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Original Article

Heliopsis longipes: anti-arthritic activity evaluated in a Freund's adjuvant-induced model in rodents

Q1 Carolina Escobedo-Martínez[a,*], Silvia Laura Guzmán-Gutiérrez[b], María de los Milagros Hernández-Méndez[a], Julia Cassani[c], Alfonso Trujillo-Valdivia[a], Luis Manuel Orozco-Castellanos[a], Raúl G. Enríquez[d]

[a] *Departamento de Farmacia, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Guanajuato, Mexico*
[b] *Catedrática CONACyT, Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, DF, Mexico*
[c] *Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana Unidad Xochimilco, México, DF, Mexico*
[d] *Instituto de Química, Universidad Nacional Autónoma de México, México, DF, Mexico*

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ABSTRACT

This study assesses the anti-arthritic effect of the affinin-enriched (spilanthol, main alkamide) hexane extract from the roots of *Heliopsis longipes* (A. Gray) S.F. Blake, Asteraceae, on a Freund adjuvant-induced arthritis model in rodents. The extract was orally administered at a dose of 2, 6.6, or 20 mg/kg; a significant edema-inhibitory activity in the acute and chronic phases was observed with a dose of 2 and 20 mg/kg, respectively. The extract showed higher anti-inflammatory and anti-arthritic effects than the reference drug phenylbutazone (80 mg/kg). Moreover, the extract prevented the occurrence of secondary lesions associated to this pharmacological model.

Introduction

Rheumatoid arthritis (RA) is an idiopathic auto-immune disease, characterized by symmetrical synovitis in large and small joints that may lead to progressive articular damage and disability (Mendoza-Vázquez et al., 2013). Analgesic and anti-inflammatory drugs, including steroids, are used to suppress the symptoms. New therapies, like those targeting the tumor necrosis factor alpha (infliximab) or the anti-CD20 therapy (rituximab), which inhibit the underlying immune process, have been proposed. However, all these drugs have several undesired effects. Recent research aims to discover long-acting anti-inflammatory drugs with minimal side effects (Ekambaram et al., 2010). A number of animal models are used to evaluate potentially useful compounds against RA, and the choice of a particular model depends on the immunological properties being observed in the model and their relation with the human disease. Among the available models is collagen-induced or adjuvant-induced (*e.g.*, Freund's adjuvant) arthritis in rodents, spontaneous arthritis induced by TNF-α or genetic models, like the transgenic animal model. While no RA model can be considered ideal, Freund's adjuvant-induced rat arthritis shares several traits with human arthritis, and its sensitivity to evaluate anti-arthritic agents was the basis for its choice as the model to assess the activity of the *Heliopsis longipes* (A. Gray) S.F. Blake, Asteraceae, hexane extract (Ekambaram et al., 2010; Tanushree and Saikat, 2013).

RA prevalence in developed countries is 0.5–2%, with an annual incidence of 12–1200 per 100,000 inhabitants. The female:male ratio is 2–3:1, and the peak age range of onset is 30–55 years old, but it could occur at any age. In Mexico, RA affects 1.6% of general population, being the main reason for consultation at the Rheumatology Service (Mendoza-Vázquez et al., 2013; Guía de Práctica Clínica, Diagnóstico y Tratamiento de Artritis Reumatoide del Adulto, México: Secretaría de Salud, 2009).

H. longipes is a perennial herb, endemic in a region comprising parts of Sierra de Alvarez and Sierra Gorda, near the triple border of San Luis Potosi, Guanajuato and Queretaro, in Central Mexico. The common names for this plant are chilcuague, pelitre, golden root, and Aztec root (Cilia-López et al., 2008). The paralyzing and toxic action against flies and other insects of *H. longipes* root extracts was discovered as similar as that of pyrethrins during World War II (Acree et al., 1945).

Fortunately, chilcuague did not become extinct after the exportation boom, even though its wild population was severely reduced. To date, land plots solely dedicated to the cultivation of this species,

* Corresponding author.
E-mail: c.escobedo@ugto.mx (C. Escobedo-Martínez).

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disseminated either by seed or by cutting, can be found between Guanajuato and Queretaro; the root is fully developed after 2–3 years (García-Chávez et al., 2004). It is the most economically important species in its genus; its root is used in the region as a cooking condiment, and it is added to alcoholic beverages to improve taste. In traditional medicine, chilcuague root is used to treat respiratory diseases, as an anesthetic to treat tooth and muscular aches, to treat buccal lesions, and as an anti-parasitic (Cilia López, 2007). Several alkamides have been isolated from *H. longipes*, but affinin, also known as spilanthol ((2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide), was identified as the main alkamide in the plant (Molina-Torres et al., 1996; García-Chávez et al., 2004; Hernández et al., 2009). The oily substance causes intense lip numbness and tingling within a few minutes after placed in the mouth, and it also stimulates salivation (Correa et al., 1971). *H. longipes* is also reported to produce analgesia and anti-inflammation in human dental and oral pathologies (Colvard et al., 2006). Additionally, it has been reported that *H. longipes* dichloromethane extract showed analgesic activity, as demonstrated *in vitro* by releasing of gamma-aminobutyric acid (GABA) in mouse brain slices; the increase in GABA release in a region of cerebral cortex leads to sustained analgesia, favoring a descendent inhibition of nociceptive spinal neurons (Ríos et al., 2007).

With regard to anti-inflammatory activity, Hernández et al. (2009) evaluated *H. longipes* ethanolic extract and purified affinin on mouse ear edema induced by either arachidonic acid (AA) or phorbol 12-myristate 13-acetate (PMA), demonstrating a significant anti-inflammatory effect. DE_{50} values of 0.8 mg/ear and 1.2 mg/ear were obtained, respectively, in the AA-induced edema model; nimesulide (1 mg/ear) was used as reference drug. In the PMA-induced edema model, the ethanolic extract and affinin showed a dose-dependent anti-inflammatory effect with DE_{50} values of 2 mg/ear and 1.3 mg/ear, respectively, using indometacin (3 mg/ear) as reference drug.

On the other hand, Cilia-López et al. (2010) evaluated the analgesic activity of *H. longipes* ethanolic extract and affinin and their effect on the central nervous system on a mouse model; both affinin (1 mg/kg) and *H. longipes* root ethanolic extract (10 mg/kg) showed an analgesic action similar to ketorolac (6 mg/kg), as assessed by pain induction with acetic acid and thermal stimulation (hot plate); also, *H. longipes* extract and affinin showed a stimulant effect on the central nervous system comparable to caffeine, as measured by the Irwin test. Cariño-Cortés et al. (2010) evaluated the cytotoxic potential of *H. longipes* ethanolic extract by recording the variability in the count of micronucleated polychromatic erythrocytes induced by the extract and the ratio of polychromatic erythrocytes with respect to normochromatic erythrocytes; no significant cytotoxic effect was observed. However, brain histopathological studies showed necrotic changes in the gray matter, described as polioencephalomalacia and neurophagy, at a dose of 1000 mg/kg of ethanolic extract. No damage or histopathological change was observed in other organs (liver, heart, kidney, spleen, and lung).

Among the recent studies on *H. longipes* is the assessment of ethanolic extract and affinin as potential anti-mutagenic and anti-carcinogenic agents. The anti-mutagenic properties of affinin were evaluated by adding it to several mutagens, either with or without S9 metabolic activation, in *Salmonella typhimurium* (TA98, TA100, and TA102 strains) cultures. Affinin significantly decreased the point mutations induced by 2-aminoanthracene (2AA) (40%) and decreased the DNA oxidative damage induced by norfloxacin (NOR) (37–50%). Additionally, it showed antioxidant properties capable of reducing the rate of 2AA- and NOR-induced mutation in *S. typhimurium* TA98 and TA102, respectively, which could be relevant to treat some pain symptoms related to anti-radical activity (Arriga-Alba et al., 2013).

Materials and methods

Plant material

H. longipes (A. Gray). S.F. Blake, Asteraceae, roots were collected on February 16, 2014, 2:14:59 pm, within the boundaries of a land plot in Beltran community (N 21°16.427′, W 100°0.2672′) at 1780 m above sea level, Xichu municipality, in Guanajuato, Mexico. After proper identification, five plant specimens were deposited in the Isidro Palacios herbarium (SLPM), at the Universidad Autónoma de San Luis (No. 048150).

Extraction

Dry, ground *H. longipes* roots (1285 g) were macerated for three consecutive times: First component extraction was performed with 2.5 l of hexane for 2 h; then, the recovered extract solution (1750 ml) was evaporated, yielding a dark yellow, viscous oil (26.386 g, labeled as Hl-A). A second maceration was performed on the same plant material after the first extraction, adding 1.250 l of hexane and left for 24 h; extract solution (1 l) was recovered, and after evaporation it yielded 12.29 g of viscous oil, labeled as Hl-B. A third and last maceration was performed as described above, using 1.5 l of hexane for 4 h, recovering an extract solution (900 ml) and yielding 3.469 g of viscous oil, labeled as Hl-C. Each maceration was monitored by thin-layer chromatography. The Hl-A sample yielded 26.386 g of affinin (**1**), with a 95% purity.

HPLC and NMR analysis

High performance liquid chromatography (HPLC) analysis of the Hl-A sample was performed in a Waters 1525 Binary HPLC pump equipment, controlled by Breeze software. A 15 cm × 4.6 mm, C18 Kromasil 100-5 column was used, coupled to a diode array detector (maximum absorbance was set to 230 nm). A water-acetonitrile gradient from 60:40 to 30:70 in 25 min was used for elution, at a flow rate of 1 ml/min (Fig. 1).

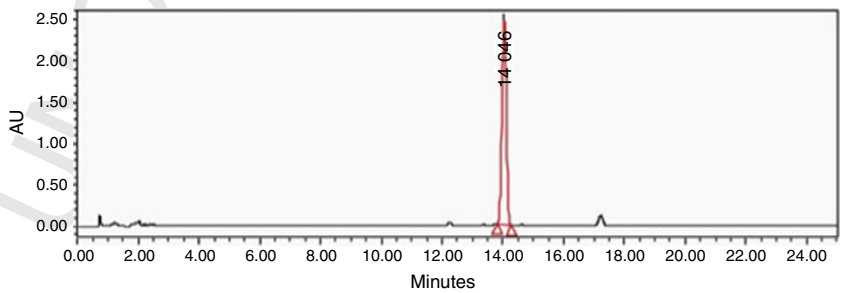

Fig. 1. Chromatogram of the Hl-A sample affinin (**1**), injected at a concentration of 1 mg/ml and read at a wavelength of 230 nm.

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Table 1
^{1}H NMR spectral data of Hl-A component (affinin) (600 MHz).[a]

Proton[a]	Chemical shift	Multiplicity	Area	Coupling constant (Hz)
CH_3-3′ and CH_3-4′	0.92	d	6	J = 6.8
CH_3-10	1.77	d	3	J = 7.2
H-2′	1.80	m	1	J = 6.0
CH_2-4 and CH_2-5	2.24, 2.32	dc	4	J = 18.0, 6.0
CH_2-1′	3.13	t	2	J = 6.9
H-6	5.26	dt	1	J = 12.0, 6.0
H-9	5.69	dc	1	J = 13.7, 6.7
H-2	5.86	d	1	J = 15.0
H-7	5.96	t	1	J = 11.0
H-8	6.28	dd broad signal	1	J = 18.0, 12.0
H-3	6.82	dt	1	J = 15.2, 6.7
N-H	6.0	Broad signal	1	

[a] Data recorded in $CDCl_3$. Chemical shifts (δ) are expressed in ppm with respect to tetramethylsilane (TMS). Coupling patterns are labeled as: d, doublet; dd, doublet of doublets; dt, doublet of triplets; t, triplet; dc, doublet of quartets; m, multiplet. All assignments were based on the following experiments: ^{1}H-1D, ^{13}C-1D, COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence; coupling of ^{1}H—^{13}C to a bond), and HMBC (heteronuclear multiple bond correlation; coupling of ^{1}H—^{13}C to 2 and 3 bonds).

Magnetic nuclear resonance (NMR) measurements for the Hl-A sample were acquired in an Agilent 600 MHz, DD2, Oneprobe equipment for ^{1}H y ^{13}C in $CDCl_3$ solution, using tetramethylsilane as internal standard (Tables 1 and 2). Chemical shift readings were compared with those reported in the literature (Correa et al., 1971).

Pharmacological assay

Animals

All experiments were performed in accordance with ethical standards for experimental pain research in animals (Zimmermann, 1983) and the Mexican Official Standard for animal care and handling (NOM-062-ZOO-1999). The experimental protocol (CIBIUG-P01-2016) was approved and overseen by the Institutional Ethics Committee for Care and Use of Laboratory Animals of the Universidad de Guanajuato. Inbred Wistar rats (250–300 g) and Balb/c mice (20–25 g), obtained from the Natural and Exact Science Vivarium at Universidad de Guanajuato, were used. Animals were housed in groups of six, under controlled temperature (23 ± 2 °C) and humidity (55 ± 10%), with a 12-h light/darkness cycle, and were familiarized with the environment one week before the experiments. Animals were allowed food and water *ad libitum*. Immediately after the experiments, all animals were sacrificed in a CO_2 chamber.

Table 2
^{13}C NMR spectral data of Hl-A component (affinin) (125.7 MHz).[a]

Carbon[a]	Chemical shift
C-10	18.30
C-3′ and C-4′	20.16
C-5	26.42
C-2′	28.58
C-4	32.12
C-1′	46.91
C-6	124.26
C-8	126.70
C-7	127.63
C-2	129.43
C-3	129.89
C-9	143.36
C-1	166.17

[a] Data recorded in $CDCl_3$. Chemical shifts (δ) are expressed in ppm with respect to tetramethylsilane (TMS). All assignments were based on the following experiments: ^{1}H-1D, ^{13}C-1D, COSY, HSQC, and HMBC.

Acute toxicity assays

The DL_{50} value *per os* was determined as reported by Lorke (1983). Mice were treated with Hl-A doses of 10, 100, or 1000 mg/kg. Animals were maintained under close observation over a 14-day period. The weight of the animals was monitored throughout the experiments, and the death or survival of the animals was registered.

Anti-inflammatory activity

Arthritis syndrome was induced by a plantar intradermic injection (through a 20-gauge needle) in the right hind paw of 0.05 ml of complete Freund's adjuvant (Sigma Aldrich). This method, reported by Newbould (1963), has been widely used to detect potential anti-inflammatory drugs with anti-arthritic activity. Among the several adjuvant types available, the most commonly used in experimental animals is Freund's adjuvant; two varieties of Freund's adjuvant are used: Incomplete Freund Adjuvant (IFA) and Complete Freund Adjuvant (CFA). IFA consists in a mixture of surfactant and mineral oil, usually Arlacel and Drakeol; CFA also contains killed mycobacteria, usually *Mycobacterium bovis* BCG at a concentration of 1 mg/ml or less (Rojas-Espinosa, 2006).

Edema degree was evaluated by the volume displacement method, using a PAN LAB digital plethysmometer. Volume displacement was recorded 24 h before and 8, 24, 48, 72, and 96 h after CFA injection. To complete the assessment of activity on arthritis induction, volume variations in the hind paw were recorded up to 25 days after injection. Phenylbutazone (reference drug, Sigma Aldrich, 80 mg/kg) or the Hl-A sample (in a dose of 2, 6.6, or 20 mg/kg) were administered *p.o.* 24 h before CFA injection and daily for 14 days after. The severity and progression of secondary lesions in test animals were also compared.

The percentage of edema inhibition was calculated for all animals in each treatment group with respect to a vehicle-treated control group. Differences between control and treatment groups were analyzed by Tukey's test. A p-value lower than 0.05 was regarded as statistically significant.

Results

Acute toxicity assays

The DL_{50} value *p.o.* for the Hl-A sample was 775 mg/kg.

Anti-inflammatory activity

Progression of Freund's adjuvant-induced arthritis in rats

CFA injection in the right hind paw caused inflammation, which peaked within the first 8 h. Then, inflammation slowly decreased until day 6 post-injection, had a slight increase after day 7, and showed a sustained increase until the end of the experiment. At day 16 post-CFA-injection and until the end of the period of study, inflamed lesions (secondary lesions) in the left hind paw were detected, as well as deformed front paws and increasing thickness in ears and tail (Fig. 2).

Dosing

Each tested concentration of the Hl-A sample and of phenylbutazone was administered daily for 15 days. The first dose was administered 1 day before CFA injection in the right hind paw pad (Newbould, 1963). Frequent measurements in the hind paws with a plethysmometer showed that the treatment with phenylbutazone and with the tested Hl-A concentrations (2, 6.6, or 20 mg/kg) inhibited inflammation in the hind paw during the dosing period.

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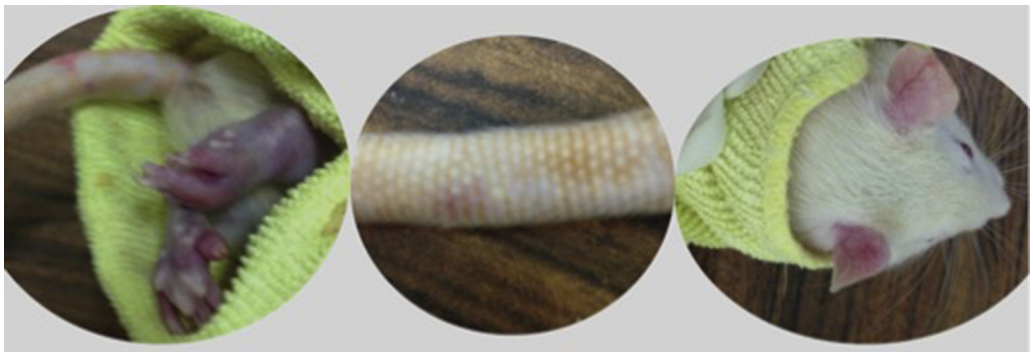

Fig. 2. Secondary lesions observed on the day 22 after plantar injection of complete Freund's adjuvant in a rat from the control, untreated group.

Table 3
Effect of Hl-A on Freund's adjuvant-induced edema in rats.

Time (h)	Sample	Dose (mg/kg)	% Inhibition ± SEM
8	Phenylbutazone	80	46.5 ± 6.8*
8	Hl-A	2	18.9 ± 7.5
8	Hl-A	6.6	14.5 ± 8.1
8	Hl-A	20	17.5 ± 8.6
24	Phenylbutazone	80	47.8 ± 5.6*
24	Hl-A	2	42.3 ± 4.2*
24	Hl-A	6.6	29.7 ± 11.3
24	Hl-A	20	31.7 ± 10.3

As shown in Fig. 4, prophylactic treatment with the reference drug (phenylbutazone, 80 mg/kg) significantly inhibited the inflammatory effect of CFA injection 8 h after the stimulus, during the acute inflammation phase. This was observed again 24 h after injection; additionally, administration *p.o.* of Hl-A at a dose of 2 mg/kg inhibited the progression of acute-phase edema by 42.3% ($F_{4,20} = 4.7$) (Table 3, Fig. 3).

During inflammation chronic phase, significant values of inhibition percentage after administration *p.o.* of Hl-A at a dose of 20 mg/kg were observed only on days 19, 20, 23, and 25 after CFA injection, with inhibition percentages of 31.1 ($F_{4,20} = 5.012$), 36.8 ($F_{4,20} = 3.17$), 34.2 ($F_{4,20} = 3.56$), and 31.3 ($F_{4,20} = 4.30$), respectively (Table 4 and Fig. 4). On the other hand, Hl-A administration at the same dose showed a higher inhibition percentage than phenylbutazone in all chronic-phase days. Secondary lesions did not occur during the treatment period at all Hl-A doses (Fig. 5), and rats from Hl-A-treated groups showed a better joint mobility.

No significant difference was observed in corporal weight between phenylbutazone-treated nor Hl-A-treated rat groups at any dose with respect to the control, untreated group.

Discussion

A number of animal models have been proposed to evaluate anti-inflammatory activity, like the use of substances such as formalin of dextran, which cause acute inflammation when injected in rat hind paws. However, these models require administering high doses of anti-inflammatory drugs like phenylbutazone, often near toxic values, to significantly inhibit inflammation (Domenjoz, 1960). Another method is the subcutaneous implant of cotton

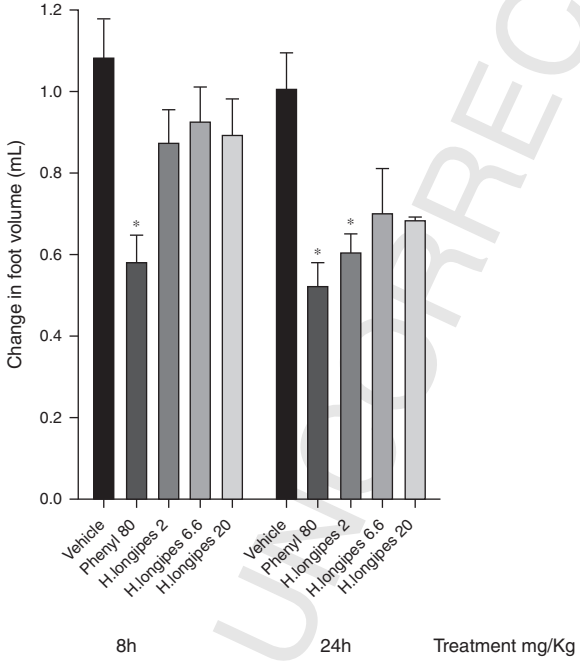

Fig. 3. Acute effect of administration (*p.o.*) of phenylbutazone (80 mg/kg) or *Heliopsis longipes* Hl-A sample (2, 6.6, and 20 mg/kg) on Freund's adjuvant-induced inflammation in the hind paw of the rodent model (rat). Each bar shows the mean of 5 lectures ± SEM. Data were analyzed by ANOVA followed by Tukey's test. $p < 0.05$.

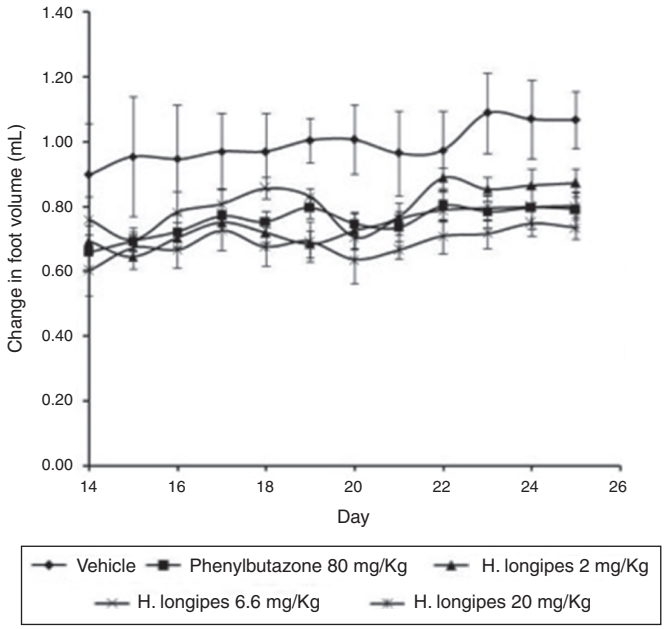

Fig. 4. Effect administration (*p.o.*) of phenylbutazone (80 mg/kg) or *Heliopsis longipes* Hl-A sample (2, 6.6, and 20 mg/kg) on the long-term, Freund's adjuvant-induced inflammation phase in the hind paw of the rodent model (rat). Each point in time-course shows the mean of five lectures ± SEM. Data were analyzed by ANOVA followed by Tukey's test. $p < 0.05$.

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Table 4
Effect of Hl-A on Freund's adjuvant-induced edema in rats.

Time (days)	Sample	Dose (mg/kg)	% Inhibition ± SEM
16	Phenylbutazone	80	23.8 ± 3.0
16	Hl-A	2	25.8 ± 3.0
16	Hl-A	6.6	17.9 ± 6.8
16	Hl-A	20	29.6 ± 5.9
17	Phenylbutazone	80	20.5 ± 0.8
17	Hl-A	2	15.8 ± 2.7
17	Hl-A	6.6	16.8 ± 4.8
17	Hl-A	20	25.2 ± 6.2
18	Phenylbutazone	80	22.4 ± 3.4
18	Hl-A	2	26.0 ± 2.9
18	Hl-A	6.6	13.5 ± 3.5
18	Hl-A	20	30.3 ± 5.3
19	Phenylbutazone	80	20.6 ± 3.2*
19	Hl-A	2	31.9 ± 4.0*
19	Hl-A	6.6	17.39 ± 2.5
19	Hl-A	20	31.1 ± 6.3*
20	Phenylbutazone	80	25.8 ± 3.2
20	Hl-A	2	27.8 ± 5.5
20	Hl-A	6.6	29.9 ± 3.9
20	Hl-A	20	36.8 ± 7.2*
21	Phenylbutazone	80	23.8 ± 4.4
21	Hl-A	2	20.5 ± 2.1
21	Hl-A	6.6	21.0 ± 5.1
21	Hl-A	20	30.9 ± 2.7
22	Phenylbutazone	80	27.41 ± 4.5
22	Hl-A	2	8.6 ± 3.1
22	Hl-A	6.6	19.0 ± 3.4
22	Hl-A	20	27.2 ± 5.4
23	Phenylbutazone	80	27.9 ± 4.6
23	Hl-A	2	21.5 ± 3.4
23	Hl-A	6.6	26.9 ± 3.6
23	Hl-A	20	34.2 ± 4.1*
24	Phenylbutazone	80	25.5 ± 6.1
24	Hl-A	2	19.2 ± 4.9
24	Hl-A	6.6	25.3 ± 4.9
24	Hl-A	20	30.1 ± 3.7
25	Phenylbutazone	80	26.0 ± 5.7*
25	Hl-A	2	18.3 ± 3.8
25	Hl-A	6.6	25.0 ± 3.8
25	Hl-A	20	31.3 ± 3.2*

pellets, a model that is more sensitive to steroid anti-inflammatory drugs but is relatively insensitive to non-steroid anti-inflammatory drugs like phenylbutazone (Winter et a.l, 1963). The method used in this study is based on a syndrome more similar to rheumatoid arthritis, which is capable of detecting anti-inflammatory activity in a wide range of drug types useful to treat rheumatoid arthritis in humans (Pearson et al., 1961).

Phenylbutazone, previously used in other models, was a suitable reference drug to inhibit inflammation caused by CFA injection in rat hind paw; no weight-loss was observed as reported with other drugs (Newbould, 1963). In our study, phenylbutazone inhibited CFA-induced inflammation by 46.5% 8 h after injection (Table 3), a very similar result to that reported by Abad et al. (1996), who recorded an inhibition rate of 40% 24 h after stimulus, increasing to over 70% at 72 h and decreasing again to 40% at 96 h, remaining constant until the day 15. In our study, the control drug was discontinued on the chronic phase, but inhibition rate remained in a range of 23–27%, indicating a long-term effect. On the other hand, an inhibition range of 27.2–36.8% was observed in the Hl-A-treated group (20 mg/kg), higher than that of the phenylbutazone-treated animals.

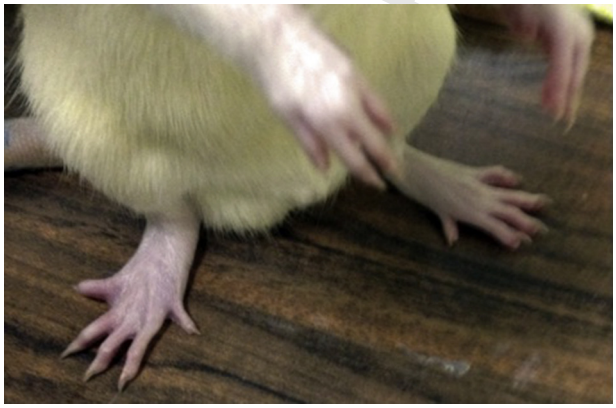

Fig. 5. Status of the right hind paw of a subject rat on day 22 after prophylactic treatment with Hl-A (*p.o.*) at a dose of 20 mg/kg.

The process underlying the occurrence of secondary lesions in the model herein used is non-infectious, and it is suggested to be result of a generalized immune response to the components of tubercle bacilli, disseminated after local administration (Warsman and Sharp, 1960). Neither phenylbutazone-treated nor Hl-A-treated rats at any dose (2, 6.6, or 20 mg/kg) showed secondary lesions, in contrast with control, untreated animals (Fig. 6); this indicates that treatments modify the immune response.

On the other hand, the highest extract dose (20 mg/kg) was set to much lower value than the DL_{50} value herein reported (775 mg/kg, *p.o.*), which allows us to regard it as a low-toxicity substance (Lorke, 1983). Extract accumulation due to a daily administration for 15 days failed to cause any toxicity sign in the period of study. Therefore, herein we provide information supporting the potential anti-inflammatory usefulness of *H. longipes* hexane extract (Hl-A), which has affinin as its main constituent as shown by HPLC and NMR analysis. The potential usefulness as anti-inflammatory agent of the extract at a low dose (2 mg/kg) was demonstrated in the acute-phase inflammation of the model, while its anti-inflammatory and anti-arthritic activity at higher but secure doses (20 mg/kg) was demonstrated in the chronic-phase inflammation of the model, with no occurrence of secondary lesions.

Conclusions

The hexane extract of *H. longipes*, of which affinin is the main constituent, has potential usefulness as an anti-arthritic agent. This study contributes to provide information supporting the traditional use of this plant species. It is noteworthy that the available information about this species would suffice to create a monograph and suggest the production of a phytopharmaceutical, which would help encouraging a standardized, high-quality cultivation of this plant species and improve the economy of peasants in Sierra Gorda, Mexico.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CEM designed the study, supervised the laboratory and drafted the paper. SLGG analyzed data and contributed to draft the paper.

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MMHM evaluated the Anti-arthritic activity, running the laboratory work. JC contributed to record NMR spectra. ATV and LMOC contributed to critical reading of the manuscript. RGE contributed to the chromatographic analysis of plant material and the isolation and identification of affinin. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Uncited references

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Pearson and Wood (1963) and Winder et al. (1962).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.09.003.

References

Abad, M.J., Bermejo, P., Carretero, E., Martínez-Acitores, C., Noruega, B., Villar, A., 1996. Antiinflamatory activity of some medicinal plant extracts from Venezuela. J. Ethnopharmacol. 55, 63–68.
Acree, F., Jacobson, M., Haller, H.L., 1945. An amide possessing insecticidal properties from the roots of Erigeron affinis DC. J. Org. Chem. 10, 236–242.
Arriga-Alba, M., Ríos, Y.M., Déciga-Campos, M., 2013. Antimutagenic properties of affinin isolated from *Heliopsis longipes* extract. Pharm. Biol. 51, 1035–1039.
Cariño-Cortés, R., Gayosso-De-Lucio, J.A., Ortíz, N.I., Sánchez-Gutiérrez, M., García-Reyna, P.B., Cilia-López, V.G., Pérez-Hernández, N., Moreno, E., Ponce-Monter, H., 2010. Antinociceptive, genotoxic and histopatological study of *Heliopsis longipes* S.F. Blake in mice. J. Ethnoparmacol. 130, 216–221.
Cilia López, V.G., (Tesis de doctorado) 2007. Biología y utilización del chilcuague (*Heliopsis longipes* S.F. Blake). Universidad Autónoma de San Luis Potosí, San Luis Potosí, S.L.P. México, pp. 1–104.
Cilia-López, V.G., Aguirre-Rivera, J.R., Reyes-Agüero, J.A., Juárez-Flores, B.I., 2008. Etnobotánicas de *Heliopsis longipes* (asteraceae: Heliantheae). Bol. Soc. Bot. México 83, 81–87.
Cilia-López, V.G., Juárez-Flores, B.I., Aguirre-Rivera, J.R., Reyes-Agüero, J.A., 2010. Analgesic activity of *Heliopsis longipes* and its effect on the nervous system. Pharm. Biol. 48, 195–200.
Colvard, M.D., Cordel, G.A., Villalobos, R., Sancho, G., Perkowits, K.M., Michel, J., 2006. Survery of medical ethnobotanicals for dental and oral medicine conditions and pathologies. J. Ethnopharmacol. 107, 134–142.
Correa, J., Roquet, S., Díaz, E., 1971. Multiple NMR analysis of the affinin. Org. Magn. Reson. 3, 1–5.
Domenjoz, R., 1960. The pharmacology of phenylbutazone analogues. Ann. N.Y. Acad. Sci. 86, 263–291.
Ekambaram, S., Selvan Perumal, S., Subramanian, V., 2010. Evaluation of antiarthritic activity of *Strychnos potatorum* Linn seeds in Freund's adjuvant induced arthritic rat model. BMC Complement. Altern. Med. 10, http://dx.doi.org/10.1186/1472-6882-10-56.
García-Chávez, A., Ramírez Chávez, E., Molina-Torres, J., 2004. El género Heliopsis (Heliantheae: Asteraceae) en México y las alcamidas presentess en sus raíces. Acta Bot. Mex. 69, 115–131.
Guía de Práctica Clínica, 2009. Diagnóstico y Tratamiento de Artritis Reumatoide del Adulto, México: Secretaria de Salud. CENETEC-IMSS-195-08 México, DF.
Hernández, I., Márquez, L., Martínez, I., Dieguez, R., Delporte, C., Prieto, S., Molina-Torres, J., Garrido, G., 2009. Anti-inflammatory effects of ethanolic extract and alkamides-derived from *Heliopsis longipes* roots. J. Ethnopharmacol. 124, 649–652.
Mendoza-Vázquez, G., Rocha-Muñoz, A.D., Guerra-Soto, A.J., Ramírez-Villafaña, M., González-Sánchez, A.G., Gámez-Nava, J.I., Nava, A., 2013. Artritis reumatoide y dislipidemias. El Residente 8, 12–22.
Molina-Torres, J., Salgado Garciglia, R., Ramírez-Chávez, Del Río, R.E., 1996. Purely olefinic alkamides in *Heliopsis longipes* and Acmelia (Spilanthes) oppositifolia. Biochem. Syst. Ecol. 24, 43–47.
Newbould, B.B., 1963. Chemotherapy of arthritis induced in rats by micobacterial adyuvant. Br. J. Pharmacol. 21, 127–136.
Lorke, D., 1983. A new approach to practical acute toxicity testing. Arch. Toxicol. 54, 275–287.
Pearson, C.M., Waksman, B.H., Sharp, J.T., 1961. Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. V. Changes affecting the skin and mucous membranes. Comparison of the experimental process with human disease. J. Exp. Med. 113, 485–509.
Pearson, C.M., Wood, F.D., 1963. Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. VII. Pathologic details of the arthritis and spondylitis. Amer. J. Path 42, 73–95.
Ríos, M.Y., Aguilar-Guadarrama, B., Gutiérrez, M.C., 2007. Analgesic activity of affinin, an alkamide from *Heliopsis longipes* (Compositae). J. Ethnopharmacol 110, 364–367.
Rojas-Espinosa, O., 2006. Inmunología. Ed. Panamericana México, DF., pp. 100–101.
Tanushree, R., Saikat, G., 2013. Animal models of rheumatoid arthritis: correlation and usefulness with human rheumatoid arthritis. IAJPR 3, 6131–61342.
Warsman, B.H., Sharp, J.T., 1960. Immunologic nature of arthritis induced in rats by mycobacterial adjuvant. Fed. Proc. 19, 210.
Winder, C.V., Wax, J., Scotti, L., Sherrer, R.A., Jones, E.M., Short, F.W., 1962. Antiinflammatory, antipyretic and antinociceptive properties of N-(2,3-xylyl) anthranilic acid (mefenamic acid). J. Pharmacol. exp. Ther. 138, 405–413.
Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animal. Pain 16, 109–110.