

Spasmolytic and anti-inflammatory effects of *Aloysia triphylla* and citral, *in vitro* and *in vivo* studies

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Abstract

Aloysia triphylla is traditionally utilized for the treatment of menstrual colic (primary dysmenorrhea) in Mexico. Citral is the main chemical component found in *Aloysia triphylla* leaves extract. Primary dysmenorrhea is a very frequent gynecological disorder in menstruating women, affecting 30–60% of them. It is usually treated with non-steroidal anti-inflammatory drugs (NSAIDs); although their effect is rapid, they possess many side effects. Due to these shortcomings, Mexican folk therapy is considered as a feasible alternative. The effects of the hexane extract of *Aloysia triphylla* and citral on uterine contractions were evaluated *in vitro* as well as their anti-inflammatory properties and gastric wound capabilities were assessed *in vivo*. The inhibitory effects on the contractions were analyzed using isolated uterus strips from estrogen primed rats. Contractions were induced by KCl 60 mM, oxytocin 10 mIU/mL, charbacol 10 μ M and PGF_{2 α} 5 μ M. The anti-inflammatory effect was assessed on carrageenan-induced rat hind paw edema model. The inhibitory concentration-50 (IC₅₀) of the hexane extract of *Aloysia triphylla* upon each contractile response was for KCl 44.73 \pm 2.48 μ g/mL, oxytocin 42.16 \pm 3.81 μ g/mL, charbacol 41.87 \pm 1.73 μ g/mL and PGF_{2 α} 28.70 \pm 2.40 μ g/mL in a concentration-dependent way. The extract of *Aloysia triphylla* produced a significant inhibitory effect on PGF_{2 α} -induced contraction compared to its inhibitory actions on the others. Citral exhibited the same inhibitory effect on the contraction induced by PGF_{2 α} . The oral administration of the extract (100–800 mg/kg) and citral (100–800 mg/kg) showed anti-inflammatory activity; furthermore, the maximal dose utilized did not produce gastric injury. These results were compared with anti-inflammatory effects and gastric damage produced by 30 mg/kg of indomethacin p.o. The spasmolytic and anti-inflammatory effects support the traditional use of *Aloysia triphylla* leaves in the treatment of the primary dysmenorrhea in Mexican communities.

Key words: *Aloysia triphylla*, citral, spasmolytic effect, anti-inflammatory effect, dysmenorrhea

Introduction

Dysmenorrhea is defined as cramping pain in the lower abdomen occurring just before or during menstruation, and it is one of the most common gynecological disorders in young women (Doubova *et al.*, 2007). Typical menstrual contractions can be reproduced in human by a long-term infusion of prostaglandin- $F_{2\alpha}$ ($PGF_{2\alpha}$) (Roth-Brandel *et al.*, 1970). $PGF_{2\alpha}$ levels are elevated in women with primary dysmenorrhea compared to asymptomatic controls (Ghodgaonkar *et al.*, 1979; Rosenwaks *et al.*, 1981). In addition, women with primary dysmenorrhea have been found to produce higher levels of $PGF_{2\alpha}$ in their endometrium, which in turn, increases uterine tones and cause exaggerated uterine contractility (Speroff *et al.*, 1999). PG synthesis is initiated by lysosomal enzymes that are released in the late luteal phase of the menstrual cycle. Rapidly synthesized PGs exert a direct effect on the myometrium, causing the uterine musculature to contract resulting in constriction of small endometrial blood vessels, tissue ischemia, endometrial disintegration, bleeding and pain; that may be the underlying cause of dysmenorrhea (Maxson and Rosenwaks, 1993). The prevalence of dysmenorrhea was recently evaluated in two Mexican student groups: one of 1152 High School students (Ortiz *et al.*, 2009) and another group of 1,539 University students (Ortiz, 2010); it was found a prevalence of dysmenorrhea of 48.4% in the High School students, and 62.4% in the University students. Dysmenorrhea is usually treated with non-steroidal anti-inflammatory drugs (NSAIDs) in clinical medicine. In spite of their effectiveness, the side effects of these drugs on the liver, kidney and digestive system in long-term therapy limit their clinical uses (García-Rodríguez and Jick, 1994). Because of these effects, traditional medicine is considered as a feasible alternative treatment. A number of medicinal plants, including *Aloysia triphylla*, are usually employed for the treatment of menstrual colic (primary dysmenorrhea) in many rural Mexican communities (Pérez-Escandón *et al.*, 1995; Villavicencio-Nieto *et al.*, 1998; Pérez-Escandón *et al.*, 2003). In this study we evaluated the spasmolytic effects of the *Aloysia triphylla* hexane extract (ATHE), and its main chemical component citral, on the uterine contraction of the isolated rat strips *in vitro*. The contractions were induced by high-potassium depolarizing solution (KCl 60 mM), oxytocin 10 mIU/mL, charbacol 10 μ M and $PGF_{2\alpha}$ 5 μ M. Additionally, we studied the anti-inflammatory effects and the gastric wound capacity of the hexane extract of *Aloysia triphylla* and citral *in vivo*. Indomethacin was used to compare the anti-inflammatory and gastric damage effects.

Materials and Methods

Compounds used

The following compounds were used: 17 β -estradiol benzoate, charbacol chloride and $PGF_{2\alpha}$, propranolol, cimetidine, glibenclamide, N^G-nitro-L-arginine (L-NAME), indomethacin, ethanol, DMSO, carrageenan, carboxymethylcellulose (Sigma Chemical Co.), and oxytocin (Syntex). All of them and others were acquired of the best quality available in the market.

Plant material and extract preparation

Aloysia triphylla (L'Herit) Britton, synonyms *Aloysia citriodora* Ortega ex Pers., *Verbena*

triphylla L'Héritier and *Lippia citriodora*, Kunth (family *Verbenaceae*), is original from America and popularly known in Mexico as “cedrón”, “limón verbena”, “hierba de la princesa” or “hierba Luisa” (Argueta *et al.*, 1994; Gupta, 1995). The vegetable material was collected in the community of Santa María Atipac, Municipality of Axapusco, State of Mexico, in summer of 2007; the plant was authenticated by Dr. Abigail Aguilar. The voucher specimen (herbarium number 15286-IMSSM) of the plant was kept in the “Herbario de Plantas Medicinales del Centro Médico Nacional Siglo XXI del IMSS”, Mexico. ATHE from dried leaves (114 g) was prepared by maceration with 500 mL of hexane at room temperature for 24 h. After filtration, the solvent of the macerated material was eliminated by rotary evaporator Büchi RE-111, to yield a 3.5 % (w/w) of extract. The literature reports that extracts of *Aloysia triphylla* have been examined by gas chromatography-mass spectrometry and pointed out that the main component of this plant is citral, reaching up to 32-41 %; that is a natural mixture of the isomeric acyclic aldehydes geranial (trans citral, citral A) and neral (cis citral, citral B) (Stashenko *et al.*, 2003; Kim and Lee, 2004; Díaz *et al.*, 2007).

Animals and isolation of uterine preparations

Virgin female Wistar rats (180–220 g) from our own breeding facilities were used in this study. All experiments followed the Guidelines on Ethical Standards for Investigation in Animals (Zimmermann, 1983) and the Mexican Official Norm (NOM-062-ZOO-1999) regarding technical specifications for production, care and use of laboratory animals as well as according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals [National Institutes of Health Publ. 86–23, rev. 1985]. They were pretreated with 17 β -estradiol benzoate (40 μ g/kg, subcutaneously) 48 h prior to the experiment. The estrous stage was confirmed by microscopic examination of fresh vaginal smears. Animals were euthanized with pentobarbital (50 mg/kg, intraperitoneally), and myometrial tissue was removed and trimmed from the surrounding connective tissue. The detailed method utilized for tension studies has been previously described (Ponce-Monter *et al.*, 2008). Briefly, uterine strips (4 \times 10 mm) were placed longitudinally in 3 mL organ bath containing Ringer Krebs Bicarbonate (RKB) solution with the following composition (mM): NaCl 120, NaHCO₃ 20, KCl 4.6, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.5 and glucose 11. RKB solution pH 7.4 was maintained at 36°C and gassed continuously with a 5% mixture of CO₂ in O₂. Depolarizing solution (KCl 60 mM) was prepared by equimolar substitution of NaCl by KCl.

Each uterine strip was placed under optimum resting force of 1 g and allowed to equilibrate for 1 h before experimental protocol; during this equilibration period, tissues were washed with 3 mL of fresh RKB every 10 min. The contractile responses were recorded isometrically with a Grass FT03 force transducer connected to a polygraph Grass Telefactor; the data were digitalized and analyzed by mean of software PolyView. Tissues were stimulated with KCl 60 mM until obtaining two similar contractile responses.

Study on the uterine spontaneous activity and KCl-, oxytocin-, charbacol- and PGF_{2 α} -induced uterine contractions

After one hour of incubation, the uterine strips showed a spontaneous rhythmic activity; while activity was constant in amplitude and frequency, ATHE was added in a cumulative fashion (3, 10, 30 and 56 μ g/mL) at intervals of 8 min in between every addition. Subsequently, tissues were

washed, then the spontaneous phasic contractions appeared; that recovery suggests no toxic effects by the extract.

After the maximum contractile response to a single concentration of KCl 60 mM, oxytocin 10 mIU/mL, charbacol 10 μ M, and PGF_{2 α} 5 μ M, was obtained, the uterine strip was then washed with fresh RKB solution every 10 min until it returned to the resting level. The contractile process was repeated two times until stable response. Subsequently, the effects of the ATHE (3–230 μ g/mL) were determined by pre-incubation the uterine strip with a single concentration of the extract 15 min before the addition of the uterotonic substances. Citral compound was only tested on PGF_{2 α} -induced uterine contraction. The extract and citral were dissolved in ethanol-DMSO (2:1), the final concentration of solvent mixture was less than 0.1 % and did not significantly affect the uterine response. In order to assess the participation of some intracellular signaling routes during the evaluation of ATHE and citral, some known drugs were added to the isolated tissue chambers: propranolol 10 μ M (β_2 -adrenergic antagonist), cimetidine 10 μ M (H₂-antagonist), glibenclamide 10 μ M (ATP-sensitive K⁺ channel blocker) and L-NAME 100 μ M (a non-selective nitric oxide (NO) synthase (NOS) inhibitor).

Carrageenan-induced paw inflammation

Virgin female Wistar rats (of mixed estrous phases) aged 7–9 weeks (weight range, 180–220 g) were also used in this study. The carrageenan-induced paw edema model (Winter *et al.*, 1962; Bignotto *et al.*, 2009) was used to evaluate the anti-inflammatory effect of ATHE and citral. The right hind paw of the rats were marked to a point on the skin at a position that was located over the lateral malleoli, and the initial paw volume was recorded using an Ugo Basile Model 7140 plethysmometer (Comerio, Italy). Rat groups were administered orally with vehicle, ATHE (100–800 mg/kg) or citral (100–800 mg/kg) or indomethacin (30 mg/kg). ATHE and citral were emulsified as well as indomethacin was suspended in a 0.5% carboxymethylcellulose solution (vehicle, 1 mL). After 30 min, carrageenan (100 μ L of a 1% w/v solution, prepared in sterile saline) was injected subcutaneously in the plantar region of the right hind paw of each rat. Following the injection of carrageenan the paw volumes of each rat were measured at the third and sixth hour later (Bignotto *et al.*, 2009). The person performing these measurements was unaware of the treatments that the rats had received. Efforts were made to minimize animal suffering and to reduce number of animals used. Each rat was used in only one experiment and at the end of the experiments they were sacrificed in a CO₂ chamber.

Gastric damage

Following completion of the inflammation experiments (6 h after), the same rats from the groups treated with the highest doses of ATHE and citral (800 mg/kg) as well as those administered with indomethacin (30 mg/kg) were euthanized in a CO₂ chamber. The stomach was removed and the extent of hemorrhagic damage was scored by one observer who was unaware of the treatments that each rat had received. The length (in mm) of all hemorrhagic lesions was measured and the gastric damage score for each rat stomach was calculated by summing values (Wallace *et al.*, 2000; Chávez-Piña *et al.*, 2008).

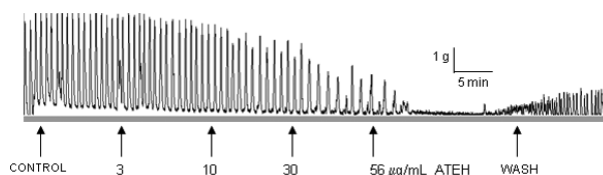


Fig. 1. Typical record of the inhibitory effect of cumulative concentrations of ATHE (3–56 $\mu\text{g}/\text{mL}$) on the spontaneous contractile activity of isolated uterine smooth muscle.

Data analysis

Data from uterus relaxing effect experiments are expressed as percentage of relaxation of the maximal contraction induced by KCl 60 mM, charbacol 10 μM , oxytocin 10 m IU/mL or PGF_{2 α} 5 μM and are the mean \pm standard error of mean (SEM) of six experiments. The relaxation was assessed by maximal response (R_{max}) and inhibitory concentration 50 (IC₅₀, concentration of ATHE or citral causing 50 % inhibition of uterine tension development). These values were calculated from linear regression analysis with Sigma Plot software, version 10. The rest of experimental data obtained are expressed as means \pm SEM. Differences in responses among the groups were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA), followed by Bonferroni test. In all cases, value of $P < 0.05$ was considered statistically significant.

Results

Inhibitory effect of hexane extract and citral

The ATHE evidenced an inhibitory effect on the spontaneous phasic contraction of myometrial smooth muscle (Fig. 1). Such activity was observed in a concentration-dependent manner by addition of cumulative concentrations of ATHE (3–56 $\mu\text{g}/\text{mL}$) to the bath of isolated uterine strips. Interestingly, at the maximal concentration ATHE achieved a 100 % of inhibition.

Figure 2 shows the inhibitory effect exerted by the extract upon the contractions induced by different uterotonic stimuli. The ATHE inhibited the maximal contraction induced by the high-K⁺ depolarizing solution, oxytocin, charbacol, and PGF_{2 α} , in a concentration-dependent manner, although with different potency. Indeed, ATHE inhibited the contraction induced by PGF_{2 α} in a more effective manner than those induced by high-K⁺ depolarizing solution, oxytocin or charbacol. The values of IC₅₀ and R_{max} are shown and summarized in Table 1; as it can be seen, ATHE achieved a significantly lesser IC₅₀ on PGF_{2 α} -induced contraction, and this suggests that its activity is more specific for such inducer. Citral concentrations (3–230 $\mu\text{g}/\text{mL}$) also inhibited the contractile response induced by PGF_{2 α} in a concentration-dependent way in uterus, with both a curve profile and IC₅₀ = 20.16 \pm 2.20 $\mu\text{g}/\text{mL}$ comparable to that one of ATHE (Fig. 3).

The inhibitory effects induced by ATHE and the citral were not altered by the presence of propranolol (10 μM), cimetidine (10 μM), glibenclamide (10 μM) and L-NAME (100 μM). However, the relaxant effect of both the ATHE and citral was reversed when the concentration of CaCl₂ (0.5, 1.0 and 3.0 mM) was gradually increased in the incubation medium of uterine tissue (data not shown).

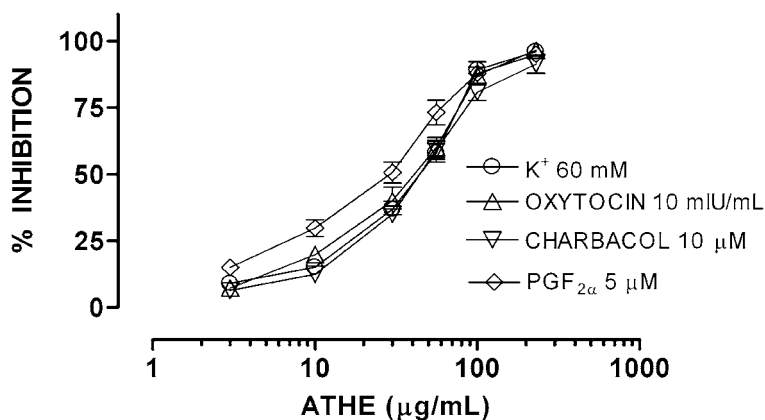


Fig. 2. Concentration-effect curves of ATHE on potassium chloride (K^+ , 60 mM), oxytocin 10 mIU/mL, charbacol (10 μ M), $PGF_{2\alpha}$ (5 μ M)-induced contractions on uterine strip preparations. Each point represent the mean of 6 experiments, vertical bars indicate standard error of the mean (SEM).

Table 1. Inhibitory concentration-50 (IC_{50}) and maximal effect (R_{max}) induced by hexane extract of *Aloysia triphylla* (ATHE) on different contraction inducers

Inducer	IC_{50} (μ g/mL)	R_{max} (%)
KCl 60 mM	44.73 \pm 2.48	95.76 \pm 2.07
Oxytocin 10 mIU/mL	42.16 \pm 3.81	94.93 \pm 1.59
Charbacol 10 μ M	41.87 \pm 1.73	88.20 \pm 3.33
$PGF_{2\alpha}$ 5 μ M	28.70 \pm 2.40*	92.66 \pm 3.28

Each IC_{50} value is the mean value \pm SEM from 6 different experiments. E_{max} are expressed as the mean % \pm SEM from for 6 different experiments. *, $P < 0.05$ vs. KCl, oxytocin and charbacol.

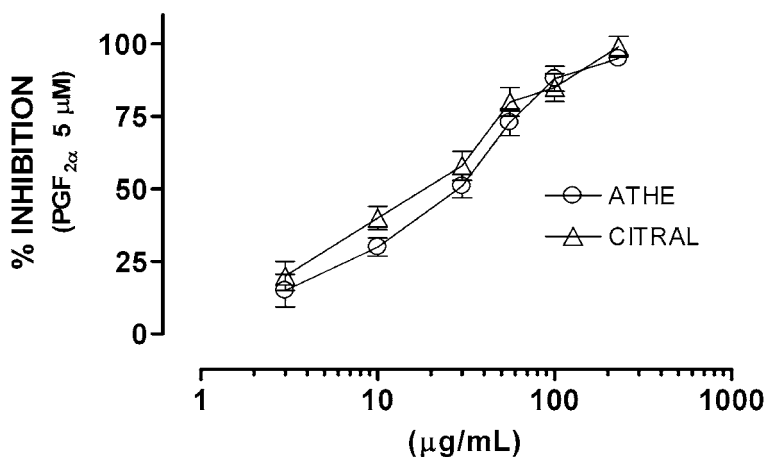


Fig. 3. Concentration-effect curves of ATHE and citral on $PGF_{2\alpha}$ (5 μ M)-induced contractions on uterine strip preparations. Each point represent the mean of 6 experiments, vertical bars indicate standard error of the mean (SEM).

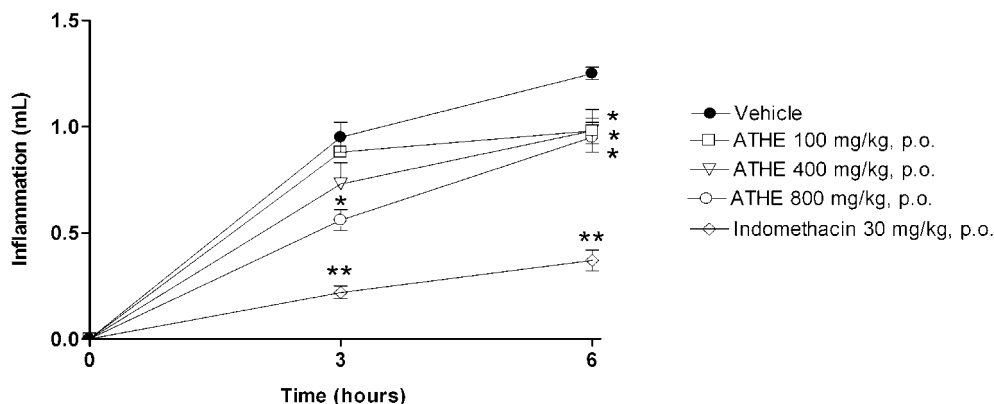


Fig. 4. Effect of *Aloysia triphylla* hexanic extract (ATHE) (100–800 mg/kg) and indomethacin (30 mg/kg) on carrageenan-induced paw edema in the rat. Each point represents the mean of 6 rats, vertical bars indicate standard error of the mean (SEM). * and **, significantly different from vehicle (*, $P < 0.05$; **, $P < 0.001$), as determined by analysis of variance followed by Bonferroni's test.

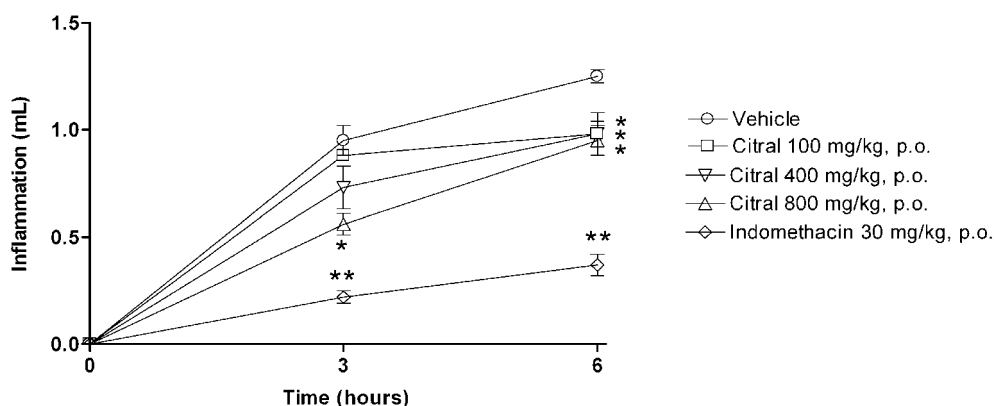


Fig. 5. Effect of citral (100–800 mg/kg) and indomethacin (30 mg/kg) on carrageenan-induced paw edema in the rat. Each point represents the mean of 6 rats. Vertical bars indicate standard error of the mean (SEM). * and **, significantly different from vehicle (*, $P < 0.05$; **, $P < 0.001$), as determined by analysis of variance followed by Bonferroni's test.

Anti-inflammatory effect of hexane extract, citral and indomethacin

Injection of carrageenan into the hind paw of rats resulted in a marked time-dependent increase in paw volume as a consequence of edema formation. Augmented inflammation was significantly inhibited by ATHE as well as by citral, in a dose-dependent manner ($P < 0.05$) (Figs. 4 and 5); additionally, ATHE evidenced a better anti-inflammatory outcome 6 h after carrageenan injection than citral. On the other hand, indomethacin was drastically more potent than ATHE and citral as an anti-inflammatory agent.

Gastric damage

At the sixth hour, oral administration of indomethacin used as a positive control, resulted in

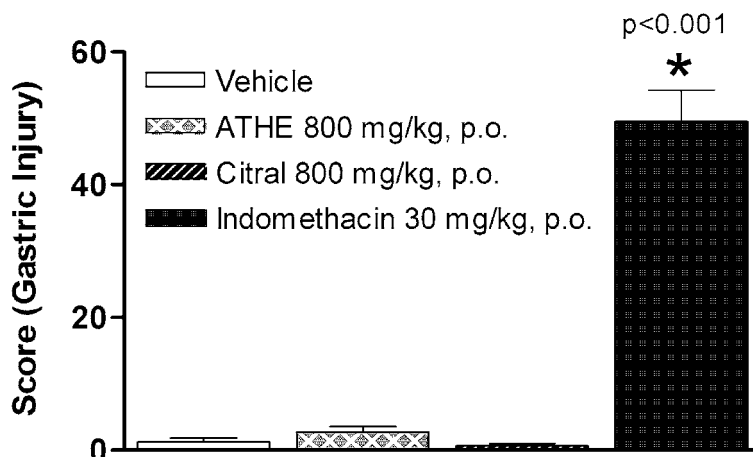


Fig. 6. Gastric damage score 6 h after administration of *Aloysia triphylla* hexanic extract (ATHE, 800 mg/kg), citral (800 mg/kg) and indomethacin (30 mg/kg). Bars are the means of 6 animals. Vertical bars indicate standard error of the mean (SEM). *, significantly different from vehicle ($P < 0.05$), as determined by analysis of variance followed by Bonferroni's test.

the formation of hemorrhagic erosions in the corpus of the stomach, while neither ATHE nor citral caused gastric damage; furthermore, their effects were not different regarding the vehicle (Fig. 6). Thus, although indomethacin was markedly more effective as an anti-inflammatory agent, it produces great gastric damage but ATHE and citral did not ($P < 0.01$).

Discussion

The present results are the first evidence that describes the spasmolytic and anti-inflammatory effects of *Aloysia triphylla*, popularly known in Mexico as “cedrón” o “hierba Luisa” and its main chemical component citral. It was notable that ATHE showed an inhibitory effect on the spontaneous contraction of myometrial smooth muscle, since it is known that such an activity may participate in the development of painful feeling in menstruating women.

It is interesting that the hexane extract of *Aloysia triphylla* relaxed more effectively the uterine contraction induced by $\text{PGF}_{2\alpha}$ than those caused through oxytocin, carbacol or high KCl, as shown in the Table 1. The foregoing data agree with reports that during the menstrual cramping the release of mostly $\text{PGF}_{2\alpha}$ as well as other pro-inflammatory substances increases (Speroff *et al.*, 1999). Also, it was important to find that citral, the main component of the plant, inhibited the contraction induced by $\text{PGF}_{2\alpha}$ in the same way and extent when compared to ATHE. This suggests an advantage by using this plant or its major secondary metabolites to obtain an effective relaxant outcome on that colic leader promoter.

The relaxing effects of ATHE and citral were not affected by propranolol, a non selective β -adrenoceptor blocker, suggesting that their inhibitory effects are not evoked through the activation of

β -adrenoceptor. Glibenclamide, an ATP-sensitive potassium channel blocker, also did not antagonize the inhibitory effects of ATHE or citral, indicating that their relaxant activities are not due to the opening of ATP-sensitive potassium channels. On the same way, neither cimetidine, an antagonist of H_2 -receptors, nor L-NAME, an inhibitor of NOS, modify the relaxing effect induced by ATHE and citral, this pointed out that the H_2 -receptors and NO are not involved in their relaxing action. On the other hand, although the exact mechanism as smooth muscle relaxants was not established in this study, the fact that the relaxing effect of ATHE and citral was reversed by increasing calcium in the incubation medium of uterine tissue, suggests that such activity might be via inhibition of L-type calcium channels. Furthermore, the relaxing effect of ATHE and citral was completely reverted after washing several times, demonstrating that their capacity is reversible. These observations regarding the relaxing activity of this plant are in agreement with one report, which has shown the spasmolytic effect of *Aloysia triphylla* on rat duodenum; nevertheless, that study claims that such an outcome may be mostly associated to the increase in cGMP levels and the activation of K^+ -channels (Ragone *et al.*, 2007), conversely to the effect by glibenclamide herein observed, that did not antagonize the spasmolytic action of either ATHE or citral. That discrepancy may be related to the differences between both smooth muscles, uterus and duodenum. In addition, citral possesses a significant inhibiting effect on ileum contractions induced by KCl, indicating that citral can block the L-type calcium channels (Buddhakala *et al.*, 2008). The ATHE significantly inhibited the uterine contraction induced by $PGF_{2\alpha}$, what may explain its spasmolytic effect on the menstrual cramps.

Also both, ATHE and citral, showed a significant anti-inflammatory effect in the carrageenan model; in fact, citral has a very important anti-inflammatory activity in the test of croton oil-induced ear edema in mice (Lin *et al.*, 2008). Moreover, the synergistic effect of the interaction between naproxen and citral on inflammation has been recently reported, this also in the carrageenan model in rats (Ortiz *et al.*, 2010 in press). In addition, the clear advantage by using ATHE or citral as spasmolytic and anti-inflammatory agents was evidenced, inclusive at high doses, but without the aggressive gastric injury that the administration of NSAID causes during the common treatment for primary dysmenorrhea.

Taken together, the uterus-relaxing effect and the anti-inflammatory activity showed by ATHE, may likely explain the mode of action of the medicinal plant *Aloysia triphylla* in the folk treatment of menstrual colic (primary dysmenorrhea). However, it is necessary a further study on the mechanism of action of ATHE and citral as relaxants and anti-inflammatory agents. In conclusion, this work revealed that the hexane extract obtained from *Aloysia triphylla* leaves and its main component citral, have uterine relaxing *in vitro* and anti-inflammatory *in vivo* activities, but without causing gastric damage. Thus, the combined anti-spasmodic and anti-inflammatory effects of this extract observed in the present study, support the ethnomedicinal use of this plant for the treatment of menstrual cramps.

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