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Antinociceptive effect of extracts and compounds from *Hofmeisteria schaffneri*☆

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ABSTRACT

Ethnopharmacological relevance: Hofmeisteria schaffneri (Asteraceae) is a medicinal plant widely commercialized in the most important Markets of Mexico City for the treatment of gastro-intestinal complaints and skin afflictions.
Aim of the study: The main goals of this study were to establish the potential acute toxicity and the antinociceptive activity in animal models of several preparations and compounds from *Hofmeisteria schaffneri*.
Materials and methods: The aqueous and organic extracts as well as the essential oil of *Hofmeisteria schaffneri* were prepared by infusion, maceration and hydrodistillation, respectively. Investigation of the acute toxicity was accomplished by the Lorke method. The antinociceptive effect was assessed using the writhing and the hot plate tests. Natural compounds were isolated by standard phytochemical procedures. In addition, a few thymol esters were prepared by chemical synthesis. The stability of natural and synthetic esters was qualitatively analyzed by measuring their susceptibility to hydrolysis by pig liver estearase and mouse plasma at 37 °C.
Results: The LD_{50} for each preparation tested was higher than 5000 mg/kg revealing that they were not toxic to mice after exposure for short space of time. On the other hand, the extracts showed significant antinociceptive effect when tested in the hot plate model. The most active natural product as antinociceptive agent was hofmeisterin III (**1**) which also was the most stable in the stability study. Its pharmacological effect seems to be partially mediated by an opioid mechanism since naloxone inhibits its action. Using compound **1** as a lead molecule, several synthetic thymol esters were prepared and only compounds **13**, **15** and **17** were antinoceptive at the dose of 1 mg/kg.
Conclusions: The present investigation provided evidence of the efficacy of several preparations of *Hofmeisteria schaffneri* as antinociceptive agents. The most active preparation was the essential oil which contained large amount of hofmeisterin III (**1**) and other thymol derivatives. Some novel synthetic analogs of hofmeisterin III with antinociceptive properties were discovered. The nature of the ester chain of these analogs did not have a clear impact on the antinociceptive activity. The phyto-preparations analyzed in this study were not toxic to mice according to the Lorke's test; therefore considering their long term use of the plant they might be secure for human consumption.

1. Introduction

In Mexico an important segment of the population relies on botanical raw materials for primary health care, therefore it is very important to investigate these herbs from the pharmacological and toxicological points of view to establish their real efficacy and safety. The results of such investigations will be also useful to integrate the scientific monographs of these plants in order to promote their rational use. Consequently, the present investigation was undertaken to initiate the preclinical pharmacological and toxicological analyses of *Hofmeisteria schaffneri* (A. Gray) R.M. King & H. Robinson (Asteraceae), a widely commercialized medicinal species in Central Mexico. The infusion prepared from the fresh or dried aerial parts of the plant is highly valued for treating gastro-intestinal complaints, including stomach aches, related or not with irritable bowel syndrome and dyspepsia, and bleeding diarrhea (King, 1967; Mendoza-Castelán et al., 1997; Pérez-Vásquez et al., 2005, 2008). In addition, the plant is highly valued as a topic antiseptic agent.

Previous chemical work of the plant allowed isolation of several thymol and northymol derivatives (Pérez-Vásquez et al., 2005,

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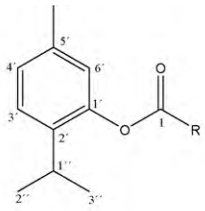

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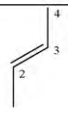
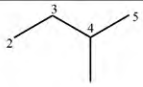
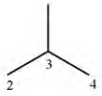
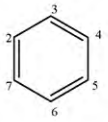
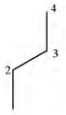
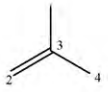
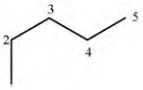
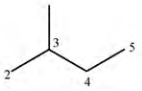
1		**11**	
4		**12**	$-CH_2CH_2CH_3$
5		**13**	$-CH_2CH_2CH_2CH_2CH_3$
6		**14**	
7		**15**	$-CH_2CH_2CH_2CH_3$
8	$-CH_2CH_3$	**16**	
9		**17**	$-CH_3$
10			

Fig. 1. Structures of thymyl esters.

2008) including hofmeisterin III (**1**), thymol itself (**2**) and 8,9-epoxy-10-acetoxythymyl angelate (**3**) (Figs. 1 and 2). Furthermore, the essential oil and infusion of the plant revealed significant antimicrobial properties against Gram+ bacteria. The antimicrobial essential oil of the plant harvested at different seasons during a year period was also chemically analyzed by GC and capillary GC–MS. Forty four compounds representing ~90% of the total constituents were identified. Compounds **1**–**3**, thymyl isovalerate (**4**), thymyl isobutyrate (**5**) were the major components of the oils but only **3** and **5** were active against *Staphyloccocus aureus* and *Bacillus subtilis* (Pérez-Vásquez et al., submitted for publication).

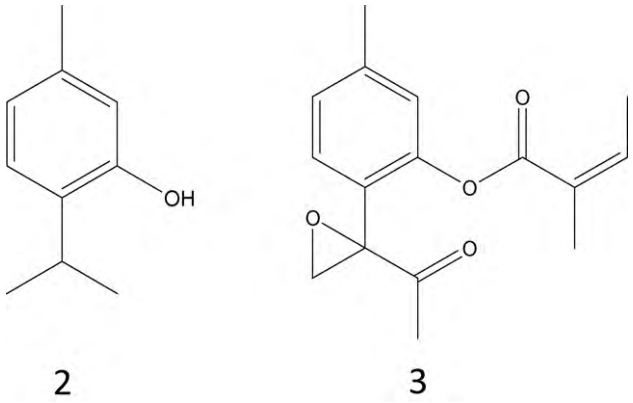


Fig. 2. Structures of thymol (**2**) and 8,9-epoxy-10-acetoxythymyl angelate (**3**).

2. Materials and methods

2.1. Plant material

The aerial parts of *Hofmeisteria schaffneri* (4 kg) were collected in San Luis Potosi, Mexico on November 2002. An authenticated

voucher (Bye & Linares 31018) specimen is deposited in the National Herbarium (MEXU), Instituto de Biología, UNAM.

2.2. General chemistry procedures

IR (films) spectra were recorded in a Perkin Elmer 59913 spectrophotometer. NMR spectra were registered on a Varian Mercury 300 spectrometer in $CDCl_3$, at 400 MHz (^1H) and 100 MHz (^{13}C) with tetramethylsilane (TMS) as internal standard. Electron impact (EI) mass spectra (MS) were obtained on a JEOL SX 102 mass spectrometer. Open column chromatography: silica gel 60 (0.063–0.200 mm), 70–230 Mesh (Merck). TLC analyses were carried out on silica gel 60 F_{254} plates (Merck) using a ceric sulphate (10%) solution in H_2SO_4 as color reagent.

2.3. Preparation of organic extract

The organic extract of *Hofmeisteria schaffneri* was obtained from dried and shredded aerial parts (750 g) macerating with CH_2Cl_2–MeOH (1:1) (2 l × 3) during 14 days at room temperature. After filtration, the extract was concentrated *in vacuo* to yield 172 g of a greenish residue. Hofmeisterin III (**1**) (100 mg), thymol (**2**) (79 mg), and 8,9-epoxy-10-acetoxythymyl angelate (**3**) (20 mg) were isolated as previously described (Pérez-Vásquez et al., 2008).

2.4. Preparation of the essential oil

Dried aerial parts (500 g) cut in small pieces were hydrodistilled during 3 h using a Clevenger-type apparatus. When the condensed material cooled down, the essential oils were separated from the water by liquid–liquid extraction with CH_2Cl_2 (2 l × 3). After removal of the solvent, 0.4 g of the oil was obtained. The oil was stored at 4 °C until analysis (WHO, 1998).

2.5. Preparation of the aqueous extract

The dried aerial parts (900 g) were extracted with boiling water (25 l) during 30 min. The resulting aqueous extract was partitioned with CH_2Cl_2 (25 ml × 3). The organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a brown residue (3.43 g).

2.6. Chemicals and drugs

Morphine (MOR) was a donation from Alfredo Covarrubias Gómez, MD, and Luis Antonio Reyes Vallejo, MD. Dipirone (DIP) was purchased from Laboratorios Pisa (Mexico City); the vehicle (VEH) was prepared with isotonic solution of NaCl and tween 80 (0.05%); pig liver esterase, 4-nitrophenyl acetate (4-NPA), glibenclamide (GLIB), N(G)-nitro-L-arginine methyl ester (L-NAME), naloxone (NLX), acetic anhydride, valeroic, tiglic, isovaleric, propionic, 3-methylvaleric, 4-methylvaleric, 2-methylbutiric and 3,3-dimethylacrylic acids as well as 2-methylvaleric, butyric, hexanoic, benzoic and isobutyric acid chlorides were purchased from Sigma–Aldrich (St. Louis. MO, USA). Compounds **4**–**17** were prepared by synthesis as describe below.

2.7. Synthesis of thymol derivates

2.7.1. Preparation of acyl chlorides of isovaleric, tiglic, 2-methylbutiric, propionic, 3-methylvaleric, 4-methylvaleric, valeric and 3,3-dimethylacrylic acids

Thyonyl chloride (2.5 ml; 0.034 mol) was added drop wise to a stirred solution of appropriate carboxylic acid (0.002 mol) in CH_2Cl_2 (1 ml). The resulting mixtures were refluxed gently for 1 h. In all cases, the excess of thyonyl chloride was distilled and the resulting products were used to prepare esters **4**, **6**, **7**, **8**, **10**, **11**, **15** and **16**, respectively.

2.7.2. Thymyl esters ***4*–*16***

To a stirred mixture of thymol (0.5 g, 0.003 mol) and 10% aqueous NaOH (4.5 ml) the acid chloride previously obtained was slowly added. In each case, the reaction mixture was stirred for 30 min at room temperature. Every reaction mixture was extracted with CH_2Cl_2 (3× 10 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure; the crude products were purified by open column chromatography [silica gel, hexane–EtOAc (98:2)] to yield pure **4**–**16** (Fig. 1). Compound **14** was obtained as colorless oil in a yield of 70%. The remaining products **4**–**13** and **15**–**16** were yellow oils and the yields were 46, 61, 50, 41, 48, 65, 41, 35, 62, 71, 45 and 52%, respectively. The spectral properties of compounds **4**–**8**, **12**, **15**, and **16** were identical to those previously described (Mathela et al., 2008; Martinez et al., 1988; Paolini et al., 2005, 2007; Schmitz et al., 1979; Rice and Coats, 1994; Viana et al., 1981; Ateeque et al., 2002; Sheng et al., 1984; Kumar et al., 2008, respectively). Compounds **9**, **10**, **11** and **13** were new chemical entities therefore their NMR (Table 1) and other spectral data are presented in this paper.

Thymyl 2-methylvalerate (**9**): UV (MeOH) λ_{max} nm ($\log \varepsilon$): 222 (4.71), 263 (4.36), 271 (4.34), IR (film) ν_{max} cm^{-1}: 2962, 2934, 2873, 1756, 1621, 1506, 1458, 1149, 1127, 815, 730, EI-MS *m*/*z* (rel. intensity): 248 [M^+ (12)], 150 (34), 135 (45), 115 (10), 91 (25), 71 (100).

Thymyl 3-methylvalerate (**10**): UV (MeOH) λ_{max} nm ($\log \varepsilon$): 222 (4.81), 263 (4.46), 271 (4.44), IR (film) ν_{max} cm^{-1}: 2963, 2930, 2874, 1759, 1621, 1505, 1461, 1148, 954, 815, 577, EI-MS *m*/*z* (rel. intensity): 248 [M^+ (11)], 150 (49), 135 (87), 115 (15), 91 (60), 71 (100).

Thymyl 4-methylvalerate (**11**): UV (MeOH) λ_{max} nm ($\log \varepsilon$): 222 (4.71), 263 (4.36), 271 (4.23), IR (film) ν_{max} cm^{-1}: 2960, 2930, 2871, 1760, 1621, 1505, 1467, 1150, 950, 815, EI-MS *m*/*z* (rel. intensity): 248 [M^+ (20)], 150 (70), 135 (100), 115 (10), 105 (20), 91 (40).

Thymyl hexanoate (**13**): UV (MeOH) λ_{max} nm ($\log \varepsilon$): 224 (4.75), 263 (4.40), 271 (4.38), IR (film) ν_{max} cm^{-1}: 2960, 2931, 2871, 1760, 1621, 1505, 1458, 1151, 815, 728, 577, EI-MS *m*/*z* (rel. intensity): 248 [M^+ (15)], 150 (75), 135 (100), 115 (10), 105 (15) 91 (35).

2.7.3. Thymyl acetate (*17*)

In a 50 ml round-bottomed flask, thymol (0.5 g, 0.003 mol), acetic anhydride (1.5 ml, 0.015 mol) and sulphuric acid (0.1 ml, 0.001 mol) were mixed and stirred for 45 min. The reaction mixture was then extracted with CH_2Cl_2 (3× 10 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated *in vacuo*. The residue was purified by open column chromatography on silica gel, eluting with hexane–EtOAc (98:2) to yield a yellow oil (72%). Its spectral properties were identical to those previously described by Viana et al. (1981) and Ateeque et al. (2002).

2.8. Qualitative stability test of thymol esters

The stability of esters **1**, **4** and **5** toward pig liver esterase (2 mg suspended in 10 ml of distilled water) was estimated using the procedure described by Schlacher et al. (1998). The enzyme activity was qualitatively corroborated by monitoring the production of 4-nitrophenol, yellow, from the colorless 4-nitrophenyl acetate (4-NPA) (Innocenti et al., 2008). The stock solution, in water–acetonitrile (99:1), of 4-NPA (0.1 mM) was freshly prepared before use. For the analysis, 10 μl solution acetate buffer (pH 5.0) or phosphate buffer (pH 7.4), 100 μl (0.1 mM) of test compound (**1**, **4** and **5**) or positive control (4-NPA, 1 mM) and enzyme preparation (10 μl) were added to Eppendorf tubes (Jewell et al., 2007). The resulting reaction mixtures were shaken for 1 h at 37 °C. Suitable blanks using compounds **1**, **4**, **5** and **2** were also prepared in

Table 1
^{1}H and ^{13}C (400 MHz and 100 MHz, respectively) NMR spectroscopic data of compounds **9**, **10**, **11** and **13**.

Nuclei	Compounds							
	9		**10**		**11**		**13**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	–	175.6	–	171.7	–	172.4	–	172.7
2	2.73 *m*	39.8	2α 2.35 *dd* (15.2, 8), 2β 2.6 *dd* (14.8, 6)	41.1	2.59 *m*	32.2	2.5 *t* (7.6)	34.6
2-CH_3	1.32 *d* (7.2)	36.0	–	–		–	–	–
3	1.52 *m*	20.7	1.38 *m*	31.7	1.68 *m*	33.5	1.78 *m*	24.9
3-CH_3	–	–	1.17 *d*	19	–	–	–	–
4	1.45 *m*	17.3	4α 1.45 *m* 4β 1.35 *m*	29.1	1.71 *m*	27.4	1.38 *m*	31.5
4-CH_3	–	–	–	–	0.98 *d* (7.2)	21.9	–	–
5	0.98 *t* (7.2)	14.2	0.97 *t* (7.4)	10.9	0.98 *d* (7.2)	21.9	1.38 *m*	22.5
6	–	–	–	–	–	–	0.92 *t* (7.2)	14.1
1′	–	148.2	–	147.6	–	147.6	–	148.1
2′	–	137.2	–	136.7	–	136.7	–	137.2
3′	7.19 *d* (8)	126.4	7.18 *d* (8)	126.0	7.19 *d* (8)	126.0	7.17 *d* (8)	126.5
4′	7.01 *br d* (8)	127.1	7.0 *br d* (8)	126.7	7.0 *br d* (8)	126.7	7.0 *br d* (8)	127.2
5′	–	136.6	–	136.2	–	136.18		136.7
6′	6.77 *d* (0.8)	122.8	6.78 *d* (0.8)	122.4	6.79 *d* (0.8)	122.4	6.78 *d* (0.8)	122.9
5′-CH_3	2.30 *s*	21.0	2.30 *s*	20.5	2.31 *s*	20.5	2.30 *s*	21.0
1″	2.97 *h* (6.8)	27.1	2.97 *h* (6.8)	26.7	2.95 *h* (6.8)	26.7	2.95 *h* (6.8)	27.2
2″	1.19 *d* (6.8)	23.1	1.20 *d* (6.8)	22.7	1.18 *d* (6.8)	22.7	1.17 *d* (6.8)	23.2
3″	1.19 *d* (6.8)	23.1	1.20 *d* (6.8)	22.7	1.18 *d* (6.8)	22.7	1.17 *d* (6.8)	23.2

J values are in parentheses in Hz.

the same conditions but in the absence of the enzyme. In all cases, the test was carried out by triplicate. After incubation, the mixture of each tube was partitioned with 100 μl CH_2Cl_2; organic phase aliquots were withdrawn and immediately analyzed by TLC on silica gel (hexane–AcOEt 8:2) to detect the presence of **2**. For each compound, the procedure was repeated using incubation periods of 4.5 and 24 h. Finally, the same set of experiments was repeated using mouse plasma instead pig liver esterase.

2.9. Pharmacological and toxicity studies

2.9.1. Animals

ICR male mice weighing 20–25 g were used in the experiments. Mice were purchased from Centro UNAM-Harlan (Harlan México, S.A. de C.V.). They were housed under standard laboratory conditions and maintained on standard pellet diet and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. After the experiments, all animals were sacrificed in a CO_2 chamber. Furthermore, clearance for conducting the studies was taken from the Ethics Committee for the Use of Animals in Pharmacological and Toxicological Testing, Facultad de Química, UNAM, which in turn is coordinated by UNAM Central Committee of Ethics for Animal Care and Handing. For the pharmacological studies groups of six animals were used, whereas for the toxicological tests the groups were of three mice. All doses are in mg/kg of body weight.

2.9.2. Acute toxicity study in mice

The treatments were orally administrated in two phases, in both phases four groups with three animals were used; in the first phase, mice were treated with doses of 10, 100 and 1000 mg/kg of the organic extract, essential oil or aqueous extracts; these treatments were suspended in the VEH. In the second phase, the animals received 1600, 2900 and 5000 mg/kg of the treatment according to the Lorke procedure (Lorke, 1983). In both stages, the animals were observed during 14 days. After this period of time, the lungs, heart, stomach and intestines were removed under dissection to detect any macroscopic injury.

2.9.3. Writhing test

The acetic acid-induced writhing test was performed in mice as previously described (Zimmerman, 1983; Williamson et al., 1996). The organic extract (10–316 mg/kg) suspended in the VEH was orally administered 30 min before intraperitoneal (*i.p*) injection of 0.6% acetic acid. Control animals received a similar volume of VEH or dipirone (DIP, 100 mg/kg) *p.o*. Animals were placed in an observation box, and the abdominal constrictions were counted cumulatively over a period of 30 min. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions. The results corresponded to the area under the curve.

2.9.4. Hot plate test

The hot plate apparatus (Ugo Basile, Italy) was used to measure the antinociceptive effect (Zimmerman, 1983; Williamson et al., 1996). Mice were placed into an acrylic cylinder over a heated surface (55.5 ± 0.2 °C), the time between placement and shaking or licking paws or/and jumping was recorded as latency response. The organic extract (10–562 mg/kg), essential oil (1–100 mg/kg), aqueous extract (10–316 mg/kg), thymol (**2**) (10–100 mg/kg) or thymol derivates (**1**, **3** and **4**–**17**) (0.1–17.7 mg/kg) were orally administered 30 min before beginning each experiment. Mice were observed before and 30, 60, 90, 120 and 150 min after each drug administration. A cut off of 30 seconds was used to avoid injury. MOR (3.16 and 5 mg/kg, *i.p*) was used as positive control. The cumulative antinociceptive effect was determined measuring the area under the curve of the course time (AUC) analysis. Curves were plotting with AUC (trapezoidal method) and doses.

2.9.5. Preliminary studies on the mode of action of hofmeisterin III (*1*)

Naloxone (NLX, 1 mg/kg, in VEH), glibenclamide (GLIB, 10 mg/kg, in DMSO 2% and VEH) or L-NAME (L-NAME, 30 mg/kg, in VEH) were *i.p* administrated 15 min before the oral treatment with compound **1** (1 mg/kg). In each case, the antinociceptive effect was recorded as describe above (Déciga-Campos et al., 2007).

2.9.6. Statistical analysis

Data are expressed as the mean ± SEM for the number ($n = 6$) of animals in all groups. In all cases the results of treated-animal (com-

pounds, extracts and reference drugs) groups were compared with the VEH groups. Statistical analysis was performed with one-way ANOVA followed by Dunnett *post hoc* to determine the source of significant differences where appropriate; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered statistically significant. Prisma GraphPad (version 4.0) software was used for statistics and plotting.

3. Results and discussion

As part of our investigations dealing with the preclinical efficacy, quality control and safety of selected Mexican medicinal plants, the present study was undertaken to determine the acute toxicity and the antinociceptive action of *Hofmeisteria schaffneri*. The antinociceptive activity was selected on the basis of its extensive use for treating painful complaints. In all cases the primary target for testing was the plant crude extracts considering their similarity with the preparations used in traditional medicine, although some major pure compounds isolated from the plant were also evaluated in order to find out the active principles.

3.1. Acute toxicity

Investigation of the acute toxicity is the first step in the toxicological investigations of herbal drugs. Lorke's method is perhaps the most widely used approach since the experiments are carried out with a minimum number of experimental animals (Lorke, 1983). Thus, to asses any potential toxic effects of the plant mice were treated orally with *Hofmeisteria schaffneri* essential oil as well as with the organic and aqueous extracts using doses in the range of 10–5000 mg/kg. After 14 days, treated mice did not present any visible toxic effect. Furthermore, no lesions or bleedings were observed in internal organs such as lungs, kidneys, liver, heart and stomach. Since no death or damage was observed throughout the experiments, the LD_{50} for each preparation tested was higher than 5000 mg/kg revealing that they were not toxic to mice after exposure for short space of time.

3.2. Antinociceptive effect using writing and hot plate tests

The potential antinociceptive effect produced by extracts and essential oil was assessed using two well known models of nociception, the writhing and the hot plate tests. The former is an inflammatory visceral pain model and is very useful to detect painful complaints due to inflammatory disorders of internal organs such as the stomach or intestines (Al-Chaer and Traub, 2002). This method shows good sensitivity for weak analgesics, but shows poor specificity because abdominal constrictions could be suppressed by smooth muscle relaxants so results could be misunderstood. This problem can be overcome using other models of nociception such as the hot plate test. Human preclinical pain tests have frequently employed heat stimulation for physiological, pathological and pharmacological assessments (Arendt-Nielsen and Chen, 2003). In addition, this model measures animal behavior and has good sensitivity and specificity.

Graded doses of the organic extract (10–562 mg/kg, *p.o.*) did not decrease acetic acid induced contortions in mice (Table 2). Therefore, the essential oil and aqueous extracts were not further evaluated.

On the other hand, the organic extract significantly increased ($P<0.001$) the latency to thermal stimuli when tested at the doses of 177 and 562 mg/kg; the effect was dose-dependent. The essential oil and aqueous extract were also very active in the hot plate test at doses ranging from 1 to 316 mg/kg (Table 3), however, the effect was not dose dependent (Table 3).

Since the essential oil was the most active its major components (Pérez-Vásquez et al., submitted for publication), namely hofmeisterin III (**1**), thymol (**2**), 8,9-epoxy-10-acetoxythymyl angelate (**3**), thymyl isovalerate (**4**), thymyl isobutyrate (**5**) were tested at different doses. For this endeavor, compounds **4** and **5** were synthesized by condensing thymol with isovaleryl and isobutyryl chlorides, respectively. The spectral properties of **4** and **5** were identical to those described by Mathela (Mathela et al., 2008). Compounds **1**–**3** were isolated from the plant material as previously described (Pérez-Vásquez et al., 2008).

Compound **1** (0.1–17 mg/kg) was the most active increasing significantly the latency to thermal stimulis in a dose dependent manner up to the dose of 1 mg/kg. At higher doses (3.16 and 17.7 mg/kg) the effect remains constant (Table 4). The pharmacological effect of **1** was also assessed after pretreatment (15 min before) of mice with NLX (1 mg/kg), a non-selective opiate receptor antagonist, GLIB (10 mg/kg), a Na^{+} channel blocking, or L-NAME (30 mg/kg), an inhibitor of nitric oxide synthase. According to the results in Table 5, L-NAME and GLIB were not able to decrease the antinociceptive activity of **1**. However, the effect of **1** was significantly reversed by the treatment with NLX suggesting that its mode of action involves opioids receptors and/or an increment of endogenus opioids.

Compounds **4** and **5** (0.1–17 mg/kg) showed similar activity than hofmeisterin III (**1**) while **2** (10–100 mg/kg) was less active; finally, compound **3** (0.316–17.7 mg/kg) did not show any activity suggesting that the esterification of the free phenolic group of thymol improved the activity (Table 4). Altogether this information reveals

Table 2
Effect of the organic extract of *Hofmeisteria schaffneri* in the contortions induced by acetic acid.

Treatment	Doses (mg/kg)	AUC ± E.E
VEH		3725 ± 100
Organic extract	316	3440 ± 289.1
	100	3202.5 ± 1033.8
	31.6	4162.5 ± 855.8
	10	3842.5 ± 595
DIP		770 ± 269.9*

Data are expressed as the mean ± SEM for the number ($n=6$) of animals in every group. ANOVA followed by Dunnett post hoc $^{*}P<0.05$.

Table 3
Antinociceptive effect of extracts and essential oil from *Hofmeisteria schaffneri* as detected in the hot plate test.

Treatment	Doses (mg/kg)	AUC ± E.E
VEH		983.1 ± 39.6
Organic extract	31.6	1136.4 ± 62.3*
	56.2	1268.1 ± 65.6*
	100	1381.1 ± 79.9*
	177	1661.6 ± 100.8***
	562	1914.6 ± 117.8***
MOR	3.16	2638.1 ± 200.1***
VEH		983.1 ± 39.6
Aqueous extract	10	1788.7 ± 172.9***
	56.2	1974.4 ± 116.3***
	100	2212.5 ± 61.2***
	177	2107.6 ± 89.3***
	316	2071.9 ± 124***
MOR	3.16	2638.1 ± 200.1***
VEH		1494.5 ± 97.6
Essential oil	1	2280 ± 158**
	3.16	2328.7 ± 152.5***
	17.7	2353.1 ± 124.7***
	31.6	1755.9 ± 138.7
	100	1618.3 ± 126.8
MOR	3.16	3078.7 ± 177.8***

Data are expressed as the mean ± SEM for the number ($n=6$) of animals in every group. ANOVA followed by Dunnett post hoc $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Table 4
Antinociceptive effect of compounds **1**–**5**, **13**, **15** and **17** as detected in the hot plate test.

Treatment	Doses (mg/kg)	AUC ± E.E
VEH		1494.5 ± 97.6
Hofmeisterin III (**1**)	0.1	1775.6 ± 85.7
	0.316	1976.2 ± 78*
	1	2460 ± 83.7***
	3.16	2465.6 ± 61.3***
	17.7	2546.2 ± 114.4**
MOR	3.16	3078.7 ± 177.8***
VEH		1726.9 ± 84.5
Thymol (**2**)	1	1427.4 ± 169.5
	10	2116.9 ± 68.1
	56.2	2096.2 ± 133.5
	100	2420.6 ± 102.6**
MOR	3.16	2783.1 ± 148.3***
VEH		1547.3 ± 116.7
8,9-Epoxy-10-acetoxythymyl angelate (**3**)	0.316	1778.3 ± 108.7
	1	1804 ± 107.7
	3.16	1662.7 ± 166.5
	17.7	1790.9 ± 189.9
MOR	3.16	2371.6 ± 122.1***
VEH		1597.6 ± 52.2
4	0.1	1889.2 ± 271.4
	0.316	2196.2 ± 141*
	1	2245.4 ± 178.1*
	17.7	2114.1 ± 113.1*
MOR	5	3372.8 ± 100***
VEH		1483.3 ± 91.6
5	0.316	1474.9 ± 260.8
	1	1998.8 ± 86.1*
	3.16	1964.1 ± 207.6*
	17.7	1948.6 ± 125*
MOR	5	3221.4 ± 133.4***
VEH		1483.3 ± 91.6
13	0.316	1642.7 ± 281.8
	1	1986 ± 55.3*
	17.7	1709.8 ± 86.9
MOR	5	3221.4 ± 133.4***
VEH		1371.4 ± 120
15	0.316	1773.4 ± 122.6
	1	2253.3 ± 133.7**
	17.7	1759.6 ± 209.2
MOR	5	2900.9 ± 178***
VEH		1371.4 ± 120
17	0.316	1670.7 ± 62.1
	1	2101 ± 223.9**
	17.7	1775.1 ± 74.1
MOR	5	2900.9 ± 180***

Data are expressed as the mean ± SEM for the number ($n = 6$) of animals in every group. ANOVA followed by Dunnett post hoc $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

that the thymyl esters of *Hofmeisteria schaffneri* are responsible of the antinociceptive activity of the different preparations tested in this work.

Thymol (**2**) was only active at the dose of 100 mg/kg, however it has been previously shown that this compound partially block voltage-operated Na^+ (Haeseler et al., 2002) and K^+ (Elliot and Elliot, 1997) channels and directly activates γ-aminobutyric acid $GABA_A$ receptors (Mohammadi et al., 2001). It was also suggested that the demonstrated effects accounted for its pain-reliever properties and contributed to its sedative–hypnotic actions (Haeseler et al., 2002). On the other hand, thymol inhibited prostaglandin synthesis by microsomes of bovine tooth pulp. This inhibition was reversible and probably related with the analgesic effect of thymol in endodontic therapy (Anamura et al., 1988). Furthermore, Beer et al. (2007) speculated that thymol could have analgesic effect due to its agonistic effect on α1-, α2- and β-adrenergic receptors.

Table 5
Antinociceptive effect of hofmeisterin (**1**) from *Hofmeisteria schaffneri* in the presence of GLIB (10 mg/kg), NLX (1 mg/kg) or L-NAME (30 mg/kg).

Treatment	AUC ± E.E
VEH	1373.7 ± 78.2
1 (1 mg/kg)	1950.2 ± 123.4**
1 (1 mg/kg) + GLIB (10 mg/kg)	1716.7 ± 433.7*
1 (1 mg/kg) + NLX (1 mg/kg)	1380 ± 96.2
1 (1 mg/kg) + L-NAME (30 mg/kg)	1817.9 ± 314.6**
MOR (3.16 mg/kg)	3070 ± 96.2***

Data are expressed as the mean ± SEM for the number ($n = 6$) of animals in every group. ANOVA followed by Dunnett post hoc $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.3. Antinociceptive effect of thymol derivates (*6*–*17*) in hot plate test

Structural modification of a lead molecule could improve its efficacy since the pharmacokinetics parameters as well as the interaction with the molecular targets could be improved. Therefore, 12 analogs of compound **1**, namely **6**–**17**, were prepared condensing thymol with the corresponding acid chlorides in a single step reaction. The resulting esters were then evaluated in the same pharmacological model. Compounds **9**, **10**, **11** and **13** are new chemicals entities but compounds **6** (Paolini et al., 2005), **7** (Schmitz et al., 1979; Paolini et al., 2007), **8** (Rice and Coats, 1994; Ateeque et al., 2002), **12** and **14** (Viana et al., 1981; Ateeque et al., 2002), **15** (Sheng et al., 1984), **16** (Kumar et al., 2008) and **17** (Viana et al., 1981; Ateeque et al., 2002) have been reported as natural or synthetic products and their spectral data are in agreement with those previously described. Compounds **9**–**11** and **13** were identified by spectral means. In all cases their IR exhibited the typical phenolic ester carbonyl at ∼1760 cm^{-1}. The NMR spectra (Table 1) were very similar to those of the other thymyl esters under study with the aromatic region displaying the characteristic ABX system of the trisubstituted benzene ring of thymol (Table 1). The spectra differed only in the resonances due to the acid residues which were easily assigned to a tiglic, 2-methylvaleric, 3-methylvaleric, 4-methylvaleric and hexanic acids, respectively by comparison with the information previously reported for similar structures (Mathela et al., 2008; Martinez et al., 1988; Paolini et al., 2005, 2007; Schmitz et al., 1979; Rice and Coats, 1994; Viana et al., 1981; Ateeque et al., 2002; Sheng et al., 1984; Kumar et al., 2008).

Testing all analogs (**6**–**17**) at three different doses in the range of 1–17.7 mg/kg revealed that only compounds **13**, **15** and **17** were active. These results indicated that the nature of the ester chain did not have a clear impact on the antinociceptive activity (Table 4).

Considering that the stability of drugs in biological systems plays an important role in their activity (Di et al., 2005) and that degradation of drug esters in biological matrices is mainly attributed to their hydrolysis by esterases, the stability *in vitro* of the most active (**1**, **4** and **5**), and one inactive (**6**) thymyl esters was assessed during 24 h. The stability study was qualitatively performed measuring the susceptibility to hydrolysis of esters **1** and **4**–**6** in the presence of a commercial pig liver estearase solutions in two buffers (pH 7.4 and 5.0) as well as in mouse plasma at 37 °C (Di et al., 2005). The hydrolysis of the esters was monitored on a period of 24 h by TLC.

According to the results summarized Table 6, in general, the ester compounds were unstable in blood plasma; only compound **1** was stable during the first hour of the experiment. In the presence of the enzyme the degradation time was different for each compound. Thus, compound **1** was stable in the control solutions (pH 5.0 and 7.4) throughout the experiment; however, it was totally hydrolyzed in the enzymatic solution (pH 7.4) and mouse plasma after 4.5 h. On the other hand, at pH 5.0 this compound was partially hydrolyzed after the same period of time and completely hydrolyzed after 24 h. These results suggested that compound **1** could have an acceptable stability in the gastro-intestinal tract.

Table 6
In vitro stability of thymol esters derivatives toward pig liver estearase.

Compounds	pH 7.4						pH 5.0						Mouse plasma					
	1 h		4.5 h		24 h		1 h		4.5 h		24 h		1 h		4.5 h		24 h	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	–	–	++	–	++	–	–	–	+	–	++	–	+	–	++	–	++	–
4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
5	+	–	++	+	++	+	–	–	+	–	++	–	++	–	++	–	++	+
6	++	–	++	–	++	++	++	–	++	–	++	–	++	–	++	–	++	–
4NPA	++	–	++	–	++	–	++	–	++	–	++	–	++	–	++	–	++	–

A: esterase or mouse plasma; B: buffer or distilled water; h: hours.
–: not hydrolyzed; +: partially hydrolyzed; ++: totally hydrolyzed.

Compound **5** was stable in buffer solution at pH 5.0 but partially hydrolyzed at pH 7.4 after 4.5 h. With regard to mouse plasma and enzymatic solutions, this compound was totally hydrolyzed at 1 and 4.5 h, respectively. Therefore one would expect a similar gastrointestinal stability than for compound **1**. Finally, esters **4** and **6** were completely hydrolyzed in all test conditions; this result raised the possibility that these compounds might hydrolyze before intestinal absorption.

On the whole, the results indicated that the better antinociceptive activity of compounds **1** and **5** could be partially due to a better stability, however in the case of **4** other factors might be involved such as a better absorption *in vivo*, although substrate specificity differences *in vitro* could not be ruled out. Further work is in progress to determine the bioavailability of these esters *in vivo*.

4. Conclusions

The present investigation provided evidence of the efficacy of several preparations of *Hofmeisteria schaffneri* as antinociceptive agents. The most active preparation was the essential oil which contained large amount of hofmeisterin III (**1**) and other thymyl derivatives. According to the experimental results, ester **1** was the major antinociceptive active principle of the essential oil. Its pharmacological effect seems to be partially mediated by an opioid mechanism since naloxone inhibits its action. In addition, this compound was more effective as antinociceptive agent than several synthetic and natural analogs discovered in the present work; and although it was not possible to establish a clear structure–activity relationship, the stability tests revealed that **1** is less susceptible to enzymatic degradation and, probably more bioavailable than the remaining analogs tested in this investigation.

The phyto-preparations analyzed in this study were not toxic according to the Lorke's test, therefore the plant they might be secure for human consumption considering its long term use and the lack of acute toxicity for mice.

Altogether, the antinociceptive effect demonstrated in this work for *Hofmeisteria schaffneri* and some of its metabolites are in agreement with the popular use of this species in Mexican folk medicine for the treatment of painful stomach complaints.

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