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Anti-inflammatory effect of an ethanolic root extract of Heliopsis longipes in vitro

[Efecto anti-inflamatorio *in vitro* del extracto etanólico de la raíz de *Heliopsis longipes*]

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Abstract

Heliopsis longipes (A. Gray) Blake (Asteraceae) is a species broadly used in Mexican Traditional Medicine. The present study illustrates the effects of the root ethanolic extract from this species on the production of tumor necrosis factor alpha (TNFα) and nitric oxide (NO) by activated RAW264.7 macrophage. The extract showed an inhibitory activity on TNFα (IC_{50}= 223.0 μg/mL) and NO (IC_{50} = 136.9 μg/mL). These results represent a contribution to the elucidation of the mechanism involved in the analgesic and anti-inflammatory effects reported for the *H. longipes* extract.

Keywords: *Heliopsis longipes;* inflammation; tumor necrosis factor alpha; nitric oxide; macrophages.

Resumen

Heliopsis longipes (A. Gray) Blake (Asteraceae) es una especie ampliamente utilizada en la Medicina Tradicional Mexicana. El presente estudio ilustra los efectos del extracto etanólico de la raíz de esta especie sobre la producción del factor de necrosis tumoral alfa (TNFα) y el óxido nítrico (NO) en macrófagos RAW264.7 activados. El extracto mostró una actividad inhibitoria sobre TNFα (IC_{50}= 223.0 μg/mL) y NO (IC_{50} = 136.9 μg/mL). Estos resultados representan una contribución a la elucidación del mecanismo involucrado en los efectos analgésico y anti-inflamatorio publicado para el extracto de *H. longipes*.

Palabras Clave: *Heliopsis longipes;* inflamación; factor de necrosis tumoral alfa; óxido nítrico; macrófagos.

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INTRODUCTION

Heliopsis longipes (A. Gray) Blake (Asteraceae) is a species broadly used in Mexican Traditional Medicine for the treatment of dental pain (Gutierrez-Lugo et al., 1996) and is also used by indigenous and rural peoples of Central and South America for its analgesic, anti-inflammatory and anti-ulcerative properties (Colvard et al., 2006). Studies carried out with extracts obtained of this plant have demonstrated its capacity to inhibit the constrictions induced by intraperitoneal administration of acetic acid in a murine model of pain (Ogura et al., 1982).

It is relevant to note the presence of affinin in the extract obtained from *H. longipes* roots (Molina-Torres and García-Chávez, 2001). Also, the alkamides has been studied *in vitro* for their inhibitory action on the enzymes cyclooxygenase and 5-lipoxygenase (Müller-Jakic, 1994).

Taking in to account these reports, we have proposed to determine the anti-inflammatory effects of the ethanolic extract of of *H. longipes* roots. In this paper, we have evaluated the *in vitro* effects on tumor necrosis factor (TNFα) and nitric oxide (NO) production by activated macrophages.

MATERIALS AND METHODS

Plant Material

H. longipes (Gray) Blake (Asteraceae) specimens, as authenticated by Dr J. Rzedowski, Instituto de Ecología, Pátzcuaro, Michoacan, Mexico, were collected in Sierra Gorda in the state of Guanajuato, Mexico at an altitude of between 2000 and 2500 meters above sea level. Voucher specimens *(H. longipes* JMT, IED) were deposited at the above mentioned institution.

Preparation of the extracts

Dry roots were ground and extracted with absolute ethanol in a continuous extraction system (Tecator, Soxtec System HT 1043 Extraction Unit) for 2 h at 80°C. The ethanolic extract was then freed from solvent in a rotary evaporator at 60 °C under reduced pressure (Molina-Torres et al., 1999). Dry extract was maintain at –20 ^{o}C until its use when it was dissolved in DMEM for *in vitro* pharmacological studies.

Chemical characterization of the extract

The chemical characterization of this ethanolic extract was reported by Molina-Torres et al. (1996; 2004). It contains affinin, as the major alkamide, along with *N*-isobutyl-2*E*-decenamide, and *N*-isobutyl-decanamide. Interestingly, sesquiterpene lactones - bioactive secondary metabolites commonly present in Asteraceae- were not found in the extract.

Reagents

Dexamethasone, lipopolysaccharide (LPS, from *Escherichia coli* Serotype: 055:B5), recombinant murine gamma interferon (IFNγ), tumor necrosis factor (recombinant murine TNFα, specific activity: 10^7 U/mg), actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO, USA). N$^{\omega}$-monomethyl-L-arginine (L-NMMA) was from Cayman Chemical, Ann Arbor, MI.

Cell lines

Dulbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Pisley, UK) and RPMI 1640 (Sigma Chemical Co. St. Louis, MO, USA) were supplemented with 10% FBS, 1% de L-glutamine and 0.5% penicillin-streptomycin solution.

The murine macrophage RAW264.7 cell line and murine fibrosarcoma L929 cell line were cultured in DMEM medium and RPMI 1640, respectively, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assays

The *H. longipes* extract was not cytotoxic (≤ 500 μg/mL) according to previous studies of cytotoxicity. This was assessed by dimethyl-diphenyl-tetrazolium (MTT) incorporation for each experimental condition. The viability was consistently > 97% (Delgado et al., 1998). Dexamethasone and L-NMMA were used as positive control for TNFα and NO inhibition, respectively. Tests were repeated in at least three independent experiments and the assays were performed in triplicate.

Pro-inflammatory challenge (RAW264.7 activation)

RAW264.7 cells were washed twice with phosphate-buffered saline (PBS) and incubated with trypsin-EDTA, without calcium or magnesium, for 3 min at 37 ^{o}C to detach the cells from the culture flask. Cells were resuspended in DMEM and incubated in

24-well tissue-culture plates at a concentration of 10^5 cells/mL for 24 h in a humidified incubator at 37 °C, with 5% CO_2. Growth medium was removed and cell monolayers were stimulated with 10 ng/mL LPS and 2 U/mL recombinant murine IFNγ. To test the effects of the *H. longipes* extract, concentrations (1-200 μg/mL) were dissolved in DMEM medium and added to wells 10 min before treatment with LPS + IFNγ. Cell-free supernatants were harvested after 1 h incubation and kept at -70 °C until use to assay TNFα and NO_2 levels.

Cytokines (TNFα) determination

TNFα production by macrophages was determined by the L929 cell lysing assay as described Gomez-Flores et al. (1997) in supernatants of cell cultures in the presence of actinomycin D 1 μg/mL. Recombinant TNFα was used as standard (specific activity, 10^7 U/mg).

Nitrite determination

NO is rapidly oxidized to nitrite in culture medium, and nitrite (NO_2^-) concentration is an indicator of NO production. Cell-free culture supernatants were mixed with equal amounts of Griess reagent (1% sulfanilamide, 0.1% naphtylethylenediamide in 2.5% phosphoric acid) in 96-well ELISA plates. Samples were incubated at room temperature for 10 min and the absorbance was measured at 540 nm with the use of a microplate reader. Nitrite concentrations were calculated using a sodium nitrite standard curve (Jun et al., 1994).

Statistical analysis

Effects of *H. longipes* extract on TNFα and NO production represent the means ± SEM of three determinations. Analysis of variance followed by Dunnet's test for specific comparisons were performed. Probability values less than 0.05 ($p<0.05$) were considered significant. Regression analysis was used to calculate the effective inhibitory concentration 50 (IC_{50}), defined as the concentration necessary to produce a 50% of inhibition on TNFα and NO on *in vitro* endotoxic shock.

RESULTS AND DISCUSSION

Few studies have demonstrated the pharmacological activity of ethanolic extracts of *H. longipes*. In this paper, when macrophages RAW 264.7 were stimulated with LPS and IFNγ, the production of TNFα and NO was triggered. However, Fig. 1 shows the inhibition of TNFα production by the extract in this cell line with an IC_{50} of 223.0 μg/mL. This activity is very important for the anti-inflammatory actions of this extract. The action is in correspondence with the effects of other natural inhibitors on this cytokine in the inflammatory process (Manthey et al., 1999). The NO production (equivalent to levels of NO_2) was also reduced when stimulated cells were pre-incubated with the *H. longipes* extract (IC_{50}= 136.9 μg/mL) as shown in Fig. 2.

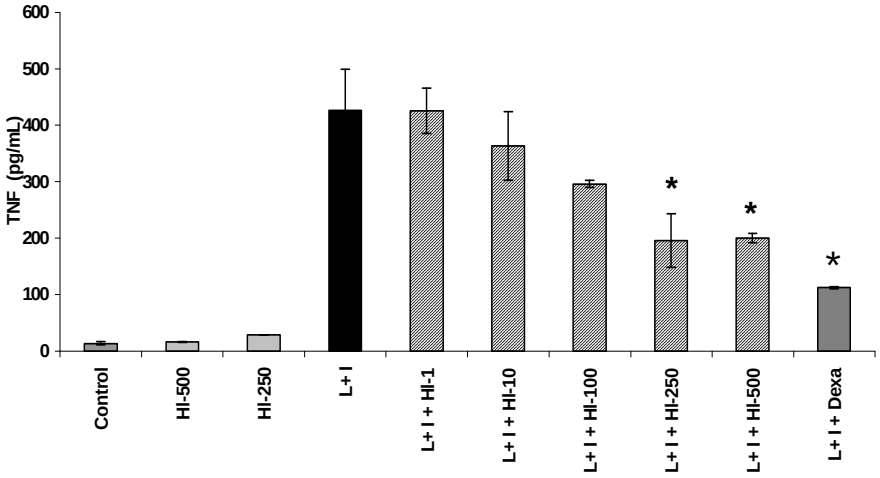
The inhibitory effects of the *H. longipes* ethanolic root extract may be explained, at least in part, by the presence of alkamides (mainly affinin) in the root extract. This family of chemical compounds has been studied as cytokine and NO inhibitors in *in vitro* models of activated macrophages (Murakami et al., 2000). Experiments using extracts from *Echinacea angustifolia* and alkylamide derivatives have reported anti-inflammatory (Tragni et al., 1985; Tubaro et al., 1987; Raso et al., 2002) or immunosuppression (Matthias et al., 2007) activities including the inhibition of TNFα and NO in macrophages.

Macrophages are important cells of the immune defense system because they participate in the processing and presentation of antigens to T cells, the phagocytosis of foreign particles, and killing of microorganisms. During these process, high levels of NO and reactive oxygen intermediates are generated contributing to intracellular destructive mechanisms, and can be activated some metabolic pathway to production of cytokines such as TNFα and IL-1β, eicosanoids like prostaglandins, leukotrienes, and other mediators of inflammatory response (Macmicking et al., 1997).

On the other hand, activated macrophages produce mediators of cytotoxicity (such as NO and TNFα), which protect the host against the infections development. One important mediator of the inflammatory process is NO of which excessive or inappropriate production can lead to tissue damage through peroxynitrites formation in a reaction that takes place between NO and the superoxide anions. It is of interest to note that the inhibitory action observed in macrophages activated with LPS and IFNγ, is directly related to the effect on the induction of the enzyme inducible NO synthase in these cells (Paul-Clark et al., 2001).

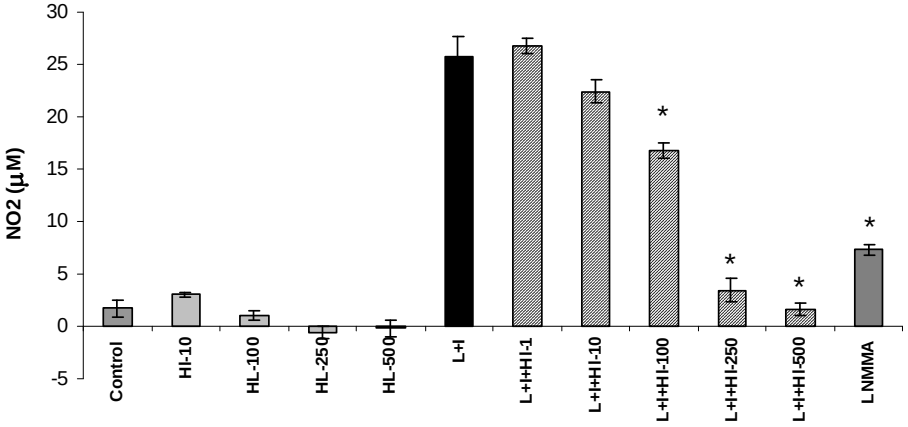
However, TNFα is one the pro-inflammatory cytokines that are primarily released by activated monocytes and macrophages (Vilcek and Lee, 1991). The results shown in this study would also explain at

Figure 1. Effect of an ethanolic root extract of *H. longipes* on tumor necrosis factor (TNFα) production in activated murine macrophages.



RAW264.7 cells (10^5 cells/mL) were activated with LPS (L, 10 ng/mL) and IFNγ (I, 2 U/mL) and treated with *H. longipes* (Hl) 1, 10, 100, 250, 500 μg/mL. Dexamethasone (DEXA, 1mM) was used as the reference drug. Each group represents the mean ± S.E.M of three independent experiments. *p< 0.05 statistical significance compared with the group treated only with LPS plus IFNγ.

Figure 2. Effect of an ethanolic root extract of *H. longipes* on nitric oxide (NO) production in activated murine macrophages.



RAW264.7 cells (10^5 cells/mL) were activated with LPS (L, 10 ng/mL) and IFNγ (I, 2 U/mL) and treated with *H. longipes* (Hl) 1, 10, 100, 250, 500 μg/mL. L-NMMA 1 mM was used as the reference drug. Each group represents the mean ± S.E.M of three independent experiments. *p< 0.05 statistical significance compared with the group treated only with LPS plus IFNγ.

least partially, the analgesic effect reported for *H. longipes,* which inhibits the abdominal writhing induced by the intraperitoneal administration of acetic acid in mice (Ogura et al., 1982) where these pro-inflammatory mediators play an important role.

CONCLUSION

The ethanolic extract of *H. longipes* shows anti-nociceptive and anti-inflammatory activities *in vitro*. We report that here they may be mediated, at least in part, by the inhibition of pro-inflammatory cytokines (such as TNFα) and free radicals (such as NO). These results represent the first contribution towards the full elucidation of the pharmacological activities exhibited by *H. longipes* extracts, which are rich in alkamides.

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