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ScienceDirect
Phytomedicine 16 (2009) 336–341
Phytomedicine
www.elsevier.de/phymed

Interaction between *Heliopsis longipes* extract and diclofenac on the thermal hyperalgesia test

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Abstract

Heliopsis longipes is an herbaceous plant found in Mexico, used traditionally for its analgesic and anesthetic activities. Plant extracts in combined use with synthetic drugs may represent a therapeutic advantage for the clinical treatment of pain, allowing the use of lower doses, and limiting side-effects. Therefore, the main objective of this study was to determine the possible pharmacological interaction between *Heliopsis longipes* ethanolic extract (HLEE) and diclofenac in the Hargreaves model of thermal hyperalgesia in the mouse. HLEE, diclofenac or fixed-dose ratio HLEE–diclofenac combinations were administered systemically to mice and the antihyperalgesic effect was evaluated using the thermal hyperalgesia test. All treatments produced a dose-dependent antihyperalgesic effect. ED_{30} values were estimated for all the treatments and an isobologram was constructed. The derived theoretical ED_{30} value for the HLEE–diclofenac combination was 54.4 ± 9.4 mg/kg body wt, significantly higher than the actually observed experimental ED_{30} value, 8.6 ± 4.0 mg/kg body wt. This result corresponds to synergistic interaction between HLEE and diclofenac in the Hargreaves model of thermal hyperalgesia. Data suggest that low doses of the HLEE–diclofenac combination can interact synergistically at the systemic level and that this association may therefore represent a therapeutic advantage for the clinical treatment of inflammatory pain.

Keywords: *Heliopsis longipes*; Diclofenac; Synergism; Thermal hyperalgesia; Mouse

Introduction

Heliopsis is a genus of herbaceous flowering plants that belongs to the Asteraceae family. It includes several species, most of them endemic to Mexico (García-Chávez et al. 2004). One of the species, *Heliopsis longipes*, is an herbaceous plant found in Mexico in the states of Guanajuato, San Luis Potosí and Queretaro (García-Chávez et al. 2004). *H. longipes* was identified over 50 years ago as having possible commercial value as a source of insecticide (Little 1948), and several literature reports on its activities exist. Several alkamides have been reported from *H. longipes*. Affinin

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doi:10.1016/j.phymed.2008.12.014

(N-isobutyl-2E,6Z,8E-decatrienamide) was identified as the main alkamide present in the plant (Molina-Torres et al. 1999, 2004; García-Chávez et al. 2004), and the antimicrobial and fungistatic properties of affinin, N-isobutyl-2E-decenamide and N-isobutyl-decanamide from *H. longipes* have been reported (Molina-Torres et al. 1999, 2004). Chewing of a piece of *H. longipes* root creates an intense numbness and tingling sensation in the lips, tongue and mouth and stimulates salivation (Correa et al. 1971; Fabricant and Farnsworth 2001); it has also been reported that *Heliopsis longipes* produces analgesia and anti-inflammation in dental and oral pathologies in humans (Correa et al. 1971; Colvard et al. 2006). An *H. longipes* extract and a pure compound of *Heliopsis longipes* also showed an antinociceptive effect in the acetic acid-induced writhing test in mice (Ogura et al. 1982). More recently, it has been reported that a solution of dichloromethane extract from *H. longipes* showed analgesic activity as determined by gamma-amino butyric acid (GABA) release in mice brain slices (Rios et al. 2007).

Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, are among the most widely used medications in the world. NSAIDs provide effective management of pain and inflammation, but a major factor limiting their use is gastrointestinal damage (Wolfe et al. 1999; Fiorucci et al. 2001). Therefore, physicians are eager to find a pain treatment with fewer side-effects for patients. For this reason, it is important to continue investigations of herbal medicine that show a profile of suitable analgesic activity with a good index of security. Hence, the purpose of the present study was to characterize the antihyperalgesic effect of the systemic administration of the *Heliopsis longipes* ethanolic extract (HLEE)–diclofenac combination in the Hargreaves model of thermal hyperalgesia.

Materials and methods

Animals

Balb/c male mice (weight range, 20–28 g each) from our own breeding facilities were used in this study. Efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were used only once. Animals had free access to food and drinking water before the experiments. At the end of the experiments, mice were sacrificed in a CO_2 chamber. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann 1983) and the protocol was approved by the Institutional Animal Care and Use Committee (CINVESTAV, IPN, México, D.F., Mexico).

Drugs and *Heliopsis longipes* ethanolic extract

Diclofenac was purchased from Sigma (St. Louis, MO, USA). Carrageenan (Type IV, Lambda) was purchased from Research Biochemical International (Natick, MA, USA). *Heliopsis longipes* (Gray) Blake (Asteraceae) specimens, as authenticated by PhD Jose García Pérez from the Herbarium of the University San Luis Potosí (SLP), were collected in the mountain zone of Río Verde in the state of SLP, Mexico, at an altitude of 1795 m above sea level. Voucher specimens (*H. Longipes* 41523) were deposited at the above mentioned institution. 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 extracts were filtered through Whatman paper no. 4 and the ethanolic extract was freed from solvent in a rotary evaporator (Büchi model R 3000) at 60 °C under reduced pressure. Diclofenac, HLEE and carrageenan were dissolved in 0.9% saline solution.

Evaluation of thermal antihyperalgesia

Antihyperalgesia was assessed using the Hargreaves model of thermal hyperalgesia (Hargreaves et al. 1988). A plantar test (Ugo Basile apparatus) was used to measure the withdrawal latencies of the hind paws from a radiant heat stimulus. Mice were manually restrained and no pre-experiment habituation to the test environment was carried out (Ortiz et al. 2007). The thermal nociceptive stimulus originated from a high-intensity projector lamp bulb (infra-red intensity: 217 mW/cm^2) was manipulated manually and positioned under each footpad before and after the intraplantar injection of saline into the left hind paw or carrageenan (25 µl; 2%) into the right hind paw. A timer was automatically actuated with the light source, and the paw withdrawal latencies (PWLs) measured was defined as the time required for the paw to show an abrupt withdrawal. A cut-off time of 25 s was used to prevent tissue damage. Measurements of PWLs were made before and 1, 2, 3, 4, 5 and 6 h after saline or carrageenan injection.

Study design

In order to assess the antihyperalgesic effect, 30 min before the carrageenan injection, animals were pre-treated with oral (p.o.) and intraperitoneal (i.p.) administrations of vehicles or increasing doses of HLEE (10–300 mg/kg body wt, p.o.), diclofenac (1–30 mg/kg body wt, i.p.) or the diclofenac (i.p.)–HLEE (p.o.) (6.8–108.7 mg/kg body wt) combinations. The injection volumes were 100 µl. Mice in all groups were observed

for behavioral or motor function changes induced by the treatments. This was assessed, but not quantified, by testing the animals' ability to stand and walk in a normal posture. All observations were carried out by a blinded investigator.

Characterization of the interaction between diclofenac and HLEE

Results are presented as mean ± S.E.M. for 6–12 animals per group. Time courses of antihyperalgesic response of individual drugs and the combination were constructed by plotting the PWLs as a function of time. The areas under the PWLs against time curves (AUC) were calculated by the trapezoidal rule. AUC was calculated and percent of antihyperalgesia was calculated. The dose–response curves were constructed and the experimental points fitted using least-squares linear regression. ED_{30} value ± standard error (S.E.M.) were calculated according to Tallarida (2000).

In the present study, we used isobolographic analysis to determine the nature of drug interaction between diclofenac and HLEE (Berenbaum 1989). Isobolographic analysis assumes that the combination of drugs is made from equipotent doses of the individual drugs (Berenbaum 1989). Thus, from the dose–response curves of each individual agent, the dose resulting in 50% of the effect (ED_{50} value) can be determined. However, considering a maximal effect of 100% as the total suppression of thermal hyperalgesia, it appeared that HLEE was unable to achieve a 50% response, and thus the calculation of the ED_{50} value was not feasible. Therefore, we estimated a minor effective dose (ED_{30} value) instead of the ED_{50} value (Tallarida 1992; Jiménez-Andrade et al. 2003; Ortiz et al. 2007). Subsequently, a dose–response curve was obtained by concurrent delivery of diclofenac and HLEE in a fixed ratio mixture (1:1) based on the ED_{30} values of each individual agent. To construct this curve, group of animals received one of the following doses of the combination: diclofenac ED_{30} value + HLEE ED_{30} value; (diclofenac ED_{30} value + HLEE ED_{30} value)/2; (diclofenac ED_{30} value + HLEE ED_{30} value)/4; (diclofenac ED_{30} value + HLEE ED_{30} value)/8; (diclofenac ED_{30} value + HLEE ED_{30} value)/16. The experimental ED_{30} value for the combination was calculated from this curve.

The theoretical additive ED_{30} value was estimated from the dose–response curves of each drug administered individually, i.e., considering that the observed effect with the combination is the outcome of the sum of the effects of each of the individual drug. This theoretical ED_{30} value was then compared with the experimentally derived ED_{30} to determine if there is a statistically significant difference (Tallarida 2002; Tallarida et al. 1999). The theoretical and experimental ED_{30} values of the studied combination were also contrasted by calculating the interaction index (γ) as it follows: $\gamma = ED_{30}$ value of combination (experimental)/ED_{30} value of combination (theoretical) (Berenbaum 1989).

An interaction index not significantly different from unity corresponds to an additive interaction whereas values higher and lower than unity imply an antagonistic and synergistic interaction, respectively (Berenbaum 1989; Tallarida 2002; Jiménez-Andrade et al. 2003).

Statistical analysis

Dose–response data were analyzed by one-way analysis of variance (ANOVA) using Dunnet's test for post hoc comparison. Statistical significance between the theoretical additive ED_{30} value and the experimentally derived ED_{30} values was evaluated using Student's *t*-test (Tallarida 2000). An experimental ED_{30} value significantly lower than the theoretical additive ED_{30} value was considered to indicate a synergistic interaction between diclofenac and HLEE. Statistical significance was considered to be achieved when $p < 0.05$.

Results

Effect of diclofenac, HLEE and their combination

Intraplantar carrageenan (25 µl, 2%) into the right hind paw, but not saline in the contralateral paw, produced a time-dependent thermal hyperalgesia (Fig. 1). Administration of diclofenac, HLEE or

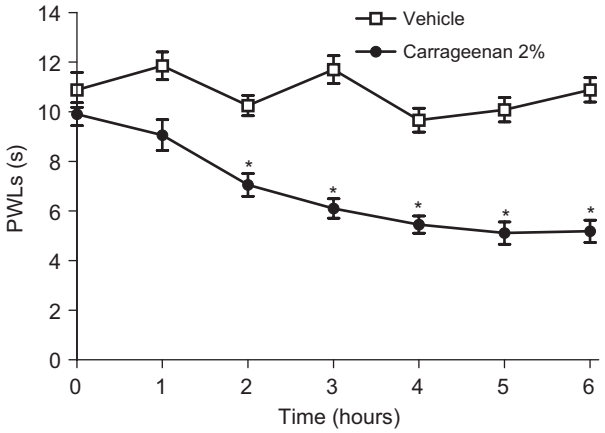

Fig. 1. Time course of paw withdrawal latencies (PWLs) induced by exposure to radiant heat in mice injected with saline into the left hind paw (contralateral) or carrageenan (2%, 25 µl) into the right hind paw (ipsilateral). Data are the means ± S.E.M. for 6–12 animals. *Significantly different from saline ($p < 0.05$), as determined by analysis of variance followed by Dunnett's *t*-test.

diclofenac–HLEE combination, but not vehicles, produced a reduction in the hyperalgesic effect induced by carrageenan ($p<0.05$, Fig. 2). None of the assayed treatments produced a significant alteration of ambulation or motor activity.

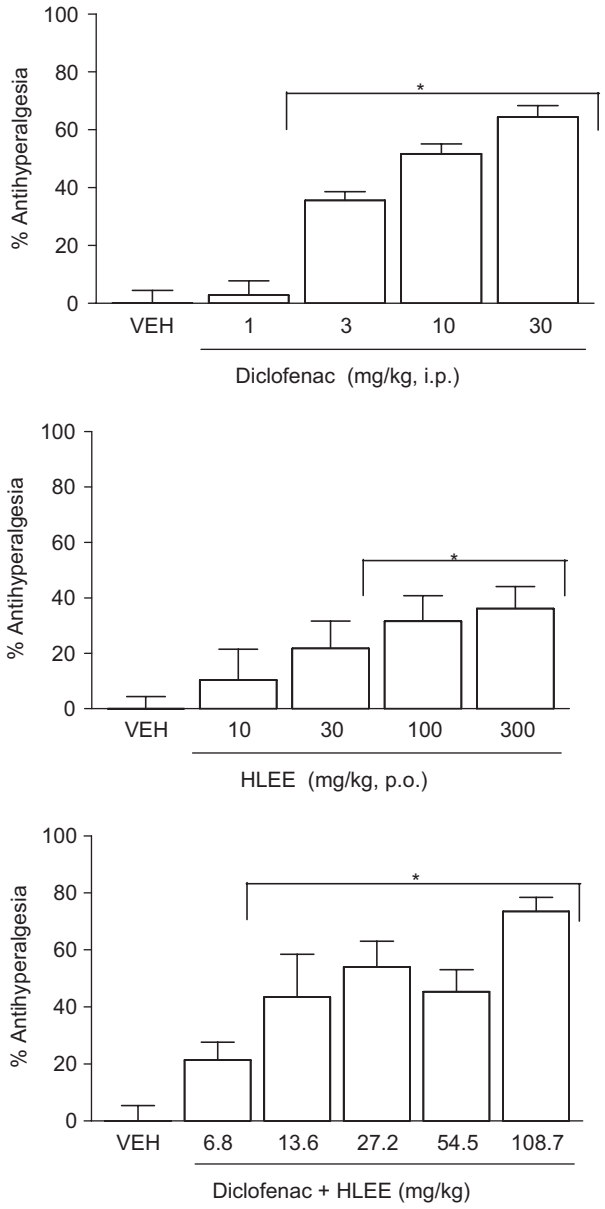

Fig. 2. Effect of the systemic administration of diclofenac (1–30 mg/kg body wt, i.p.), *Heliopsis longipes* ethanolic extract (HLEE; 10–300 mg/kg body wt, p.o.) or the diclofenac–HLEE combination (6.8–108.7 mg/kg body wt) in carrageenan-induced thermal hyperalgesia. Mice were pretreated with vehicle, diclofenac, HLEE or diclofenac–HLEE combination 30 min before carrageenan injection. Data are expressed as the percentage of antihyperalgesia. Bars are the means ± S.E.M. for 6–12 animals. *Significantly different from vehicle ($p<0.05$), as determined by analysis of variance followed by Dunnett's *t*-test.

Synergistic interaction between diclofenac and HLEE

The ED_{30} values for diclofenac and HLEE on the thermal hyperalgesia test were 3.4 ± 0.8. and 105.3 ± 18.7 mg/kg body wt, respectively. Fixed-dose ratio combinations were prepared, as described above, and assayed for construct the dose–response curve for the combination and calculate the corresponding experimental ED_{30} value, which was 8.6 ± 4.0 mg/kg body wt for diclofenac–HLEE combination. This value was significantly lower ($p<0.05$) than the theoretical ED_{30} value expected for a purely additive interaction, which was 54.4 ± 9.4 mg/kg body wt for the diclofenac–HLEE combination (as can be clearly seen in Fig. 3, where the experimental ED_{30} value appears below the additive dose line). Furthermore, the interaction index (γ) was 0.2 ± 0.08 for diclofenac–HLEE combination, being statistically different from unity. Data thus strongly suggest that the interaction between the antihyperalgesic actions of diclofenac and HLEE at systemic level are synergistic.

Discussion

Many plant products are being evaluated to ascertain the actuality of their purported anti-inflammatory and

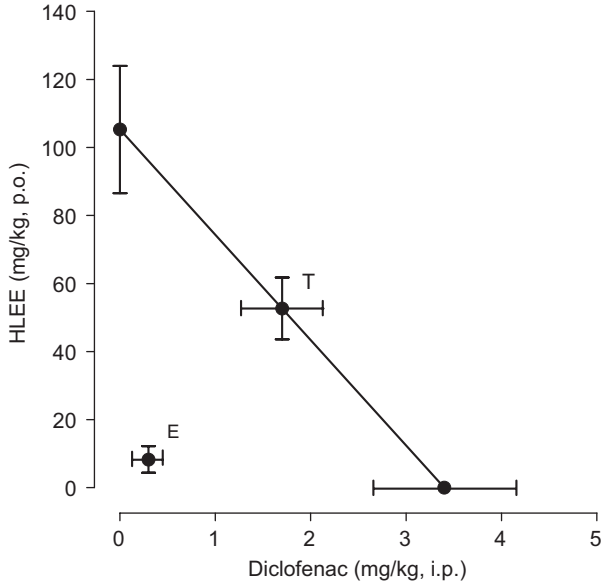

Fig. 3. Isobologram showing the systemic interaction between diclofenac and *Heliopsis longipes* ethanolic extract (HLEE) on the Hargreaves model of thermal hyperalgesia. The oblique line between the *x* and *y* axes is the theoretical additive line. The point in the middle of this line, indicated by "T", is the theoretical additive point calculated from the individual drug ED_{30} values. The experimental point indicated by "E" is the actually observed ED_{30} value with the combination. Horizontal and vertical bars indicate S.E.M.

analgesic effects. In traditional systems of medicine, the Mexican plant *Heliopsis longipes* (commonly known as *chilguague*) is used as an oral anti-inflammatory and dental analgesic (Colvard et al. 2006). Rios et al. (2007) reported that the dichloromethane extract from *H. longipes* was able to release GABA in mice temporal cortex slices. In the same work, *H. longipes* extract was divided in six fractions. Several components of the active fractions were identified and assayed in mice cortex slices. Affinin (N-isobutyl-2E,6Z,8E-decatrienamide) was the main active compound, and evoked the GABA release at 1×10^{-4} M concentration (Rios et al. 2007). The authors suggested a possible analgesic activity of *H. longipes* extract and affinin based on the neural mechanisms underlying the cortical modulation of pain by GABA (Millan 2002). However, *H. longipes* extract was not evaluated *in vivo*. Therefore, it was necessary to demonstrate its possible analgesic activity in an animal model of pain different from the acetic acid-induced writhing test (Ogura et al. 1982). In our study, systemic administration of HLEE was able to decrease the hyperalgesic effect induced by carrageenan in the mouse. Therefore, it is likely that the antihyperalgesic effect observed in our study could result from GABA liberation and its inhibition of excessive excitation of nociceptive circuits in the thalamus and cortex evoked by tissue injury (Millan 2002).

In many industrialized and developing countries, herbal agents are widely used as one of medicinal treatments for pain or inflammation. In general, these herbal products are used as total extracts. Several reports have shown the pharmacological superiority of the total extract over the active fractions (Williamson 2001; Wagner 2006; Ulrich-Merzenich et al. 2007). Therefore, in the present study we decided to examine the antihyperalgesic effect of the HLEE, and not just some of its components. In addition, according to literature reports, the main active compound of *H. longipes* is affinin (García-Chávez et al. 2004; Rios et al. 2007). It could be hypothesized that the antihyperalgesic effect observed in our work would be due to affinin. However, additional experiments are warranted to establish the role of affinin and other constituents in the antihyperalgesic effect induced by the HLEE.

The association of an NSAID to an opioid is often favorable as it allows a reduction in opioid dosing (Fletcher et al. 1997; Jiménez-Andrade et al. 2003; Litkowski et al. 2005; Strobel 1992), leading to a decrease in the incidence and intensity of side-effects (Curatolo and Sveticic 2002). However, not all the opioid–NSAID combinations are clinically successful in all cases. For example, the association of weak opioids, such as dextropropoxyphene, to acetaminophen does not significantly increase pain relief with respect to acetaminophen alone (Li Wan Po and Zhang 1997). Likewise, the combination of codeine with paracetamol results in additional pain relief but may be accompanied by an increase in drowsiness and dizziness (Moore et al. 2000). Therefore, several other combinations of analgesic agents must be evaluated experimentally to gain insight into their potential clinical use. In the current work, isobolographic analysis demonstrated a significant synergistic interaction between diclofenac and HLEE at systemic level. It has been suggested that a synergistic interaction can be obtained when two drugs or compounds with different and complementary mechanisms of action are associated. It has been demonstrated previously that HLEE was able to liberate GABA in mouse cortical slices. On the other hand, diclofenac, like all NSAIDs, inhibits prostaglandin synthesis at the site of inflammation (Vane and Botting 1998), while it also activates the nitric oxide (NO)–cyclic GMP-K^+ channel pathway at the peripheral (Ortiz et al. 2002) and spinal (Ortiz et al. 2008) levels and inhibits H^+-gated channels (Voilley et al. 2001) in sensory neurons. Therefore, it is likely that the observed synergistic interaction of diclofenac and HLEE at the systemic level involves a participation of the NO-cGMP-K^+ channel pathway, prostaglandin synthesis and H^+-gated channels inhibition, in addition to the probable liberation of GABA at cortical level. The exact mechanism of action of this interaction warrants further investigation.

In summary, diclofenac and HLEE combination produced an antihyperalgesic effect in the Hargreaves model of thermal hyperalgesia. Data suggest that low doses of the diclofenac–HLEE combination can interact synergically at systemic level and that this association may therefore represent a therapeutic advantage for the clinical treatment of inflammatory pain. Therefore, the efficacy and benefits of this combination in clinical situations await supplementary validation.

Acknowledgements

Authors greatly appreciate the technical assistance of Martha Martínez-Corona, Marta Patricia González-García and Patricia González-Ramírez.

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