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Anti-allodynic and anti-oedematogenic properties of the extract and lignans from *Phyllanthus amarus* in models of persistent inflammatory and neuropathic pain

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Abstract

This study investigated the anti-allodynic and anti-oedematogenic effects of the hexanic extract, lignan-rich fraction and purified lignans from a plant used in the traditional medicine, *Phyllanthus amarus*, in the inflammatory and neuropathic models of nociception. The hexanic extract inhibited the allodynia and the oedema induced by the intraplantar injection of complete Freund's adjuvant (CFA). The inhibition observed was 76 ± 7% (ipsilateral paw), 64 ± 7% (contralateral paw), and 41 ± 2% (oedema). Otherwise, the lignan-rich fraction or the pure lignans did not affect CFA-induced allodynia. Administered chronically, the lignan fraction reduced CFA-induced paw oedema (39 ± 9%). When evaluated in the model of neuropathic pain caused by partial ligation of sciatic nerve, the hexanic extract inhibited the mechanical allodynia (77 ± 7%), with a similar efficacy to the gabapentin (71 ± 10%). The anti-allodynic effects of hexanic extract of *P. amarus* seem not to be associated with the impairment of motor co-ordination or with the development of tolerance. Finally, the treatment with hexanic extract inhibited the increase of myeloperoxidase activity, either following intraplantar injection of CFA or after sciatic nerve injury. It is concluded that, apart from its anti-inflammatory actions, which are probably linked to the presence of lignans, another as yet unidentified active principle(s) present in the hexanic extract of *P. amarus* produces pronounced anti-allodynia in two models of inflammatory and neuropathic pain. Considering that few drugs are currently available for the treatment of chronic pain, especially of the neuropathic type, the present results may have clinical relevance and open new possibilities for the development of new anti-allodynic drugs.

Keywords: *Phyllanthus amarus*; Lignan; Allodynia; Anti-oedematogenic; Myeloperoxidase; Inflammatory pain; Neuropathic pain

1. Introduction

The plants belonging to the genus *Phyllanthus* (Euphorbiaceae) comprise more than 600 species, which are widely distributed in most tropical and subtropical countries. A substantial amount of evidence indicates that several species from the genus *Phyllanthus* are widely used in traditional medicine, in many countries, for the treatment of numerous disturbances, such as flu, dropsy, diabetes, jaundice and bladder calculus (for review see Unander et al., 1995; Calixto et al., 1998). We have investigated the basis of the medicinal use of the plants from the genus *Phyllanthus*, and have demonstrated that several extracts, as well as some purified active principles obtained from different species of *Phyllanthus*, exhibit pronounced systemic antinociceptive properties, particularly when assessed in neurogenic, but not in thermal models of nociception (Gorski et al., 1993; Santos et al., 1994; 1995a,b, 1999, 2000; Cechinel Filho et al., 1996; Miguel et al., 1996; for review see Calixto et al., 1998).

Concerning the pharmacological actions of *Phyllanthus amarus*, both experimental and clinical evidence have indicated that the extracts from this species are able to inhibit the DNA polymerase of hepatitis B and related hepatitis viruses (Venkateswaran et al., 1987; Unander et al., 1990; Wang, 2000). In addition, evidence also suggests that *P. amarus* extracts inhibit HIV-1 replication in HeLa $CD4^+$cells (Notka et al., 2002). Furthermore, simultaneous administration of *P. amarus* extract along with a carcinogen

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has been reported to inhibit the development of hepatocellular carcinoma (Rajeshkumar and Kuttan, 2000).

We have recently shown that the hydroalcoholic extract of *P. amarus*, similar to many other extracts of relative species of *Phyllanthus* collected in Brazil, caused a dose-dependent block of the pain response to acetic acid, formalin and capsaicin in mice (Santos et al., 2000; for review see Calixto et al., 1998). Furthermore, it has been shown recently that the extract of this plant, given systemically, has a pronounced anti-inflammatory action (Kiemer et al., 2002).

Phytochemical studies have revealed the existence of several classes of lignans in the extract of *P. amarus*, including hypophyllantin, phyllantin, nirantin, nirtetralin and phyltetralin. Interestingly, hypophyllantin, phyllantin and nirtetralin have also been found in *P. niruri* and have the ability to inhibit [^{125}I]-endothelin-1 binding to the recombinant human ET_A receptor expressed in Chinese hamster ovary cells, although they were inactive against the recombinant ET_B receptor (Hussain et al., 1995). Accumulated evidence now suggests that endogenous endothelins play a key role in the nociceptive and inflammatory processes (Raffa et al., 1991; Jarvis et al., 2000; Piovezan et al., 2000; Wilson et al., 2001).

Based on the abovementioned literature, we sought to investigate in the present study whether different extracts obtained from aerial parts of *P. amarus*, as well as some purified lignans isolated from this, such as hypophyllantin, phyllantin, nirantin, nirtetralin and phyltetralin, have oral anti-allodynic and/or anti-inflammatory properties. This was achieved by means of the complete Freund's adjuvant (CFA) model of inflammation and the persistent neuropathic model of pain caused by partial ligation of the sciatic nerve in mice. For the purposes of comparison, we also analysed the anti-allodynic action of gabapentin, an anticonvulsant drug that is effective in alleviating neuropathic pain in both rodents and humans (Rosner et al., 1996; Patel et al., 2001).

2. Materials and methods

2.1. Plant material

The aerial parts of *P. amarus* were collected from an experimental field of the Multi-disciplinary Center of Chemical, Biological and Agricultural Research-CPQBA/UNICAMP, Campinas, SP and authenticated by Prof. Dr. Grady L. Webster (University of California Davis, USA). A voucher specimen (number UEC 127.411) of *P. amarus* Schum and Thonn is deposited in the herbarium of the Biology Institute of UNICAMP, Campinas.

2.2. Extraction, fractionation and purification of lignans

The leaves were allowed to dry under air circulation (40 °C) for 3 days. The resulting powder (1000 g) was submitted to dynamic maceration with hexane. Concentration of the extracts under reduced pressure provided 55 g of crude hexanic extract (HE). The HE (20 g) was absorbed on silica gel and fractionated by five-dried column chromatography, using silica gel 60 with hexane/ethyl acetate (Hx/EtOAc) (70:30) as the eluent. Five fractions were obtained and extracted with ethyl acetate, analysed by TLC in H/A (70:30) (anisaldehyde detection). Fraction F4–F5 was enriched in lignans.

The F4–F5 fraction enriched in lignans (nirtetralin 1, nirantin 2, hypophyllantin 3, phyltetralin 4 and phyllantin 5), was purified on a flash chromatography column (5 cm) using silica gel 60 (0.04–0.063 mn) and a hexane–ethyl acetate mixture (Hx/EtOAc) as eluent, in a variable gradient of Hx/EtOAc (90:10), (89:11), (88:12) and (85:15). The lignans 1 to 5, presented the following Hx/EtOAc (30:10) values of Rf: 0.27 (1), 0.24 (2), 0.21 (3), 0.17 (4) and 0.5 (5) by TLC (Somanabandhu, 1993; Anjaneyulu et al., 1973).

2.3. Pharmacological studies

2.3.1. Animals

The experiments were conducted using male Swiss mice (25–35 g) ($n = 132$) kept in chambers with controlled temperature (22 ± 1 °C), under a 12 h light–dark cycle. Food and water were freely available. Animals were acclimatised to the laboratory for at least 2 h before testing and were used only once throughout the experiments. The studies reported in this manuscript were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

2.3.2. Drug and extract administration

The hexanic extract (HE) and the lignan-rich fraction obtained from *P. amarus* (100 mg/kg) were administered orally to mice according to the treatment schedules presented below. The purified lignans (30–100 mg/kg) were administered by i.p. route. Control animals were treated with vehicle (0.3% Tween 80 in saline; 10 ml/kg) by the same route and at the same time intervals. The doses used were selected based on preliminary experiments.

To assess the effects of the acute treatment, animals received a single dose of the HE, the lignan-rich fraction (100 mg/kg, p.o.) or the purified lignans (30 to 100 mg/kg, i.p.) 24 h following Complete Freund's Adjuvant intraplantar injection or 5 days after the sciatic nerve partial ligation. Developments of allodynia and oedema formation were evaluated in CFA-injected animals in 1, 2, 4 and 8 h after treatment to verify the time that the HE was effective to inhibit the oedema or allodynia. Only allodynia was assessed in nerve-injured mice in 2, 4 and 8 h after treatment to verify in what point of the HE was effective to inhibit the allodynia.

To investigate the effects of the long-term treatment, the HE or the lignan-rich fraction (both 100 mg/kg, p.o.) was administered orally to mice, twice a day (every 12 h). The allodynic responses and the increase in paw oedema were

evaluated only 6 h after treatments to discriminate the acute effects of the extract and the lignan fraction. The treatment was extended until the maximum inhibitory effect was achieved, and it was then interrupted. Next, the treatment was re-initiated to assess the development of tolerance. In the neuropathic pain model, the effects of the chronic treatment with the HE or the lignan fraction were compared to that produced by the long-term administration of the anticonvulsive drug gabapentin (70 mg/kg, p.o.).

2.3.3. CFA-induced inflammation

Mice were lightly anaesthetised with ether and received 20 μl of CFA (1 mg/ml of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate), subcutaneously in the intraplantar surface of the right hindpaw. The contralateral paw (left paw) received the same volume of phosphate buffered saline (PBS; mmol/l: NaCl 137, KCl 2.7 and phosphate buffer 10). The control groups received 20 μl of PBS in the ipsilateral paw. The dose of CFA produced a significant increase in paw volume and allodynia development (Ferreira et al., 2001; Bortolanza et al., 2002).

2.3.4. Partial sciatic nerve injury

Mice were anaesthetised with 7% chloral hydrate (0.6 ml/kg, i.p.). A partial ligation of the sciatic nerve was performed by tying the distal 1/3 to 1/2 of the sciatic nerve, according to the procedure described in rats by Seltzer et al. (1990) and adapted to mice by Malmberg and Basbaum (1998). In sham-operated mice, the nerve was exposed using the same procedure, but without ligation. As reported previously (Bortolanza et al., 2002), mice did not present paw drooping or autotomy.

2.3.5. Mechanical allodynia

The mechanical allodynia was measured as described before (Bortolanza et al., 2002), as the withdrawal response frequency to 10 applications of 0.4 g von Frey filaments (VFH, Stoelting, Chicago, USA). Mice were further acclimatised in individual clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The frequency of withdrawal was determined before and after nerve injury or CFA injection. In order to obtain data purely derived from the treatments in CFA or nerve injury-induced allodynia, the maximal inhibition (MI) values were represented as the difference between the basal values of vehicle or drug-treated animals and respective controls.

2.3.6. Anti-inflammatory activity

In another set of experiments, the effects of the HE from *P. amarus* or the lignan-rich fraction were evaluated against the paw oedema caused by the intraplantar injection of CFA. Oedema was measured by use of a plethysmometer (Ugo Basile) at several time-points and was expressed (in μl) as the difference between the right (CFA-injected paw) and the left paw (De Campos et al., 1996; Ferreira et al., 2001).

The neutrophil infiltration was evaluated indirectly by measuring the myeloperoxidase activity, as previously described (De Young et al., 1989). Naive animals were pretreated with a single dose of the HE (100 mg/kg) 1 h before CFA injection. Other groups of naive mice were chronically treated with the HE (100 mg/kg) twice a day (each 12 h). The treatment was initiated 24 h after CFA injection or 5 days following the sciatic nerve partial ligation. Animals were sacrificed 2 days after CFA injection or 12 days after nerve ligation. The subcutaneous tissue of the paws injected with CFA or the sciatic nerves from operated mice were placed in an Eppendorf tube containing 0.75 ml of 80 mM sodium phosphate buffer (pH 5.4) and 0.5% hexadecyltrimethyl ammonium (HTAB). Enzyme activity was determined colorimetrically using an Ultra microplate reader (absorbance 520 nm). Data were expressed as mOD (optical density units × 10^3)/mg of protein.

2.3.7. Measurement of motor performance

In order to evaluate the possible non-specific effects of the HE of *P. amarus*, on the motor co-ordination, mice were tested on the rota-rod (Dunham and Miya, 1957) apparatus.

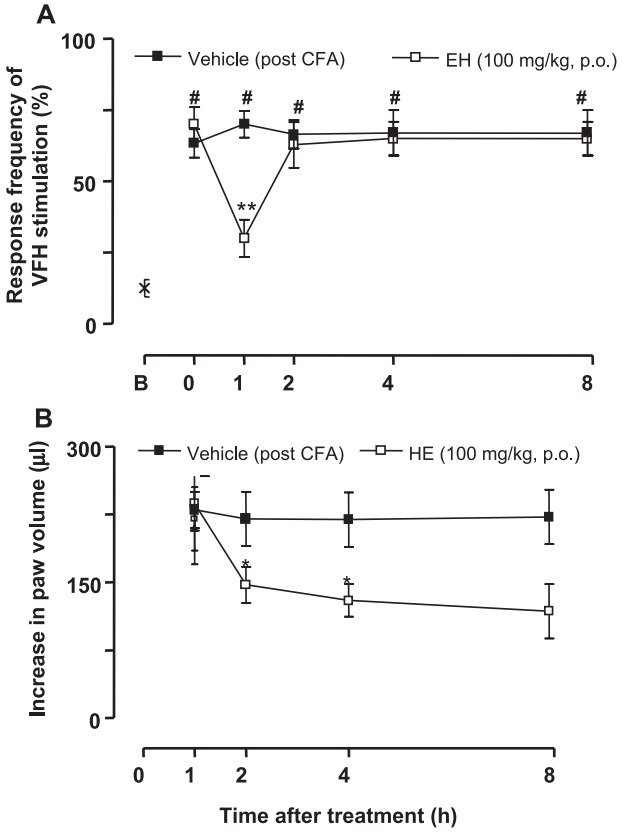

Fig. 1. Effects of acute p.o. administration of hexanic extract (HE) of *P. amarus* on mechanical allodynia (response frequency of von Frey filament (VFH) (A) and paw oedema formation after Complete Freund's adjuvant (CFA) injection into the paw (B). Baseline (B) values were obtained before CFA injection. Data are expressed as means ± S.E.M.; $n = 6–7$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.001$ versus baseline (B) values, Student's *t*-test.

The apparatus consists of a bar, 2.5 cm in diameter, subdivided into five compartments by disks of 25 cm diameter (Rota Rod Tread-mill for mice 7600, Ugo Basile, Varese, Italy). The bar rotated at a constant speed of 22 rpm. The animals were selected 24 h before by eliminating those mice that did not remain on the bar for two consecutive periods of 50–60 s. After the selection, animals were treated with HE (100–200 mg/kg, p.o.) or received the same volume of vehicle (10 ml/kg, p.o.) 60 min before the test. The results were expressed as the time for which animals remained on the rota-rod. The cut-off time used was 60 s.

2.3.8. Drugs and reagents

Gabapentin was obtained from Park-Davis (neurotin®, Brazil), Complete Frend's adjuvant (CFA), phosphate-buffered saline (PBS), hexadecyltrimethyl ammonium bromide (HTAB), were all obtained from Sigma (St. Louis, MO, USA); chloral hydrate were purchased from Vetec (Rio de

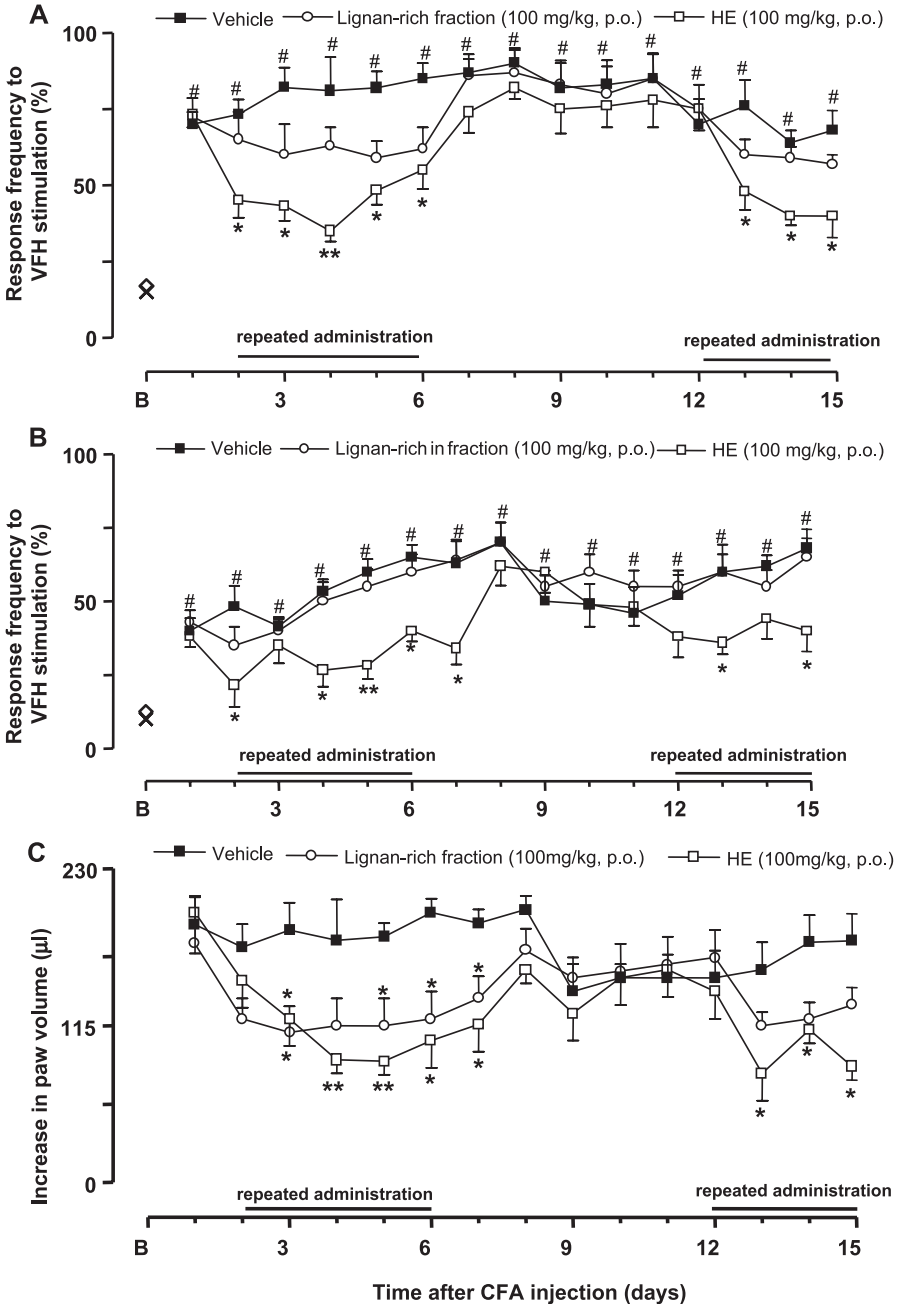

Fig. 2. Effects of chronic p.o. administration of hexanic extract (HE) of *P. amarus* and lignan-rich fraction on mechanical allodynia in ipsilateral (A) and contralateral (B) paws and on paw oedema formation (C) and in the complete Freund's adjuvant (CFA) injected paw (C). Data are expressed as means ± S.E.M.; $n = 6–7$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, Dunnett's multiple comparison test, $^{\#}P < 0.001$ versus baseline (B) values, Student's *t*-test.

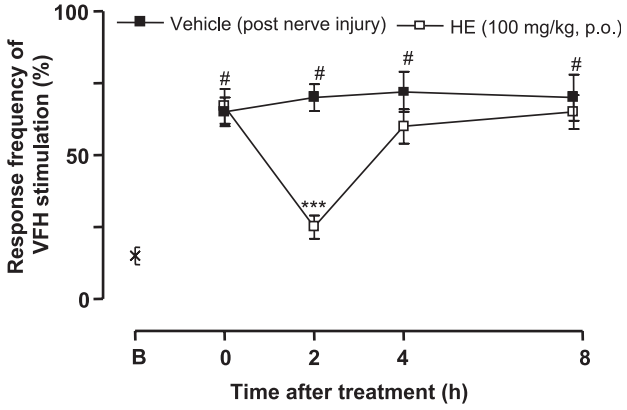

Fig. 3. Effects of acute p.o. administration of hexanic extract (HE) of *P. amarus* on mechanical allodynia in nerve-injured mice. Baseline values were obtained before the nerve-injury (NI). Data are expressed as means ± S.E.M.; $n = 6–7$ mice per group. $^{***}P < 0.01$ versus control, Dunnett's multiple comparison test, $^{\#}P < 0.001$ versus sham-operated, Student's *t*-test.

Janeiro, Brazil) and Tween 80 was acquired from Synth (Rio de Janeiro, Brazil).

2.3.9. Data analysis

Results are expressed as the mean ± standard error of the mean (S.E.M.). The statistical difference between groups was determined by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, or by means of Student's *t*-test when appropriate. *P* values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

3. Results

3.1. Effects of hexanic extract of P. amarus and lignan-rich fraction on inflammatory reaction induced by CFA injection

The intraplantar injection of CFA produced a profound and long-lasting mechanical allodynia on the ipsilateral side, and to a lesser extent on the contralateral side (Fig. 2A and B). Acute treatment with hexanic extract (HE) of *P. amarus* (100 mg/kg, p.o.) significantly decreased the mechanical allodynia, when assessed 1 h after the administration of the extract (72 ± 7%) (Fig. 1A). The HE also significantly decreased the oedema formation (41 ± 2%) (Fig. 1B). On the other hand, the acute treatment with the lignan-rich fraction (100 mg/kg, p.o.) or with the purified lignans (30 to 100 mg/kg, i.p.) did not produce any significant anti-allodynic effects (results not shown). The increase in response frequency to von Frey filament stimulation of the CFA-injected paw appeared on day 2 and persisted until day 15, while in the contralateral paw the increase in response appeared on day 3, and persisted up to 15 days (Fig. 2A and B, respectively).

The long-term treatment with the HE of *P. amarus* (100 mg/kg, p.o.) twice a day (every 12 h) decreased markedly the paw withdrawal response on the ipsilateral side (percentage of inhibition: 76 ± 7%). This effect was evident from day 2, and had a peak 4 days later (Fig. 2A). When analysed on the contralateral side, the long-term oral treatment with HE produced an inhibition of 64 ± 7% of the initial allodynia (Fig. 2B), while the oedema was reduced by 53 ± 3% (Fig. 2C). When the treatment was suspended, the anti-allodynic properties of *P. amarus* were not observed on the ipsilateral side, although on the contralateral side the effect was still observed. The anti-oedematogenic effect of the HE of *P. amarus* persisted for 2 additional days. When *P. amarus* treatment was re-initiated on day 12, the same anti-allodynic and anti-oedematogenic responses were detected (Fig. 2A), showing that the HE from *P. amarus* did not induce tolerance.

In marked contrast to the HE, the lignan-rich fraction (100 mg/kg, i.p.) from *P. amarus* failed to exhibit significant anti-allodynic actions when assessed in acute or in long-term treatment schedule (Fig. 2A and B). Otherwise, in the same experimental conditions, the lignan-rich fraction caused pronounced and significant inhibition of oedema formation (39 ± 9%) (Fig. 2C).

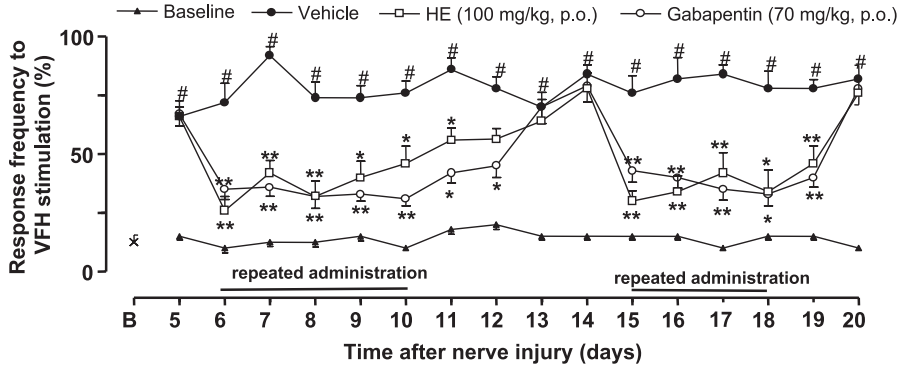

Fig. 4. Effects of chronic p.o. administration of hexanic extract (HE) of *P. amarus* or gabapentin on mechanical allodynia in nerve-injured mice. Baseline values were obtained before the nerve-injury. Data are expressed as means ± S.E.M.; $n = 6–7$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, Dunnett's multiple comparison test, $^{\#}P < 0.001$ versus sham-operated, Student's *t*-test.

3.2. Effects of hexanic extract of P. amarus on the mechanical allodynia induced by partial nerve ligation in mice

Partial sciatic nerve ligation produced a marked and long-lasting development of allodynia on the ipsilateral (injured) side (Fig. 4), but not on the contralateral side (results not shown). The allodynia developed on day 5, reached a maximum on day 7, and persisted up to 20 days (Fig. 4). The acute oral treatment with the HE of *P. amarus* (100 mg/kg, p.o.) significantly decreased the paw withdrawal response (percentage of inhibition: 77 ± 7%) (Fig. 3). Similar to gabapentin (70 mg/kg, p.o.), the administration of the HE of *P. amarus* (100 mg/kg, p.o., twice a day) significantly decreased (71 ± 10%) the paw withdrawal response when analysed on the second day of treatment. This inhibition was observed on the first day after treatment, and persisted until 2 days after the treatment was interrupted (Fig. 4). The same treatment did not affect the paw withdrawal response of sham-operated animals (data not shown).

The suspension of treatment with the HE of *P. amarus* or with gabapentin at day 10 reduced their anti-allodynic effects on the 12th day. However, the results in Fig. 4 show that there is no evidence for the development of tolerance. The continuation of long-term treatment, with both HE and gabapentin starting at day 15, produced essentially the same anti-allodynic effects, which remained stable until the treatment was suspended again (day 18) (Fig. 4).

3.3. Effects of hexanic extract of P. amarus on the myeloperoxidase activity

The intraplantar injection of CFA (20 μl/paw) caused a significant (about sevenfold) increase in the myeloperoxidase levels (12 h post-injection), when compared with the myeloperoxidase values in PBS (20 μl)-injected paws. The acute treatment with HE (100 mg/kg, p.o.) caused a 22 ± 10% inhibition of myeloperoxidase activity, although the statistical analysis did not indicate significance (Fig. 5A). When mice were treated twice a day (every 12 h), for 2 days, with the HE of *P. amarus* (100 mg/kg p.o.) the myeloperoxidase activity was significantly reduced (27 ± 3%) (Fig. 5B).

On the ipsilateral side of nerve-injured mice, a significant increase (about sixfold) was observed in the myeloperoxidase activity, in comparison to sham-operated mice (Fig. 5C). The increase in myeloperoxidase activity was significant after 7 days (Fig. 5C, black column). The chronic treatment of animals with the HE of *P. amarus* also caused an inhibition of myeloperoxidase activity (64 ± 3%) (Fig. 5C, open column).

3.4. Effects of HE of P. amarus on locomotor activity

The HE from *P. amarus* (100 or 200 mg/kg, p.o.), given 60 min before, did not significantly affect the motor response of the animals on the rota rod. The response presented by control animals was 56.7 s, versus 57.6 and 56.9 s after treatment with the HE (100 mg/kg, p.o. and 200 mg/kg, respectively) (results not shown).

4. Discussion

There is substantial evidence from both in vitro and in vivo studies of the beneficial effect of the extracts of *P. amarus* against the hepatitis B virus (Venkateswaran et al., 1987; Unander et al., 1995; Lee et al., 1996; Ott et al., 1997), although clinical studies have produced somewhat controversial results (Thyagarajan et al., 1988; Milne et al., 1994; Doshi et al., 1994; Chang et al., 1995; Wang et al., 1995). Furthermore, similar to the extracts from other parent species, *P. amarus* extract exhibited an oral antinociceptive action when assessed in chemical, but not in thermal models of nociception in mice (Calixto et al., 1998; Santos et al., 2000). The mechanisms underlying such actions and the active principle(s) responsible for these effects are, however, still elusive.

The results of the present study extended the previous data and showed, for the first time, that active constituents

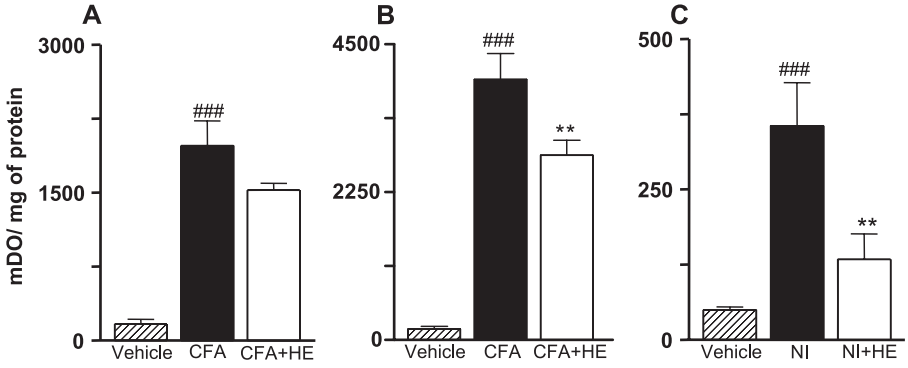

Fig. 5. Effects of acute (A) or chronic (B) p.o. administration of hexanic extract (HE) of *P. amarus* on myeloperoxidase activity in the CFA-injected paw (CFA + HE) (A and B) or injured sciatic nerve (NI + HE). mDO-milli optical density. Data are expressed as means ± S.E.M.; $n = 6-8$ mice per group. $^{**}P < 0.01$ versus control, Dunnett's multiple comparison test, $^{\#}P < 0.001$ versus normal paw or sham, Student's *t*-test.

present in the HE of *P. amarus* are effective in preventing the persistent inflammatory allodynia caused by CFA in mice. Notably, in this model, the HE from *P. amarus* was also effective in preventing both the ipsilateral and contralateral persistent nociception. Also of interest are the results showing that HE of *P. amarus* dosed orally was effective in preventing the persistent neuropathic pain caused by partial sciatic nerve constriction. In both models, the anti-allodynic action of the extract of *P. amarus* was evident early and lasted for up to 1 h. The anticonvulsant gabapentin is a relevant drug used for the clinical management of neuropathic pain (Field et al., 1997; Abdi et al., 1998). The HE of *P. amarus* presented a quite similar efficacy to that obtained with gabapentin, when assessed on mechanical allodynia in mice after the partial ligation of the sciatic nerve. Also relevant are the data showing that, in contrast to gabapentin, which is known to produce marked impairment of motor coordination in animals (Bortolanza et al., 2002), acute or prolonged oral treatment with the HE of *P. amarus* (100 to 200 mg/kg) was well-tolerated. Interestingly, the anti-allodynia caused by the extract of *P. amarus* in both models of persistent pain studied (CFA and sciatic nerve partial constriction), as well as that caused by gabapentin against the neuropathic pain, was not susceptible to tolerance. This conclusion derives from data showing that (i) the withdrawal of the extract was followed by the complete return to baseline allodynia, and (ii) a new oral treatment with the same dose of the extract or with gabapentin, twice a day, produced very similar and pronounced anti-allodynic effects.

In order to assess whether or not the lignans present in the extract of *P. amarus* are involved in its antinociceptive effect, we prepared a fraction rich in lignans and tested it against the mechanical allodynia after intraplantar injection of CFA. The results shown in Fig. 2A and B indicate that lignans (at the same dose of HE) did not prevent the ipsilateral nor the contralateral mechanical allodynia to von Frey stimulation in CFA-injected paws. Such results support the view that other active principle(s), besides the lignans, present in the HE of *P. amarus* are likely to be involved in the observed inhibition of the persistent pain.

It is now well recognised that the persistent pain caused by intraplantar injection of CFA or resulting from partial constriction of the sciatic nerve involves central sensitisation due to the release of multiple inflammatory and pain mediators, that in turn account for the increase in sensitivity of both peripheral sensory afferents at the site of the injury, and in the central nervous system (for review see Tracey and Walker, 1995; Basbaum, 1999; Urban and Gebhart, 1999; Zimmermann, 2001; Samad et al., 2001). The marked increase in the myeloperoxidase activity (indirect evidence for neutrophil influx) in the sciatic nerve following its partial constriction is a new and interesting result, which further reinforces the view that the neuropathic pain observed in this model seems to be associated with the local inflammatory response. Perkins and Tracey (2000) have reported that depletion of neutrophils at the site of nerve injury significantly attenuated the induction of hyperalgesia after partial ligation of the sciatic nerve. Likewise, Bennett et al. (1998) showed that circulating neutrophils account for the mechanism by which nerve growth factor induces thermal hyperalgesia. Therefore, the anti-allodynic action of the active principle(s) present in the HE of *P. amarus* is most probably associated with its (their) anti-inflammatory action. This notion is further reinforced by the results showing that both the acute and, mainly, the long-term administration of the HE of *P. amarus* caused a pronounced inhibition of CFA-mediated paw oedema formation. To explore further this hypothesis, we investigated whether the HE of *P. amarus* was capable of preventing the increase in the myeloperoxidase activity in the ipsilateral paw injected with CFA. Our results show that prolonged, but not acute, administration of HE of *P. amarus* markedly inhibited the myeloperoxidase activity, further confirming the anti-inflammatory action of the HE.

By contrast with the results observed with the HE in the persistent models of allodynia, the fraction rich in lignans consistently inhibited the oedema formation induced by CFA, without significantly affecting the allodynic responses. These results are interesting and clearly suggest that different active principles present in the hexane extract of *P. amarus* are likely to be responsible for its anti-allodynic and anti-inflammatory properties. While the lignans present in the HE (nirtetralin, phylanthin, hypophyllantin, niranthin and phyltetralin) had no apparent significant anti-allodynic effect when given orally, the same compounds seem to be responsible for the majority, if not all of the reported oral anti-inflammatory actions observed for the extract of *P. amarus.*

The results of the present study are in line with those recently reported by Kiemer et al. (2002) who demonstrated that the ethanolic and HEs of *P. amarus* exhibited marked anti-inflammatory properties when assessed in rat Kupffer cells, RAW 264.7 macrophages, in human whole blood and in mice. The same study also revealed that the anti-inflammatory actions of the extracts of *P. amarus* are a consequence of its inhibitory effects on the induction of inflammatory enzymes, namely cyclo-oxygenase 2 and inducible nitric oxide synthase. This effect occurs through the prevention of the synthesis of pro-inflammatory cytokines, via inhibition of nuclear transcriptional factor κB. Based on these findings, the authors concluded that the anti-inflammatory properties observed for the extract of *P. amarus* might account for the reports from animal and clinical studies of its beneficial effects against hepatitis B viruses.

In conclusion, we report here that the HE from the aerial parts of *P. amarus* dosed orally in acute and, mainly, prolonged treatment regimens in mice, produces pronounced anti-allodynia when assessed against the persistent pain caused by CFA or in neuropathic pain following partial ligation of the sciatic nerve. Our results also show that the

anti-allodynic effect of *P. amarus* was, at least in part, a consequence of its anti-inflammatory action, evident by the fact that, under similar conditions, the HE of *P. amarus* produced marked inhibition of paw oedema formation and also significantly inhibited the myeloperoxidase activity, both in the paw and in the injured sciatic nerve. Taken together, the present results show for the first time that apart from its reported anti-inflammatory action, the active principle(s) present in the HE of *P. amarus*, given orally, produces pronounced anti-allodynia in CFA and in partial sciatic ligation models of persistent pain. Considering that few drugs are currently available for the treatment of persistent pain, especially of neuropathic origin, the present results may have clinical relevance and open the possibility of the development of new anti-allodynic drugs. Studies are now in progress to isolate and characterise other constituents present in the plant that could account for the reported pharmacological effects and also to further characterise their sites of action.

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