Antinociceptive, genotoxic and histopathological study of Heliopsis longipes S.F. Blake in mice


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Ethnopharmacological relevance: H. longipes S.F. Blake (Asteraceae) is a Mexican plant, whose roots are traditionally used as a condiment, as a mouth anesthetic, and as an antiparasitic. Affinin is the alkamide present in higher amounts in the roots of H. longipes.

Aim of the study: To date, there are no published studies regarding the relation between the analgesic properties, in vivo cytotoxicity, and DNA-damaging potential of H. longipes ethanol extract (HLEE).

Materials and methods: The HLEE was chromatographically fingerprinted to validate its affinin contents. Biological evaluation was conducted in sets of 6–8 CD1+ mice. Antinociceptive effect was evaluated using the writhing and hot-plate tests, and mutagenic and cytotoxic effects were evaluated with micronucleus test in CD1+ mice. For histopathological studies, biological samples from liver, heart, kidneys, spleen, lung, and brain were collected and stained.

Results: Oral administration of HLEE (3–100 mg/kg) produced a dose-dependent antinociceptive effect in both assays. In micronucleus assay, the variability in the number of micronucleated polychromatic erythrocytes (MNPE) induced, and PE/NE index, the ratio of polychromatic erythrocytes with respect to the number of normochromatic erythrocytes induced by HLEE in the evaluated schedule, were small and nonsignificant. After histopathological results, HLEE showed polioencephalomalacia with 1000 mg/kg dose.

Conclusions: This work provides evidence that HLEE exerts analgesic effects, with no genotoxic effects in vivo. These findings would be an important contribution to explain the use of H. longipes root as an effective analgesic in traditional medicine, and to establish for the first time the absence of genotoxic and cytotoxic effects of the root in bioactive doses in vivo.

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1. Introduction

Heliopsis longipes S.F. Blake (Asteraceae), locally known as “Chilcuague”, is a perennial herb endemic to Sierra de Álvarez and Sierra Gorda in Mexico (Molina-Torres et al., 1999; Cilia-López et al., 2008). This plant is traditionally used as a condiment, as a mouth anesthetic, and as an antiparasitic (Gutiérrez-Lugo et al., 1996). It is also used by indigenous and rural people of Central and South America for treating oral pain and ulcerative conditions (Correa et al., 1971; Colvard et al., 2006). Even though alkamides are characteristic secondary metabolites of families Asteraceae, Piperaceae, Solanaceae, and Rutaceae, as well as genus Capsicum (Greger, 1984), and are widely distributed among several species of these families, affinin is the main alkamide present in the five species of Heliantheae containing olefinic alkamides: Wedelia parviceps Blake, Acmella ciliata H.B.K., Acmella oleracea L., Acmella oppositifolia (Lam.) Jansen, and H. longipes S.F. Blake. This alkamide is present in higher amounts in the roots of H. longipes than in other species containing alkamides (Molina-Torres et al., 1996, 1999). Previous studies have shown that affinin has in vitro antimicrobial and fungistatic properties (Molina-Torres et al., 1999; García-Chávez et al., 2004), and that it exerts antinociceptive effects (Ogura et al., 1982). Moreover, it has been reported that a solution of dichloromethane-extract from H. longipes showed analgesic activity as determined by gamma-aminobutyric acid (GABA) release in mice brain slices.
(Ríos et al., 2007), and the interaction between H. longipes ethanolic extract (HLEE) and diclofenac on the thermal hyperalgesia test suggested that low doses of HLEE–diclofenac combination could interact synergistically at systemic level (Acosta-Madrid et al., 2009). Recently, topical anti-inflammatory effects of H. longipes, affinin and isobutyl-decanamide were confirmed (Hernández et al., 2009). Notwithstanding the above mentioned information, to date there are no published studies regarding the relation between analgesic effect and cytotoxicity of HLEE on CD1+ mice, using the micronucleus test.

The aim of this study was to examine the possible antinociceptive, mutagenic and cytotoxic effects of HLEE on CD1+ mice, using the writhing, hot-plate, histopathological and micronucleus tests.

2. Materials and methods

2.1. Reagents

Morphine (Laboratorios Pisa, Mexico City), acetylsalicylic acid (ASA) (Bayer, Mexico City, Mexico), and diclofenac (Sigma, St. Louis, MO, USA) were used as antinociceptive reference drugs. These analgesic drugs were administered per os, in a volume of 0.1 mL/10 g of mice body weight. To induce nociception, acetic acid (Merck) was used in 0.6% solution (dissolved in saline solution). Dauornubcin was acquired from Lemery Laboratories (Mexico City, Mexico), Daunorubicin was acquired from Lemery Laboratories (Mexico City, Mexico), and formaldehyde were purchased from J.T. Baker (Mexico City). Morphine (Laboratorios Pisa, Mexico City), acetylsalicylic acid (ASA) (Bayer, Mexico City, Mexico), and diclofenac (Sigma, St. Louis, MO, USA) were used as antinociceptive reference drugs. These analgesic drugs were administered per os, in a volume of 0.1 mL/10 g of mice body weight. To induce nociception, acetic acid (Merck) was used in 0.6% solution (dissolved in saline solution). Dauornubcin was acquired from Lemery Laboratories (Mexico City, Mexico), Daunorubicin was acquired from Lemery Laboratories (Mexico City, Mexico), and formaldehyde were purchased from J.T. Baker (Mexico City).

2.2. Extraction and isolation

H. longipes S.F. Blake (Asteraceae) specimens were collected on October 2008, on the rangy zone of Río Verde in San Luis Potosí, Mexico, at an altitude of 1795 m above sea level. After proper identification, the plant voucher specimens (H. longipes 41523) were deposited at the Herbarium of the University of San Luis Potosí. Air-dried roots of H. longipes (820 g) were extracted with EtOH (8 L) under reflux for 4 h. Filtration and evaporation of the extract afforded dark-yellow, viscous oil (70 g). Thirty-five grams of it was absorbed in 20 g of silica gel 60 and chromatographed over silica gel 60 (100 g) using a hexane–EtOAc gradient (1:0, 24:1, 23:2, 22:3, 13:2, 1:4, 19:6, 18:7, 17:8 and 1:1, v/v) and EtOAc as eluents. 500 mL fractions of each polarity were collected and monitored by TLC. Fraction 5 yielded 3.3 g of affinin (Fig. 1).

2.3. NMR and HPLC analysis

NMR measurements were performed at 400 MHz for 1H and 100 MHz for 13C on a JEOL Eclipse 400 spectrometer in CDCl3 solutions, using TMS as internal standard. Chromatography column was carried out on Merck silica gel 60 (Aldrich, 230–400 mesh ASTM). HPLC separations were carried out on a PerkinElmer series 200 chromatograph using a reversed-phase Microsorb 100 C18 column, i.d. 4.6 mm, length 250 mm, using a UV–vis detector at 256 nm and a flow of 1 mL/min. Fraction 5 yielded 3.3 g of affinin (Fig. 1) 1H NMR in CDCl3, δ 0.88 (6H, d; J = 6.8 Hz, CH3-3′, CH3-4′), 1.74 (3H, d; J = 6.56 Hz, CH3-10), 1.76 (1H, m; J = 6.6 Hz, H-2′), 2.24 (4H, dc; J = 18.3, 6.2 Hz, CH2-4, CH2-5), 3.1 (2H, t; J = 6.4 Hz, CH2-1′), 5.22 (1H, dt; J = 10.2, 6.9 Hz, H-6), 5.65 (1H, dc; J = 13.5, 6.6 Hz, H-9), 5.81 (1H, d; J = 15.4 Hz, H-2′), 5.93 (1H, t; J = 11 Hz, H-7), 6.25 (1H, br dd; J = 16, 12 Hz, H-8′, H-8), 6.78 (1H, dt; J = 15, 6.6 Hz, H-3). For HPLC quantification of affinin in the extract, the solvent–system consisted of: (A) H2O, and (B) MeOH (Jang et al., 2008). A gradient was set by A:B ratio: initially A:B ratio was 1:0, shifting to A:B = 0:1 in 20 min; then A:B was 0:1, shifting to A:B = 1:0 in 10 min, thus returning to initial conditions. Run-time was 30 min; retention time of affinin was 17.83 min (Fig. 2A). To plot the calibration curve, isolated and characterized affinin was dissolved in methanol and filtered through 0.45 μm membrane, to yield five concentrations: 0.8, 0.6, 0.4, 0.2, 0.1 mg/mL; injection volume was 20 μL. A correlation coefficient R = 0.995 was obtained. Crude extract was dissolved in MeOH, and filtered through 0.45 μm membrane; three concentrations were produced: 1.0, 4.0, 5.0 mg/mL (Fig. 2B); these samples were quantified against known amounts of reference affinin standard (with affinin concentrations of 0.105, 0.405, 0.503 mg/mL), and a 10% mean concentration of affinin was determined in the crude extract.

Fig. 1. Chromatograms of affinin in extract at 256 nm. (A) Affinin standard, 8 mg/mL chromatogram. (B) Crude extract, 4 mg/mL chromatogram.
2.4. Pharmacological experiments

2.4.1. Animals

All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983), and Mexican Official Norm for Animal Care and Handling (NOM, 1999). Efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male CD1 mice (body-weight range, 28–34 g) were obtained from the vivarium of Instituto de Ciencias de la Salud de la Universidad Autónoma del Estado de Hidalgo, Pachuca, Hidalgo, Mexico. Mice were housed under controlled temperature (23 ± 2 °C) with a 12 h light/dark cycle and humidity (55 ± 10%) and habituated to the environment for at least one week before experiments. Animals were provided with food and water ad libitum. Twelve hours before experiments, only food was withheld. Immediately after experiments, all animals were euthanized in a CO2 chamber.

2.4.2. Acute toxicity study in mice

Mice were treated with doses of 10, 100 and 1000 mg/kg of the crude extracts. Animals were kept under close observation over a 14 days period as described elsewhere (Lorke, 1983). Finally, the weight of the animals was monitored throughout the experiments, and death of the animals was registered. LD50 was determined as the geometric mean for which 0/3 and 3/3 deaths were found.

2.4.3. Analgesic activity

2.4.3.1. Acetic acid induced writhing test. Writhing activity was evaluated by the method of counting the number of stretches or writhe (Koster et al., 1995) (arching of the back, development of tension in abdominal muscles, elongation of the body, and extension of the forelimbs), counted through a period of 15 min, starting 5 min after the administration of 0.6% (v/v) acetic acid (10 mL/kg, i.p.). Animals were previously treated by per os administration with vehicle (TWEEN-80 plus 0.9% (w/v) NaCl), or different doses of HLEE (1–30 mg/kg), a half hour before stimulation with acetic acid. Immediately after acetic acid injection, each animal was placed in a transparent observation cage, and the number of writhes per mouse was counted by 20 min. ASA (200 mg/kg, p.o.) was used as standard drug for comparison. Each experimental group was formed by at least six animals. A significant reduction in the number of writhes by some treatment as compared to controls injected with vehicle was considered to be a positive analgesic response.

2.4.3.2. Hot-plate test. The conventional hot-plate apparatus (Ugo Basile, Italy) was used to measure nociceptive response (Woolfe and MacDonald, 1944; Eddy and Leimbach, 1953). Mice were placed into an acrylic cylinder on the heated surface (55.5 ± 0.2 °C), and time between placement of the mouse on the platform and shaking/licking of the hind paws or jumping was recorded as the response latency. Before beginning the experiments, the basal reaction time response of all animals was taken (mice with baseline latencies of more than 15 s were eliminated from the study). Mice received vehicle (0.2% TWEEN-80) or increasing doses of HLEE (3, 10, 30, and 100 mg/kg) by oral route 30 min before the thermal noxious stimuli in the hot-plate test. Diclofenac (20 mg/kg, p.o.) and morphine (10 mg/kg, p.o.) were used as negative and positive controls, respectively. Mice were observed before and at 15, 30, 45, 60, 75, 90, and 120 min after drug administration. Each experimental group was formed with at least six animals. A cut-off period of 30 s was employed; this exposition time was enough to observe the animal responses without provoking tissue damage.

2.4.4. Genotoxic assay

For the genotoxic study, animals were organized in six groups with five individuals each: a control group was administered with saline solution, four groups were administered with HLEE (3–100 mg/kg), and the last group was treated with 3 mg/kg of the cytotoxic drug daunorubicin. A blood sample from the tail of each mouse was obtained prior to the chemical administration, and smeared onto two ethanol-cleaned slides; cells were fixed for 3 min with methanol and stained for 15 min with 5% Giemsa solution diluted in phosphate buffer (pH 6.8). Then, slides were washed in tap water, dried and observed microscopically. A blood sample from the tail of each mouse was also obtained at 24, 48, 72 and 96 h post-administration, smeared on slides, fixed and stained as described above. The clastogenic and cytotoxic potential of HLEE was determined by scoring the number of micronucleated polychromatic erythrocytes (MNPE) in 1000 polychromatic erythrocytes (PE), as well as the ratio of polychromatic erythrocytes (PE) in 1000 erythrocytes per mouse, respectively (Schmid, 1975; Mac Gregor, 1990).

2.4.5. Histopathological studies

For the histopathological studies, biological samples from liver, heart, kidneys, spleen, lung, and brain from 10, 100 and 1000 mg/kg of HLEE, and control animals were collected and fixed in 10% aqueous phosphate buffered formalin by 24 h. Afterwards, they were processed by the paraffin inclusion method with an automated tissue processor, included in paraffin and 5 μm-thick microtome-sliced. Slides obtained were H&E stained (Prophe et al., 1995). These slides were observed by light microscope, and images were taken with a digital camera and saved in TIFF format.

2.4.6. Statistical analysis

All experimental results are provided as the mean ± S.E.M. for 6–8 animals per group. In the case of hot-plate results, the response latency was plotted as a function of time. The area under latency–time curves (AUC), as an expression of the effect duration, was calculated by the trapezoidal rule (Tallarida and Murray, 1986). One-way analysis of variance (ANOVA) followed by Tukey’s test was used to compare differences between treatments. Differences were considered to be statistically significant when P < 0.05.

3. Results

3.1. HPLC analysis

An HPLC profile at 256 nm for HLEE was obtained (Fig. 2B). At least six major components were identified, two of them being the most abundant; nevertheless, the main component in extract was affinin, accounting for about 10% of crude extract, as calculated by a calibration curve obtained from isolated and characterized affinin.

These results suggest that activity of crude extract is mainly due to affinin, with saline solution, four groups were administered with HLEE (3–100 mg/kg), and the last group was treated with 3 mg/kg of the cytotoxic drug daunorubicin. A blood sample from the tail of each mouse was obtained prior to the chemical administration, and smeared onto two ethanol-cleaned slides; cells were fixed for 3 min with methanol and stained for 15 min with 5% Giemsa solution diluted in phosphate buffer (pH 6.8). Then, slides were washed in tap water, dried and observed microscopically. A blood sample from the tail of each mouse was also obtained at 24, 48, 72 and 96 h post-administration, smeared on slides, fixed and stained as described above. The clastogenic and cytotoxic potential of HLEE was determined by scoring the number of micronucleated polychromatic erythrocytes (MNPE) in 1000 polychromatic erythrocytes (PE), as well as the ratio of polychromatic erythrocytes (PE) in 1000 erythrocytes per mouse, respectively (Schmid, 1975; Mac Gregor, 1990).

3.3. Analgesic effect of the HLEE

Oral administration of HLEE significantly (P < 0.05) reduced the acetic acid induced writhes. On the hot-plate assay, HLEE significantly (P < 0.05) increased latency to thermal stimuli (Fig. 3B), starting at 10 mg/kg dosage. Observed pharmacological action was lower than that of morphine (positive control).
Effect of administration of HLEE on the ratio polychromatic erythrocytes/normochromatic erythrocytes (PE/NE percent) in mice.

Table 2: PE/NE index as determined in this protocol is shown on Table 2. No significant modification by HLEE was observed on PE production; however, daunorubicin significantly reduced the index, starting at 48 and 96 h (P < 0.05).

3.5. Histopathological studies

Histopathological findings are referred to the brain. In this organ, necrotic changes at the grey substance, described as multifocal polioencephalomalacia and neurophagy, were observed at 1000 mg/kg dose of HLEE (Fig. 4). An analysis of the other organs did not demonstrate any damage or histopathological change (data not shown).

4. Discussion

The aim of this study was to examine the possible antinociceptive, mutagenic and cytotoxic effects of ethanolic extracts obtained from the root of H. longipes. The analgesic activity was assessed in mice using two well-accepted pain models, namely the writhing and hot-plate tests. Writhing test has been reported to be useful to investigate peripheral antinociceptive activity, while hot-plate test is valuable in detecting analgesic drugs acting on the central nervous system (Collier et al., 1968; LeBars et al., 2001). Results revealed that HLEE exhibited analgesic activity in mice on the writhing and hot-plate tests.

Acetic acid has been demonstrated to produce an acute peritoneal inflammation (Ness and Gebhart, 1990), and the induced nociceptive response may involve both direct stimulation of nociceptive afferent fibers due to the pH reduction and the synthesis of inflammatory mediators (Ribeiro et al., 2000). These results suggest that the mechanism of action of HLEE may be linked partly to the lipoxygenase and/or cyclooxygenase systems (Ness and Gebhart, 1990), and the induced nociceptive response may involve both direct stimulation of nociceptive afferent fibers due to the pH reduction and the synthesis of inflammatory mediators (Ribeiro et al., 2000). These results suggest that the mechanism of action of HLEE may be linked partly to the lipoxygenase and/or cyclooxygenase system, taking into account that acetic acid increases the levels of prostaglandins (PGE2 and PGF2α) in peritoneal fluid. Several alkaloids have been reported to be present in H. longipes. Affinin (N-isobutyl-2E,6Z,8E-decatenamidine) was identified as the main alkamide present in the plant (Molina-Torres et al., 1999; García-

Table 1: The indicated results correspond to the mean ± S.E.M. of 5 mice per dose. 1000 polychromatic erythrocytes scored per mouse. *Statistically significant difference with respect to the control value by ANOVA and Tukey’s tests, P < 0.05.

Table 2: The indicated results correspond to the mean ± S.E.M. of 5 mice per dose. 1000 erythrocytes per mouse scored to determine the PE/NE percent. *Statistically significant difference with respect to the control value by ANOVA and Tukey’s tests, P < 0.05.
Chávez et al., 2004; Molina-Torres et al., 2004). In this respect, it has been found that alkamides present in other species can inhibit the production of prostaglandin E2 in lipopolysaccharide-stimulated RAW264.7 mouse macrophage cells (LaLone et al., 2007).

HLEE also showed important antinociceptive effect on the hot-plate test. This assay shows activity in thermally sensitive afferent fibers, and activity of αβ and C fibers (Jinsmaa et al., 2004). The highest doses of HLEE (30 and 100 mg/kg) significantly increased (56.6–58.9%) the response latency after 15 min. Thus, it can be suggested that HLEE may show actions at spinal cord level. Supporting this hypothesis, GABA release-mediated analgesic activity of affinin has been described in mice brain slices (Ríos et al., 2007). Thus, the analgesic effect of HLEE may follow such mechanism at the spinal cord, or at higher central nervous system levels.

In mutagenic assays, daunorubicin is widely used as a topoisomerase inhibitor antineoplastic agent that produces reactive oxygen species, DNA damage, and apoptosis (Mizutani et al., 2005). It has been reported that daunorubicin induces severe chromosome damage by measuring sister chromatid exchanges (SCE) and micronuclei (MN). For this reason, it is used as a positive control in experimental studies (Bommu et al., 2008; Paniagua-Pérez et al., 2009). In contrast, HLEE produced neither genotoxic nor cytotoxic damage in the present study. In regard to genotoxic studies in mammalian cells, no previous studies on the HLEE potential have been conducted.

Alkamides are present in some species of Asteraceae, especially in H. longipes, Echinacea purpurea, Acmella radicans and Acmella millefolium, as well as in some species of Solanaceae and Piperaceae (Hegnauer, 1977; Molina-Torres and García, 2001). Alkamides present in Piper nigrum, which are isomeric compounds to the affinin, have in fact been found to possess anti-mutagenic against aflatoxin B1 (Singh et al., 1994; Reen et al., 1997), and anti-tumor effects on in vivo models (Srinivasan, 2007). These congruent results suggest the relevance of chemical structure to the biological effect of alkamides, and also indicate the importance of using various test models in order to reach a valid conclusion.

In relation to histopathological findings, results suggest that a 1000 mg/kg dose is enough for inducing brain damage, clinically observed as head and forelimb tremors. This concept correlates with previous findings of encephalomalacia and neuropathy secondary to brain trauma in humans and in surgical treatment for patients with intractable epilepsy (Handel et al., 2007; Tellez-Zenteno et al., 2009). This dose is higher than the dose usually ingested as condiment, analgesic and anti-inflammatory in dental and oral pathologies in humans (Molina-Torres and García, 2001; Colvard et al., 2006). Furthermore, the other organs did not exhibit any damage or histopathological change.

In conclusion, this work provides evidence that, when systemically administered, HLEE had antinociceptive effects on acute thermal and acute inflammation-induced nociception in mice. The antinociceptive potency of HLEE was similar for both acute thermal-induced nociception and chemical-induced nociception. This suggests that HLEE may be a good candidate for the treatment of pain without risk of genotoxic and/or cytotoxic damage.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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