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Sphaeralcea angustifolia (Cav.) G. Don extract, a potential phytomedicine to treat chronic inflammation

[Extracto de *Sphaeralcea angustifolia* (Cav.) G. Don, un potencial fitofármaco para tratar la inflamación crónica]

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Abstract

Biological properties of *Sphaeralcea angustifolia* have recently attracted attention because of the potential that this Mexican medicinal plant has as a remedy to treat inflammation. The dichloromethane extract of the plant's aerial parts inhibits pro-inflammatory molecules such as Interleukin (IL)-1, Tumor necrosis factor (TNF)-α, and IL-6 with a maximum expression in the late phase, an effect that is consistent with the findings in the present study, which showed that in rats with induced chronic inflammation by means of Complete Freund's adjuvant (CFA), the extract (100 mg/kg per day for 8 days) produced a sustained and significant inhibition of the edema (62.6%). Topical application of the dichloromethane extract produced ear edema reduction (50.6%) and a protective effect against 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced mouse ear irritation. Active fractions contain β-sitosterol, stigmasterol, α- and β-amyrins, transcinnamic acid, and scopoletin, with scopoletin the main constituent found in the more active fraction of the extract. The results obtained here demonstrated both the systemic and topic effects of *S. angustifolia* on experimental chronic-inflammation models with no evidence of toxic effects of the extract.

Keywords: *Sphaeralcea angustifolia,* scopoletin, Freund's adjuvant-induced arthritis, anti-inflammatory plant.

Resumen

Las propiedades biológicas de *Sphaeralcea angustifolia* han atraído recientemente la atención debido al potencial de esta planta medicinal mexicana como remedio para tratar la inflamación. El extracto de diclorometano de las partes aéreas inhibe moléculas pro-inflamatorias como la IL-1, TNF-α e IL-6 con una máxima expresión en la fase tardía, este efecto es consistente con los hallazgos del presente estudio que mostró que en ratas con inflamación crónica inducida con adyuvante de Freund completo, la administración del extracto (100 mg / kg por día por 8 días) produjo una inhibición sostenida y significativa del edema (62.6%).

La aplicación tópica del extracto de diclorometano produjo reducción del edema (50.6%) y un efecto protector contra la irritación producida por TPA en la oreja del ratón. Las fracciones activas contienen β-sitosterol, estigmasterol, α- y β- amirinas, ácido trans-cinámico y escopoletina, siendo escopoletina el componente principal en la fracción más activa del extracto. Los resultados obtenidos demostraron tanto efectos sistémicos como tópicos de *S. angustifolia* en modelos experimentales de inflamación crónica.

Palabras Clave: *Sphaeralcea angustifolia,* escopoletina, artritis inducida con adyuvante completo de Freund, planta anti-inflamatoria.

INTRODUCTION

The genus *Sphaeralceae* (Malvaceae) contains about 40 species found mainly in western North America, with > 50% of these distributed in Mexico. Some of these species are used in traditional medicine practices. Navajo Indians, for example, the leaves of *Sphaeralcea coccinea* are utilized in lotions to relieve skin diseases, or the fresh mashed leaves are employed as poultices on inflamed skin, sores and wounds (Wyman and Stuart, 1941). In Mexico, the representative medicinal species of the genus is *Sphaeralcea angustifolia* Cav. G. Don., a plant commonly known as *yerba del negro*, *vara de San José,* or *tlixihuitl* in the Nahuatl language, which is used as an anti-inflammatory and wound-healing remedy. According to ethnobotanical information, the fresh plant is topically applied as a poultice that covers the affected tissue; sometimes, dietary oil is added to the plant. Also, the decoction of the aerial parts of *S. angustifolia* in combination with other medicinal species is administered orally for the treatment of stomachache and diarrhea and the root has been reported for treating bladder disorders (Aguilar *et al*., 1994).

The anti-inflammatory effect of the chloroform extract was reported in a preliminary screening study in which some plant extracts were tested for acute inflammation response (Meckes *et al*., 2004). Later, it was demonstrated that in rat adjuvant-induced arthritis model, intraperitoneal (i.p.) administration of the dichloromethane (CH_2Cl_2) extract produced significant inhibition of Interleukin (IL)-1β, IL-6, and Tumor necrosis factor (TNF)-α and increased levels of the anti-inflammatory IL-10. These cytokines showed maximum expression in the late phase of the inflammatory process (from day 12 on) (Juárez-Ciriaco *et al*., 2008).

As part of ongoing research into the therapeutic potential of *S. angustifolia*, the present study was undertaken to evaluate the effect produced by repeated doses of the CH_2Cl_2 extract of the aerial parts of the plant on induced chronic-inflammation response (rat paw edema with complete Freund's adjuvant and ear edema induced topically with multiples doses of 12-O-tetradecanoyl phorbol-13-acetate [TPA] in mouse). Likewise, phytochemical analysis of the extract was carried out to identify the active principles, responsible for the anti-inflammatory effect.

Taking into account that to our knowledge no scientific reports on the toxicity of this plant are available, evaluation of acute and sub-acute toxic parameters in rodents was undertaken. Because the topic application of the plant is frequently employed, dermal toxicity of the crude extract was also assayed.

MATERIALS AND METHODS
Plant material
S. angustifolia (Cav.) G. Don was collected in the State of Hidalgo, Mexico, and was botanically identified by Abigail Aguilar, M.Sc. A voucher specimen was deposited at the Mexican Institute of Social Security Herbarium (IMSSM) with code number 14294. A CH_2Cl_2 extract was obtained by macerating the dried aerial parts of the plant (3 kg) at room temperature after these had been previously defatted with hexane. The extract was concentrated at reduced pressure to obtain 30.6 g of a solid extract (1.02%).

Experimental animals
Male Sprague Dawley rats (body weight, 200 - 225 g) bred under conventional conditions (21 $\pm$ 1° C, 50 - 70% humidity, sterilized bed), fed with Purina pellets and with sterilized water to drink were used. Housing conditions and all *in vivo* experiments complied with the guidelines established by the local Ethics Committee for Experimentation in Animals in Mexico (Ministry of Agriculture, NOM-062-ZOO-1999, Mexico), modified in 2001, and were approved by the Institutional Animal Care and Use Committee.

Adjuvant-induced chronic inflammation in rats
Adjuvant polyarthritis was induced as described by Andersen (Andersen *et al*., 2004). Briefly, eight animals per group were injected with 0.1 mL of Complete Freund's adjuvant (CFA, heat-killed *Mycobacterium tuberculosis*; Sigma F-5881) in one of each of the animals' hind paws.

Twelve days after inoculation with CFA, the animals were treated with the extract for 8 consecutive days (daily doses of 1 g/kg oral administration or 100 mg/kg intraperitoneal administration). Oral doses were administered intragastric twice daily, while when administered intraperitoneally (i.p.), the animals were injected with the extract only once. Control groups included CFA-injected animals that received the vehicle H_2O:Tween 80 (9:1). Progression of the inflammatory response in control groups and the effect produced in those receiving the plant extract was followed by measuring the ensuing edema with a plethysmometer (model 7140; Ugo Basile). In a

subsequent experimental study, the effect of the plant product was evaluated in the early phase of the inflammatory process by administering the extract (100 mg/kg i.p.) immediately prior to the sole injection of CFA, followed by eight daily treatments. Edema was measured 3 h after extract or after vehicle administration, and the percentage of inhibition was calculated with the following equation (Araruna and Carlos, 2010; Chaitanya *et al*., 2010):

$$\% \text{ inhibition} = \left(\frac{\Delta vt}{\Delta vc} \times 100\right) - 100$$

where Δvc is the increase in paw volume in the control group and Δvt is the increase in paw volume in animals treated with the extract. Edema formation in all cases was obtained respect to the paw volume measured at initial time T_0, before the application of carrageenan.

At the same time, Body weight (BW) in a group of animals injected with the adjuvant was monitored throughout the period and compared with that of a control group of normal animals injected with 0.1 mL of saline solution.

Mouse ear edema induced by multiple topical applications of 12-O-tetradecanoyl phorbol-13-acetate (TPA)

Male BALB-c mice (BW, 20–22 g, five animals per group) were used. According to Stanley (Stanley *et al*., 1991), edema was induced in the left ear by topical application of 2.5 µg of TPA dissolved in acetone for 5 alternate days. The extract (2 mg/ear) dissolved in acetone was topically applied to the same ear 30 min before TPA, simultaneously with the pro-inflammatory agent, or 60 and 180 min after TPA. The right ear received only acetone (vehicle control). Six hours after the final treatment, the mice were killed by cervical dislocation and plugs 6 mm in diameter from the central portion of both ears were taken and weighed. Extent of inflammation in a control group was determined as the difference in weights between the ear with TPA and the right ear with acetone, and edema reduction in the group of animals treated with the extract was expressed using the following equation:

$$\% \text{ inhibition} = \left(\frac{\Delta wt}{\Delta wc} \times 100\right) - 100,$$

where Δwc is the increase in ear weight in the control group and Δwt is the increase in ear weight in animals treated with extract.

Toxicological profile

Animals were maintained under standard environmental conditions at a 12-h light/dark photoperiod. All animals were supplied with food and water *ad libitum* during the testing period. Acute toxicity was determined in male BALB-c mice (BW, 22 ± 2.2 g) and in Sprague Dawley rats (BW, 220 ± 22.0 g) following the methodology previously described by Lorke (Lorke, 1983). The rodents were randomly divided into groups of three animals each. Daily doses of the extract (140-1,000 mg/kg) solubilized in H_2O:Tween 80 (9:1) were injected i.p to rats and daily doses of 140-5,000 mg/kg were injected into mice; a dose-dependent toxicity increase was determined in both animal species. Mortality was recorded within 15 days, and the 50% Lethal dose (LD_{50}) was calculated as the geometric mean by Probit. At the end of the study, the internal organs (lung, kidney, heart, spleen, and liver) were extracted and a gross pathological observation was performed.

Subacute toxicity was determined following the methodology previously described by OECD TG407 (OEDC, 2001). Sprague Dawley rats (BW, 220 ± 22.0 g) were randomly assigned to five groups with eight animals per group. Daily doses of 12, 25, and 50 mg/kg extract were administered i.p. during 14 consecutive days and two control groups (vehicle and saline solution) were included. General behavior was observed daily, and the weight of the animals was registered once a week. The animals that died during the test period were analyzed pathologically, and those that survived were examined at the end of the experimentation phase. On day 14, the rats were fasted overnight and were anesthetized with sodium pentobarbital (56 mg/kg) for blood collection from the cava vein. The biochemical parameters assessed utilizing Wiener Lab commercial kits were protein, alkaline phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, gamma-glutamyl transferase, triglycerides, glucose, cholesterol, urea, and total bilirubin. Hematological profile included hemoglobin, leukocyte and platelet counts. Urine was collected for a general analysis with URITEST 10 strips. Hematological and chemistry analyses were performed employing automated equipment (Coulter T890 and Selectra II, respectively).

The rats were sacrificed by cervical dislocation and, after a macroscopic examination of liver, lungs, heart, kidneys and spleen, necropsy was performed soon after death. Tissue biopsies from the organs were fixed in 10% formalin, and processed, and embedded in paraffin. Paraffin slides were cut at 4-5 μm with a rotary microtome and stained with hematoxylin and eosin. Samples were examined under light microscope with particular attention paid to organs that showed gross lesions.

Following the methodology described in NOM MGA0515 by Draize (Draize, 1959), the dorsal region of 24 New Zealand white rabbits (BW, 3.5 kg) was carefully shaved 24 h before the study and groups of eight animals each were considered in this assay. The animals were housed individually in stainless-steel cages and maintained on a 12 h dark/12 h light cycle. The day of the experiment, two areas of 4 cm^2 were delimited in the right dorsal region as well as in the left one, and one of these in each region was shaved again. The extract blended into vaseline in doses of 100 or 280 mg/patch was topically applied to all four areas, covering these with patches. The presence of erythema or edema was examined 24 and 72 h after extract application. A negative control group without the extract was included.

Statistical analysis
Differences between treated animals and those of the control group were evaluated statistically with the Student t test, with $p < 0.05$ considered significant. Results in toxicological studies were expressed as mean $\pm$ Standard error of the mean (SEM) and the statistical significance between the groups was analyzed by means of a one-way Analysis of variance ANOVA (ANOVA) coupled with the Student-Newman-Keuls test with $p < 0.05$ as the minimum requirement for a statistically significant difference.

Bio-guided fractionation
Mouse ear edema induced with a single dose of TPA (acute inflammation model) was employed as the biological test to monitor the activity of the CH_2Cl_2 extract and the fractions. The extract (11 g) was chromatographed in a glass column (900 $\times$ 45 mm) packed with silica gel (320 g; 0.063–0.200 mm; Merck, Darmstadt) and eluted with organic solvents of increasing polarity. A total of 179 fractions of 250 mL each were collected and combined according to their Thin layer chromatography (TLC) profiles. Elutes were analyzed by TLC with mixtures of hexane:AcOEt (7:3) and spots were visualized with Ultraviolet (UV) 240/340 nm and by spraying with 10% H_2SO_4, followed by heating the plates at 110° C. Active fractions F7 (CH_2Cl_2 100%) and F9 (CH_2Cl_2:MeOH 9:1) were the most abundant (2.50 and 6.57 g, respectively). Fraction F7 (1 g) was further fractionated in a 30 g silica gel column (600 $\times$ 15 mm) to obtain active fractions F7–5 (hexane:CH_2Cl_2 6:4) and F7–8 (hexane:CH_2Cl_2 1:1). A mixture of α- and β-amyrins (40 mg) was precipitated from F7–5 and a mixture of stigmasterol and β-sitosterol (30 mg) was precipitated from F7-8. The compounds were identified by Gas chromatography-Mass spectroscopy (GC-MS) analysis and comparative TLC with authentic samples. Further, F9 (3 g) was subjected to silica gel (50 g) column chromatography (600 $\times$ 25 mm) with a gradient of hexane, $CHCl_3$, and MeOH to yield 576 mg of the active fraction F9–8 ($CHCl_3$:MeOH 9:1). Fraction F9–8 was separated by preparative TLC to isolate scopoletin, a hydroxycoumarin that was identified by comparing GC-MS data with those reported in the literature (Murray *et al*., 1982). GC-MS spectra were obtained in a Hewlett Packard model 5890 series II gas chromatography interfaced with a JEOL JMS AX50HA mass spectrometer at 70 eV with a GC column HP-5MS (30 m - 0.25 mm i.d. and 250 μm). The carried gas was He (Praxair) with 7 psi, 1 mL/min. The temperature was programmed from 100–300° C at the rate of 10° C $\times$ min; injection temperature was 260° C.

Concentration of scopoletin in the extract was determined by High performance liquid chromatography (HPLC-UV) diode array detection (Waters). The samples were chromatographed in a reverse phase Spherisorb ODS2 column (Waters, 250 $\times$ 4 mm; 5 μm) with a mobile phase of 0.01 M phosphoric acid (A) and methanol (B). Gradient elution was carried out; initial composition was 20% B and this increased to 80% B in 22 min, subsequently returning to initial composition in 6 min. The wavelength was set at 340 nm to detect scopoletin (Retention time [Rt] = 15.8 min). Scopoletin was detected by comparison of Rt and UV spectra with those of a certificated reference compound provided by the Institute of Chemical Metrology, Chile Foundation. A negligible signal of transcinnamic acid was also detected (Rt = 22.9 min) and was identified by comparison of RT with a commercial sample (Sigma).

RESULTS
The Effect of the CH₂Cl₂ Extract in Chronic Inflammation Models
Daily doses of 100 mg/kg extract administered i.p. for 8 consecutive days, initiating administration 60 min prior to the CFA challenge, produced significant inhibition of edema only in the first 2 days (42.90 and 49.37%, respectively); afterward, edema formation was the same as that determined in the control group (Table 1).

Table 1
Effect of the dichloromethane (CH₂Cl₂) extract in the onset of the chronic inflammation response

	Increase in paw volume (mL)		
Time (days)	Control Group	Treatment Group	% Inhibition
0	1.36 ± 0.04	1.30 ± 0.02	0
1	3.06 ± 0.10	2.27 ± 0.17*	42.94
2	2.94 ± 0.18	2.10 ± 0.07*	49.37
3	2.24 ± 0.08	1.99 ± 0.07	21.59
6	2.48 ± 0.08	2.38 ± 0.07	3.57
8	2.25 ± 0.10	2.13 ± 0.07	6.74

Doses of the extract (100 mg/kg × 8 days, intraperitoneally [i.p.]) administered starting on the day of the Complete Freund's adjuvant (CFA) challenge. The vehicle was H₂0:Tween20, 9:1. Values are means ± Standard error of the mean (SEM) ($n = 8$). ***P < 0.05**.

Phenylbutazone (100 mg/kg) demonstrated a similar profile, although with lower activity (ca. 20% during the first 48 h) (data not shown). In the chronic phase of the process, administration of the extract beginning 12 days after CFA injection caused sustained decrease of the edema, ranging from 40.2–50.1% after three doses of the extract had been administered. The percentage of inhibition of edema was 62.6%, even 24 h after the last treatment (Table 2). In this same experimental model, oral administration of 1 g/kg of the plant extract failed to reduce the formation of edema (data not shown). BW of the normal rats increased 40-50% at the end of the study; in contrast, an increase of 10.8% was registered in animals treated with CFA and a 22.43% increase in those receiving the extract (data not shown).

In the chronic topical inflammation model induced with multiple applications of TPA, the extract maintained an anti-edematous effect (50.6%) even when the extract was applied 180 min after each TPA treatment (Table 3). As shown in this table, a dose of extract of 2 mg/ear applied 30 min prior to treatment with TPA reduced the induced edematous effect in 41.1%.

Table 2
Effect of the dichloromethane (CH_2Cl_2) extract in the declared chronic inflammation response

	Increase in paw volume (mL)		
Time (days)	Control Group	Treatment Group	% Inhibition
0	2.14 ± 0.07	1.85 ± 0.08	0
12	3.19 ± 0.20	3.04 ± 0.09	NE
13	3.05 ± 0.12	2.77 ± 0.15	NE
14	3.11 ± 0.13	2.43 ± 0.07*	40.2
15	3.25 ± 0.10	2.47 ± 0.07*	44.1
16	3.74 ± 0.16	2.66 ± 0.06*	49.4
19↓	3.73 ± 0.22	2.58 ± 0.06*	50.1
20	4.09 ± 0.21	2.58 ± 0.05*	62.6

Doses of the extract (100 mg/kg x 8 days; i.p.); intraperitoneally [i.p] administered starting on day 12 of the Complete Freund´s adjuvant (CFA) challenge. The vehicle in control group was H_20:Tween 20, 9:1. Values are means ±Standard error of the mean (SEM) (n=8), * $P < 0.05$ and % inhibition. NE = no effect; ↓ Last extract administration.

Table 3
Effect of the dichloromethane (CH_2Cl_2) extract on dermal inflammation in mouse ear

Extract application	Ear edema (mg)	% Inhibition
Control (vehicle)	13.10 ± 0.10	0
30 min before TPA	7.06 ± 0.31*	41.1
Simultaneously with TPA	4.92 ± 0.85*	64.5
60 min after TPA	5.56 ± 0.71*	51.3
180 min after TPA	5.06 ± 0.53*	50.6

Doses of the extract (2 mg/ear x five application) were topically applied: 1) 30 min before TPA, 2) simultaneously with TPA, or 3) 60 and 180 min after the extract. The vehicle was acetone. Values are means ± Standard error of the mean (SEM) (n = 8), * $P < 0.05$.

Toxicity of the Extract

Using the abbreviated procedure of Lorke, the acute toxicity LD_{50} index for the crude extract was estimated as 350 mg/kg (i.p.) in rats and as 2150 mg/kg (i.p.) in mice. A non significant decrease in BW was determined in all of the treated groups.

The sub-acute toxicity test in rats showed that i.p. administration of the CH_2Cl_2 extract was well tolerated by the animals, and daily doses of 12.5-50 mg/kg for 14 consecutive days did not produce mortality. In comparison with the controls, no significant differences in BW gain throughout the treatment period were determined.

In rats treated with 50 mg/kg of the extract, serum triglyceride levels increased 32.4% but High density lipoprotein concentration (HDL) was 23.8% lower than that in the control group. Likewise, a slight increase in the relative weight of the spleen was detected with the higher dose of the 50 mg/kg extract, whereas no significant differences were determined for the remainder of the organs evaluated. On the other hand, generalized, dose-dependant fibro-adhesive peritonitis occurred in the animals injected with the extract and evident treatment-related changes were identified in the liver of the rats at all dose levels.

Steatosis was detected in animals receiving 50 mg/kg of the extract. However, administration of the previously filtrated extract through Filtropur S 0.2-20 μm filters did not produce significant changes in hematological parameters. Similarly, the filtrated extract did not produce noticeable alterations in the liver compared with the control group, exhibiting normal parenchymal architecture with cords of hepatocytes and portal tracts. No further evidence of histopathological changes could be attributed to the extract and the biological evaluation of the filtrated product showed preserved activity of the original extract. In conclusion, no sign of irritation was attributed to the extract in dermal test carried out in rabbits.

Bio-guided Chemical fractionation of the extract

A single dose of 2.5 μg/ear TPA in mouse induced an edematogenic response that was evidenced by a significant increase in ear weight. The higher dose (2 mg/ear) was employed to test the crude extract and the primary fractions, while smaller doses were used to evaluate the second fractions and the pure compounds. Topical application of 2 mg/ear crude extract 30 min after TPA reduced edema formation by 73.1%, an

effect near that determined with the same dose of indomethacine (75%). The two major active fractions, F7 and F9, inhibited edema by 55.1 and 70.6%, respectively. On the other hand, subfraction F7-8 at a lower dose of 0.5 mg/ear significantly increased anti-edematous activity to 73.2%; this effect was higher than that determined with the mixture of identified compounds in F7 (a mixture of α- and β-amyrin and β-sitosterol) at the same dose. In this model, no effect was observed with pure isolated stigmasterol (Table 4). On the other hand, at a dose of 1 mg/ear, inhibitory activity of subfraction F9–8 was 55.3% and isolated scopoletin from this fraction inhibited 54.2%, approximating the effect obtained with a commercial sample of scopoletin (52.2%).

The content of scopoletin in the extract determined by HPLC with the certificated reference was 0.5 mg/g. Determined IC_{50} values were 0.37, 0.48, and 0.88 mg/ear for the extract, scopoletin, and β-sitosterol, respectively, higher than that of indomethacine ($IC_{50} = 0.10$ mg/ear).

DISCUSSION

The effect produced by the i.p. administration of repeated doses of *S. angustifolia* CH_2Cl_2 extract was evaluated in the chronic inflammatory response induced with CFA in rat, and two strategies were designed for this purpose. Administration of the extract immediately prior to injection of the adjuvant and repeating the treatment for 8 consecutive days produced a significant reduction of edema formation only in the early stage of the inflammatory response (during the first 48 h). Conversely, once the chronic phase of inflammation was already triggered (12 days after the CFA challenge), daily administration of the extract produced from the second dose onward, sustained and significant inhibition of edema, the effect persisting even 24 h after the last treatment. The results obtained are in accordance to a parallel study performed that demonstrated that in the CFA model, IL-1β, IL-6, TNF-α, and IL-10 have maximum expression in the late phase of the inflammatory response and that the *S. angustifolia* extract produce significant inhibition of proinflammatory IL-1β, IL-6, and TNF-α, but increased anti-inflammatory IL-10 levels (Juárez-Ciriaco *et al*., 2008). Although a significant anti-inflammatory effect of the extract was established when administered i.p., oral administration of this plant product lacked its inhibitory activity, even

at a high dose of 1 g/kg daily administered for 10 days. This observation allows the assumption that oral use of the plant as a decoction to treat intestinal disorders might be related with other compounds that are not present in the dichloromethane extract. The anti-inflammatory effect of the *S angustifolia* reported by

Meckes *et al*., 2004 in the acute inflammation model with carrageenan in rats, in addition to that observed in this work, permit us to know, in part, the therapeutic potential of this plant.

Table 4
Activities of the dichloromethane (CH$_2$Cl$_2$) extract, fractions and pure compounds in mouse ear edema model

Sample	Dose (mg/ear)	Mouse ear edema (mg) Control	Treated	Inhibition (%)
CH$_2$Cl$_2$ extract	2	13.21 ± 0.44	3.55 ± 0.42	73.1
F7	2	7.81 ± 0.68	3.50 ± 0.32	55.1
F7-5	1	7.90 ± 0.84	5.73 ± 0.83	27.5
*α-+β- amyrin	0.5	11.03 ± 0.75	5.30 ± 0.85	52.0
F7-8	0.5	9.97 ± 0.10	2.67 ± 0.24	73.2
*β Sitosterol+Stigmasterol	0.5	12.12 ± 0.87	9.47 ± 0.28	21.9
F9	2	8.93 ± 0.81	2.63 ± 0.65	70.6
F9-8	1	12.87 ± 0.30	5.75 ± 0.34	55.3
Scopoletin (isolated)	1	11.32 ± 0.34	5.18 ± 0.60	54.2
COMMERCIAL REFERENCES				
Scopoletin	1	11.98 ± 0.14	5.73 ± 0.22	52.2
β-sitosterol	0.5	12.75 ± 0.51	6.70 ± 0.97	47.5
Stigmasterol	0.5	11.57 ± 0.16	11.84 ± 0.58	NE
Indomethacine	0.5	11.04 ± 0.69	2.76 ± 0.52	75.0

The samples were tested in the acute mouse ear edema model (single dose of TPA). NE= no effect. * Mixture of compounds in the fraction. Values are means ± Standard error of the mean (SEM) (n = 6).

In addition to the demonstrated anti-inflammatory effect of the extract on the adjuvant-induced arthritis model, topical application of the extract suppressed ear edema in mouse. It has been established that multiple cutaneous applications of TPA to mouse ear produce a prolonged inflammatory reaction characterized by edema formation, inflammatory cell infiltration, and epidermal

hyperplasia (Stanley *et al*., 1991). In this model, the extract exhibited both strong anti-inflammatory action and a protective effect against the irritant. Chemical study of the plant extract produced active fractions containing β-sitosterol, stigmasterol, α- and β-amyrins, transcinnamic acid, and scopoletin. The latter was the main constituent found in the more active fraction of the extract, its anti-edematous effect in the

ear inflammation model was nearly comparable with that of the whole fraction. Scopoletin has been isolated from several plant species and, recently, new information related with its anti-arthritic properties has appeared in the scientific literature (Pan *et al*., 2009; Pan *et al*., 2010). It was reported that when injected i.p. in adjuvant-induced arthritic rats at doses > 100 mg/kg of the compound, the latter produced nearly normal histological architecture of the joints, downregulate the overexpression of Vascular endothelial growth factor, basic Fibroblast growth factor (bFGF), and IL-6 in the synovial tissues. In short, it reduces the numbers of new blood vessels in the synovium and the production of important endogenous angiogenic inducers (Pan *et al*., 2009).

It is well known that cytokines such as IL-1, TNF-α, and IL-6 are also proinflammatory *in vitro* and scopoletin was able to inhibit these cytokines in Lipopolysaccharide (LPS)-stimulated mouse leukemic monocyte macrophage cell line (RAW) 264.7; in addition, scopoletin inhibited the production of Prostaglandin E2 (PGE2) and Cyclooxygenase-2 (COX-2) (Meckes *et al*., 2004).

As shown in Table 3, the subfraction F7-8 has a good activity at a dose of 0.5 mg/ear (73% inhibition) and it probably contains, besides β-sitosterol, any other compound which was not detected in this study. We assume that it is a compound that acts in interaction with the phytosterol whose activity is around 47.5%; unfortunately, the amount of F7-8 obtained was low and the compound was not identified.

The toxicological studies available on i.p. administration of the extract did not indicate a severe systemic toxicity, adverse reactions, or hematological and biochemical alterations when the extract is previously filtered. Histopathologic examination performed on select tissues from the control and the high-dose group indicated no treatment-related changes considered to be of toxicological significance.

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CONCLUSION

In an experimental arthritis model, the *S. angustifolia* dichloromethane extract exerts a significant anti-edematous effect on the CFA-induced chronic inflammation phase, results that could be associated with the previously reported inhibition of IL-1β, IL-6, and TNF-α. In addition, topical application of the extract produces strong anti-inflammatory action and a protective effect against TPA mouse ear irritation. Active fractions contain β-sitosterol, stigmasterol, α- and β-amyrins, transcinnamic acid, and scopoletin, with scopoletin the main constituent found in the extract´s more active fraction. The anti-edematous effect in the ear inflammation model was nearly comparable with that of the whole fraction. No treatment-related changes were considered to possess toxicological significance when the extract was previously sterilized by filtration.

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