.o.) were needed to elicit pharmacological action. In our toxicologic assay oral doses up to 5000 mg kg^{-1} resulted in no signs of toxicity in mice, and no significant changes in daily body weight or organ weight were observed during the next 14 days (results not shown). This might be a reflection of the low concentrations of the active and toxic substances in the extract. This confirmed the widely reported low toxicity of the plant extract. Considering the high experimental dose, this probably indicates that, at the usual doses employed by man (estimated to be about 10% of the dose used here), the plant extract would probably not have toxic effects. However, it should be stressed that extrapolations of the effects of potentially toxic substances from animals to man cannot always be made (Tanira et al 1996).

We also investigated the analgesic and anti-oedematogenic effect of the hydroalcoholic extract of *N. lobata* leaves and of its partitioned fractions in mice, by use of chemical (acetic-acid induced abdominal constriction) and thermal (hot-plate test) pain models and one acute inflammatory assay.

The abdominal constriction elicited by acetic acid has been used to assess the potential analgesic activity of drugs. Morphine, dipyrone, the hydroalcoholic extract, and its chloroform- and hexane-partitioned fractions significantly inhibited acetic acid-induced abdominal constriction in mice, by 100, 95, 47, 62 and 60%, respectively, compared with control animals (Table 1). The data show that the percentage inhibition of abdominal constrictions afforded by 100 mg kg^{-1} of the chloroform- and hexane-partitioned fractions was equivalent to that afforded by 200 mg kg^{-1} dipyrone. These data

Table 1. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, on acetic acid-induced abdominal constriction in mice.

Treatment	Dose (mg kg^{-1})	Number of constrictions	Inhibition (%)
Control	–	41·0 ± 3·11	–
Morphine	10	0*	100
Dipyrone	200	2·0 ± 0·58*	95·1
Hydroalcoholic extract	1000	21·7 ± 3·09*	47·1
Hexane-partitioned fraction	100	16·4 ± 2·88*	60·0
Chloroform-partitioned fraction	100	15·4 ± 1·62*	62·4

Each value is the mean ± s.d. of results from seven animals. Significantly different from the respective control value; analysis of variance $F(4,30) = 229$ ($P < 0.05$); Dunnett's test *$P < 0.001$.

also show significant antinociceptive activity of these fractions, considering that the partitioned fractions obtained from the hydroalcoholic extract are only semi-purified. Dipyrone and morphine, used as positive reference controls, also had significant antinociceptive effects in this pain model.

Collier et al (1968) postulated that acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs, to narcotics and to other centrally acting drugs (Vaz et al 1996). Thus, the abdominal constriction elicited by acetic acid would be considered a less selective antinociceptive model.

Although the hot-plate test is commonly used to assess narcotic analgesics, other centrally acting drugs, including sedatives and muscle relaxants or psychotomimetics are active in this test (Vaz et al 1996). In the hot-plate test there was no significant difference between pretreatment latency values obtained on the day of the test (time 0) and those obtained 24 h before during animal selection (data not shown). The results presented in Table 2 show that oral administration of the hexane- and chloroform-partitioned fractions, but not of the hydroalcoholic extract, significantly increased the latency at all observation times (30, 60 and 90 min). Morphine, used as a reference drug, had the same significant antinociceptive effect at all observation times when compared with its own control values. These results show that in both experiments the active principle(s) of *N. lobata* was (were) present in the hexane- and chloroform-partitioned fractions from the leaves.

Because of the use of this plant for cancer treatment (François et al 1995; Pasrreiter 1995), we supposed a possible action of the extract or its partitioned fractions on opioid receptors and used naloxone, a non-selective antagonist of the opioid receptors, in an attempt to gain some insight into the mechanisms involved in the antinociceptive properties of the hydroalcoholic extract and its hexane- and chloroform-partitioned fractions. In some animal models naloxone apparently acts by antagonizing the actions of endogenous opioids mobilized by pain or stress (Faden 1988). The data shown in Table 3 indicate that the non-selective opioid antagonist naloxone (5 mg kg^{-1}, i.p.) did not consistently reverse the extract- and the fractions-induced antinociception when assessed against acetic acid-induced pain. Naloxone significantly reversed the morphine-induced antinociceptive effect in the chemical pain model (acetic acid-induced abdominal constriction). Our results show, however, that the organic fractions obtained from *N. lobata* did not act by interaction with the opioid system.

Pretreatment with the hexane- and chloroform-partitioned fractions significantly reduced carrageenan-induced oedema, by 44 and 68%

Table 3. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, on acetic acid-induced abdominal constriction in mice pretreated with intraperitoneal naloxone (5 mg kg^{-1}).

Treatment	Oral dose (mg kg^{-1})	Number of constrictions	Inhibition (%)
Control	–	28·0 ± 3·03	–
Morphine	10	0	100
Naloxone + morphine	10	25·8 ± 2·48	13·6
Naloxone + hydroalcoholic extract	1000	13·0 ± 1·67*	53·6
Naloxone + hexane-partitioned fraction	100	9·7 ± 1·03*	65·4
Naloxone + chloroform-partitioned fraction	100	8·5 ± 0·55*	69·6

Each value is the mean ± s.d. of results from eight animals. Significantly different from the respective control value; analysis of variance $F(5,42) = 225$ ($P < 0.05$); Dunnett's test *$P < 0.001$.

Table 2. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, in the hot-plate test in mice.

Observation time (min)	Latency (s)				
	Control	Morphine	Hydroalcoholic extract	Hexane-partitioned fraction	Chloroform-partitioned fraction
0	6·62 ± 0·92	6·75 ± 1·04	5·87 ± 0·99	6·25 ± 1·28	6·12 ± 0·99
30	7·25 ± 1·16	15·3 ± 2·90†	7·50 ± 1·31	9·50 ± 1·07*	9·75 ± 1·04*
60	6·25 ± 1·28	16·5 ± 3·59†	9·0 ± 2·33	11·1 ± 1·81*	13·9 ± 1·89†
90	7·12 ± 0·83	13·8 ± 3·49†	7·50 ± 1·07	10·8 ± 1·60*	13·6 ± 2·07†

Each value is the mean ± s.d. of results from eight animals. Significantly different from result at 0 min for each group value; analysis of variance $F(4,35)$ 30 min = 24·6; 60 min = 22·4; 90 min = 17·9 ($P < 0.05$); Dunnett's test. *$P < 0.05$; †$P < 0.001$.

Table 4. Effect of the chloroform- and hexane-partitioned fractions of *Neurolaena lobata* on carrageenan-induced paw oedema in mice.

Treatment	Dose ($mg\,kg^{-1}$)	Paw weight (mg)	Inhibition (%)
Control	–	63·7 ± 5·51	–
Indomethacin	20	23·3 ± 5·67†	63·4
Hexane-partitioned fraction	100	35·8 ± 4·89*	43·8
Chloroform-partitioned fraction	100	20·2 ± 6·18†	68·3

Each value is the mean ± s.e.m. of results from seven animals. Significantly different compared with the respective control value; analysis of variance $F(3,24) = 12{\cdot}6$ ($P < 0{\cdot}05$); Dunnett's test $*P < 0{\cdot}05$; $\dagger P < 0{\cdot}001$.

respectively, 3 h after phlogistic compound injection. These results, reported in Table 4, show that these fractions are not endowed with morphinomimetic properties; the effect might be related to anti-inflammatory action on the acute inflammatory processes.

The punctual antinociceptive mechanism of the extract and its partitioned fractions cannot be determined from these data or with these tests because they measure different types of pain, i.e. those induced by chemical- (abdominal constriction test), thermal- (hot-plate test) or inflammatory (paw oedema) agents. The mechanism underlying this analgesic effect might be related to inhibition of prostaglandin synthesis or to other endogenous mediators of the inflammatory process, such as histamine and 5-hydroxytryptamine (Emim et al 1992).

Although the chemical principles responsible for the antinociceptive effects of the fractions obtained from *N. lobata* are not known, much of the action might be related to the presence of the terpenoids, sequiterpene-lactones, flavonoids, or some still unidentified alkaloid (Germosén-Robineau 1995).

These preliminary findings lend support to the use of this plant in folk medicine for pain treatment, mainly because of its low toxicity. Further work is needed to clarify the mechanism of its antinociceptive action.

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