Antinociceptive Effect of *Heliopsis longipes* Extract and Affinin in Mice

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**Abstract**

*Heliopsis longipes* is used as analgesic in Mexican traditional medicine. The present study assesses the possible antinociceptive effect of *Heliopsis longipes* and describes the pharmacological mechanism of action of the antinociceptive effect of affinin, identified as the one active principle in *Heliopsis longipes* acetone extract. Intraperitoneal administration of *H. longipes* extract and affinin produced a dose-dependent antinociceptive effect when assessed in mice submitted to acetonic acid and capsaicin tests. Affinin-induced antinociception (30 mg/kg, i.p.) was blocked by naltrindrene (1 mg/kg, s.c.), *p*-chlorophenylalanine (80 mg/kg, i.p.) and flumazenil (5 mg/kg, s.c.) suggesting that its pharmacological effect could be due to the activation of opiodergic, serotoninergic and GABAergic systems. In addition, the antinociceptive effect of affinin was attenuated by pretreatment with 1H-[1,2,4]oxadiazolo[1,2-a]quinoxalin-1-one (1 mg/kg, s.c.) and glibenclamide (10 mg/kg, s.c.) suggesting that the nitric oxide-K⁺ channels pathway could be involved in its mechanism of action. These results suggest that affinin itself or its derivatives may have potential antinociceptive effects.

**Introduction**

*Heliopsis longipes* (A. Gray) Blake (Compositae), known in Mexico under the popular names of “chilcuague” or “chilcuan”, is used to treat complaints associated with pain, particularly toothache [1]. This plant is still widely used as a condiment in the areas of its natural occurrence. *H. longipes* is distributed in Mexico in the states of Guanajuato, San Luis Potosí and Queretaro [2, 3]. Affinin [N-isobutyl-2(E),6(Z),8(E)-decatrienamide (© Fig. 1)], is the major lipidic component of the plant [4–6]. In a previous study, it was demonstrated that affinin has fungistatic (*Sclerotium rolfsii*, *Sclerotium cepivorum*, *Phytophthora infestans* and *Rhizoctonia solana*) and bacteriostatic (*Escherichia coli* and *Bacillus subtilis*) activities [7].

In Mexico, *H. longipes* is used as a local analgesic and anesthetic [1, 5]. It produces intense numbness and a tingling sensation in the lips, tongue and mouth and it stimulates saliva [5]. The use of *H. longipes* for treating the symptoms of pain in folk medicine suggests the presence of compounds with analgesic and/or anti-inflammatory properties [1, 8, 9]. Accordingly, the *H. longipes* extract has shown to produce anti-hyperalgesic effects in carrageenan-induced thermal hyperalgesia [8]. This effect could be produced by affinin, by increasing γ-aminobutyric acid (GABA) release in mice brain slices [9]. However, the effects of affinin as well as its possible mechanisms of action are at present unknown. Thus, the aim of the present research was to assess the antinociceptive effect of the acetone extract of *H. longipes* and a compound obtained from such extract, affinin, as well as the possible mechanisms of antinociceptive activity. Two well-known animal tests were used in this study to accomplish such an endeavor, the writhing and capsaicin tests.

**Material and Methods**

**Animals**

Experiments were performed on male ICR mice (body weight range, 25–30 g) from Centro Harlan (Harlan México). All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals [10] and their care was conducted in conformity with Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999). Efforts were made to...
minimize animal suffering and to reduce the number of animals used. Animals were housed in groups of six per standard cage on climate controlled room (22 ± 2°C) with a 12-h light/dark cycle. At the end of the experiment, animals were sacrificed in a CO2 chamber.

Plant material
Fresh stems of *H. longipes* (Compositae) were collected in “Real de Xichú”, Guanajuato, México, in July 2007, and identified by Ramiro Ríos Gómez, MSc, Universidad Nacional Autonóma de México. A voucher specimen (number 5904) was deposited at the FEZA Herbarium of the Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autonóma de México, México.

Extraction and isolation
Fresh stems of *H. longipes* (4.725 kg) were extracted at room temperature with acetone (3 × 24 L, 48 h each). The extraction solvent was concentrated to dryness in vacuum to render 76.5 g of extract. Fractionation of the acetone extract by means of open column chromatography (silica gel, 100–230 mesh; Natland Int. Corp.; 11.5 × 50 cm) was performed with a step gradient of n-hexane-acetone 100 : 0 to 40 : 60, collecting 124 fractions of 250 mL each. The presence of affinin [N-isobutyl-2(Z,6(Z,8(E)-decatrienamide)] in the obtained fractions was monitored by TLC using one authentic sample of affinin (RF = 0.33, n-hexane-acetone 80 : 20) as reference, visualizing with a UV lamp (affinin develops a dark gray spot at 254 nm), and by spraying with a 1% solution of (NH₄)₂Ce(SO₄)₃/ H₂O in 2 N H₂SO₄ (affinin develops a brilliant yellow spot). On the basis of TLC the fractions 17–29 (4000–7250 mL, eluent n-hexane-acetone, 9 : 1), 30–73 (7251–18 2850 mL, eluent n-hexane-acetone, 8 : 2 to 7 : 3), and 74–107 (18 251–26 750 mL, n-hexane-acetone, 6 : 4) were combined because they afforded practically only affinin. This compound was further purified from these fractions by means of flash column chromatography: fractions 17–29 (2.39 g, silica gel, 230–400 mesh; Natland Int. Corp.; 3 cm i.d. ×10 inch, eluent n-hexane-CH₂Cl₂ 70 : 30, flow rate 2 ml/min) collecting 11 fractions of 25 mL each, yielding 1.97 g (2.58%) of pure affinin, fractions 30–73 (9.34 g, silica gel, 230–400 mesh; 6 cm i.d. ×10 inch, eluent n-hexane-CH₂Cl₂ 70 : 30, flow rate 2 ml/min) collecting 33 fractions of 50 mL each, yielding 7.34 g (9.59%) of pure affinin, and fractions 74–107 (5.55 g, silica gel, 230–400 mesh; 4 cm i.d. ×10 inch, eluent n-hexane-CH₂Cl₂ 70 : 30, flow rate 2 ml/min) collecting 21 fractions of 35 mL each, yielding 4.78 g (6.25%) of pure affinin. The total yield of pure affinin was 14.09 g (18.42%) with respect to extract weight. The structure of the compound, affinin, was established on the basis of its IR, UV, ¹H- and ¹³C-NMR, DEPT, COSY, HSQC, HMBC, and MS data and their comparison with those previously reported [1]. The purity of affinin (96.15%) was established by means of NMR and by the comparison of its retention time (20.57 min) with respect to an authentic sample on GC-MS analysis (vide infra).

**Figure 1** Chemical structure of N-isobutyl-2(Z,6(Z,8(E)-decatrienamide (affinin) obtained from the extract of Heloposis longipes.

**GC-MS analyses**
GC-MS analyses were obtained using an Agilent 6890 GC System/5973 MSD chromatograph equipped with an HP-1 capillary column (length 30 m, i.d. 0.25 mm, 0.25 μm). The carrier gas was helium, and the linear gas velocity was 36 cm/s. The detector temperature was 250°C, and the column temperature, initially at 45°C, was gradually increased at a rate of 10°C/min to 250°C. For detection, a flame ionization detector at 280°C, EI (scan 30–550 u), was used. The identification of affinin as total extract and as pure compound was based on the comparison of its retention time (20.57 min) with respect to an authentic sample, and the comparison of its mass spectrum with those contained in the N-15598 Mass Spectral Library. On GC-MS analysis, fresh stems of *H. longipes* extracted with acetone showed eight principal peaks at: 20.71 min (affinin, 80.307%), 20.81 min (affinin isomer, 1.591%), 20.91 min (2(Z,6(Z,8(E)-decatrienamide isomer, 0.523%), 21.54 min (N-(2-methylbutyl)-2(Z,6(Z,8(E)-decatrienamide isomer, 3.396%), 21.61 min (N-(2-methylbutyl)-2(Z,6(Z,8(E)-decatrienamide, 6.880%), 22.42 min (unknown, 3.489%), 22.85 min (unknown, 1.085%) and 23.31 min (unknown, 2.297%).

**Chemicals and drugs**
Affinin was isolated from the fresh stems of *H. longipes* by acetone extraction. 1H-[1,2,4]-oxadiazolo[4,2-a]quinolin-1-one (ODQ), glibenclamide, flumazenil, 4-chloro-6-deoxyphenylalanine (PCPA), naltrexone, capsaicin, metamizol and carboxymethylcellulose were purchased from Sigma. Acetic acid and morphine were purchased from Baker and Laboratorios Pisa, respectively. All drugs were of the highest-purity grade (99.9%). *H. longipes* extract, affinin, and PCPA were suspended in carboxymethylcellulose 1%. Glibenclamide, morphine, naltrexone, ODQ, metamizol and flumazenil were suspended in saline solution (0.9%). Capsaicin was dissolved in vehicle (containing 10% ethanol, 10% Tween 20, and 80% phosphate buffered saline [pH 6.2]) as previously described by Sakurada et al. [11]. All drugs were freshly prepared each time and administered (subcutaneous or intraperitoneal) in a volume of 0.1 mL/10 g body weight. Capsaicin was administered in a volume of 20 μL in the right paw. Control animals received the same volume of vehicle (carboxymethylcellulose 1% in saline solution 0.9%).

**Measurement of antinociceptive activity**
**Acetic acid-induced abdominal writhes:** The abdominal writhes were performed as previously described by Koster et al. [12]. The total number of writhes following the intraperitoneal (i.p.) administration of 0.6% acetic acid (10 mL/kg) was assessed for 30 min after the injection. Logarithmic doses were administered of the extract of *H. longipes* (0.01–1 mg/kg, i.p.) and affinin (1–1.875 mg/kg, i.p.) 15 min before acetic acid injection. Control animals received a similar volume of vehicle (carboxymethylcellulose 1%) or the morphine as a positive control (log dose 1.875 mg/kg, i.p.). Animals were then placed in a Plexiglas observation chamber for 15 min to allow them to accommodate to their surroundings, then they were removed for algesic administrations. Nociceptive behavior was induced by intraperitoneal acetic acid 0.6%. Mice were then returned to the chamber for observation. Nociceptive behavior was quantified as the number of writhes (abdominal contractions) produced in the treated animals during 5-min periods up to 30 min after algesic injection.

**Capsaicin-induced nociception:** In an attempt to provide more direct evidence concerning the possible antinociceptive effects of...
an extract of *H. longipes* and affinin on neurogenic nociception, we also investigated whether these decrease capsaicin-induced nociception in the mouse paw. The procedure used was similar to that described previously by Sakurada et al. [11], with a minor modification. Before testing, the animals were placed individually in a transparent glass cylinder, 20 cm in diameter, serving as a observation chamber. After the adaptation period, 20 µL of capsaicin (1.6 µg/paw) were injected subcutaneously into the dorsal surface of the right hind paw with a 26-gauge needle. The animal was then returned to the chamber for observation and nociceptive behavior was observed immediately after capsaicin injection. A mirror was placed behind the chamber to enable unhindered observation. Nociceptive behavior was quantified as the amount of time spent licking the injected paw which was timed with a chronometer and was considered as being indicative of nociception. Logarithmic doses of the extract of *H. longipes* (0.01–1.75 mg/kg, i.p.) and affinin (1–1.875 mg/kg, i.p.) were administered 15 min before capsaicin injection. Control animals received a similar volume of vehicle (carboxymethylcellulose 1% in saline solution 0.9%) or morphine as a positive control (log dose 1.875 mg/kg, i.p.).

**Analysis of the possible mechanism of action of affinin on acetic acid-induced writhes in mice**: To investigate the participation of the GABAergic, opioidergic and serotoninergic mechanisms in the writhing test, animals were pretreated with: naltrexone (1 mg/kg, s.c.), an antagonist of opioid receptors, PCPA (80 mg/kg, i.p., for four consecutive days), an inhibitor of serotonin synthesis, and flumazenil (5 mg/kg, s.c.), a benzodiazepine site antagonist at the GABA<sub>A</sub> receptor. These animals received affinin (30 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) 15 min before acetic acid (0.6%, i.p.) injection, and the antinociceptive effect was recorded as described above. We also investigated the possible participation of the nitric oxide pathway in the antinociceptive effect of affinin. For this, animals were pretreated 15 min before affinin administration (30 mg/kg, i.p.) with ODQ (1 mg/kg, s.c.), an inhibitor soluble guanylyl cyclase, and glibenclamide (10 mg/kg, s.c.), a blocker of ATP-sensitive K<sup>–</sup>-channels. 15 min after affinin administration the animals were injected with acid acetic (0.6%, i.p.) and the number of writhes was quantified.

**Statistical analysis**
All experimental results are given as the mean ± S.E.M. of the data obtained in 8 animals per group. Analysis of variance followed by the Tukey test was used to test the significance of differences with three or more treatments. Differences with a p < 0.05 were considered significant.

**Results**

Intraperitoneal 0.6% acetic acid administration in mice produces writhes. Systemic administration of logarithmic doses of *H. longipes* extract (0.01–1 mg/kg, i.p.) and affinin purified from *H. longipes* extract (1–1.875 mg/kg, i.p.) significantly reduced (p < 0.05) the number of abdominal contractions in a dose-dependent manner when tested in the writhing model (Fig. 2A and B). Morphine, a standard opiate analgesic drug (used as a positive control) significantly reduced the number of writhes induced by acetic acid (Fig. 2). Linear logarithmic data showed that systemic administration of *H. longipes* extract (DE<sub>50</sub> = 2.2 ± 0.2 mg/kg, i.p.) was more potent than affinin (DE<sub>50</sub> = 36 ± 5 mg/kg, i.p.) in nociception induced by acetic acid.

![Fig. 2](image)  **Antinociceptive effect of *H. longipes* extract (A) and affinin (B) in the acetic acid-induced writhes. Morphine (M, log dose 1.875 mg/kg, i.p.), used as a positive control, was given 15 min before the noxious stimulation. Data are expressed as the total number of writhes counted over a 30-min period. Bars are the means of eight mice ± S. E. M. * Significantly different from the vehicle (V) group (p < 0.05), as determined by analysis of variance followed by the Tukey test.

![Fig. 3 A and B](image)  **Fig. 3 A and B** shows that intraperitoneal administration of logarithmic doses of *H. longipes* extract (0.01–1.75 mg/kg, i.p.) and affinin (1–1.875 mg/kg, i.p.) reduced nociceptive behaviors (licking) induced by capsaicin. The maximum antinociceptive effect observed with the extract was 65.8% (log dose 1.75 mg/kg, i.p.) whereas affinin showed a maximum antinociceptive effect of 46.67% (log dose 1.875 mg/kg, i.p.). Morphine (used as a positive control) significantly reduced the licking time in capsaicin-induced nociception (Fig. 3). Thus, the results clearly reveal that extract *H. longipes* is able to produce antinociception in two different nociceptive tests. One of the compounds involved in this effect is affinin that showed part of this antinociceptive effect. Systemic pretreatment with naltrexone (1 mg/kg, s.c.), PCPA (80 mg/kg, i.p., once a day for four consecutive days) and flumazenil (5 mg/kg, s.c.) before injection of the vehicle did not modify the behavior generated by acid acetic injection. However, when naltrexone, PCPA or flumazenil was administered before affinin (30 mg/kg, i.p.), all of them were able to partially reverse the anti-
nociception caused by affinin when assessed on acid acetic-induced writhes in mice (Fig. 4).

Fig. 5 shows that pretreatment of animals with ODQ (1 mg/kg, s.c.) or glibenclamide (10 mg/kg, s.c.), given 15 min prior to affinin (30 mg/kg, i.p.), slightly modifies the antinociceptive effect caused by affinin (30 mg/kg, i.p.) when assessed against the acetic acid-induced nociception. Neither glibenclamide nor ODQ modify the behavior of vehicle administration (saline solution 0.9%).

Discussion

Animal pain models have proven to be useful both as instruments to teach us about the basic biology of pain in addition to having proven predictive value for drug discovery. Acetic acid and capsaicin test were used because the administration of these irritants provokes a very stereotyped behavior in mice that represent a nociceptive effect. In this way, the antinociceptive effect can be measured as reversal of nociception to “normal” levels.

The extracts of *H. longipes* and affinin were administered before algesic stimulus as preventive of behavior. Thus, the effect observed is characterized as prevention of nociception.

In the present experiments, systemic administration of the *H. longipes* extract and affinin, the main compound of the extract, produced significant antinociceptive effects against chemical nociception in mice induced by intraperitoneal acetic acid and subplantar capsaicin. To the best of our knowledge, this is the first report regarding the antinociceptive effect of affinin. Our results in the writhing test agree with previous observations in this model [13]. Therefore, our data confirm the antinociceptive effect of the...
H. longipes extract in acetic acid-induced writhing. The acetic acid-induced writhing test has been widely used for the evaluation of antinociceptive activity. Transmission of visceral nociceptive information in this model is related with the spinal cord, parabrachial nucleus and certain hypothalamic nuclei [14]. Thus, it is likely that the H. longipes extract and affinin may exert their effects at these sites. Moreover, our data extend these observations in the writhing test by showing that the extract as well as affinin are also effective in mice previously injected with capsaicin. The later effect suggests that extract and affinin may reduce the release of neuropeptides in both central and peripheral afferents otherwise transmitting nociceptive signals to the spinal cord and brain as there is evidence that capsaicin promotes neurogenic inflammation, plasma protein extravasation, neuropeptide release and vasodilatation [15,16]. Thus the data obtained in the capsaicin test suggest that the extract of H. longipes and affinin may produce antinociception suppressing neurogenic as well as the inflammatory nociception. In support of our data, it has been reported that the H. longipes extract is also able to reduce thermal hyperalgesia induced by carrageenan [8].

Our results showed that doses of the extract were lower than those of affinin to reduce nociception in both tests. This may be explained by the fact that the extract has a complex mixture of olefinic alkamides [6] that may synergize the antinociceptive effect. Of these, affinin was identified as the main alkamidine present in the plant [4–6].

In order to assess the possible mode of action of affinin, its antinociceptive effect was determined in mice pretreated with different antagonists using the acid-acetic induced nociception. Pretreatment with naltrexone, a nonselected opioid receptor antagonist, significantly reversed the affinin-induced antinociception suggesting that affinin activates opioid receptors and/or increases endogenous opioid system. Moreover, in the present study we were able to observe that pretreatment with PCPA significantly reversed affinin-antinociception in the writhing test. Since PCPA is an inhibitor of serotonin synthesis [17], our data suggest a role of the serotoninergic system in the antinociceptive effect of affinin. Likewise, flumazenil, a benzodiazepine site antagonist at the GABA_A receptor [18], blocked the antinociceptive effect of affinin. This result suggests that GABA_A receptors might participate in the antinociceptive effect of affinin. Accordingly, there is evidence that H. longipes increases GABA release in mice brain slices [9]. Taken together, these data suggest that affinin may activate three of the main mechanisms to produce antinociception. However, the way that affinin activates these mechanisms remains to be determined.

The antinociceptive effect is related to the elevated levels of cyclic GMP and NO activates guanylate cyclase and the subsequent production of cGMP. We confirm that cGMP is associated with affinin antinociception because pretreatment with ODQ, a soluble guanylyl cyclase inhibitor, decreased the antinociceptive effect. Thus, these results suggest the participation of the NO–cGMP pathway in this antinociceptive effect of affinin, as is the case for other NSAIDs [19–21]. On the other hand, NO can activate different types of K+ channels in different types of tissues by an increase in cGMP [22]. It has been reported that glibenclamide, an ATP-sensitive K+ channel blocker with no effect on Ca2+ or voltage dependent K+ channels [22–26], reduces the antinociceptive effects of the NO donor, sodium nitroprusside [27], suggesting a link between activation of the arginine–NO–cGMP pathway and potassium channel opening. Thus this work showed that ODQ and glibenclamide were able to decrease affinin-induced antinociceptive effects. In this way, the antinociceptive effect of NO may be explained by its interaction with guanylyl cyclase and the subsequent production of cGMP; this latter, in turn, would activate ATP-sensitive K+ channels. Hence, our data suggest that cyclic GMP and K+ channels partially participate in the antinociceptive effect of affinin as proposed for several drugs [28–30].

The information generated in this study indicates that H. longipes and one of its major metabolites, namely affinin, have antinociceptive effects in the writhing and capsaicin tests in mice. The precise mechanisms and sites by which affinin induces antinociception are currently under investigation, but an interaction with the opioidergic, GABAergic and serotoninergic systems as well as the cyclic GMP–K+ channel pathway has an important modulatory role in its antinociceptive action. Thus, H. longipes extract or its alkamides have antinociceptive effects in mice and rats. These results might support the popular use of the species in folk medicine for the treatment of painful complaints.

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