

Effect of Dichloromethane-Methanol Extract and Tomentin Obtained from *Sphaeralcea angustifolia* Cell Suspensions in a Model of Kaolin/Carrageenan-Induced Arthritis



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Supporting information 1H NMR spectra of tomentin, HPLC chromatograms of SaDM extract, tomentin, and sphaeralcic acid, and a curve of tomentin doses and joint edema size are available online at <http://www.thieme-connect.de/products>.

ABSTRACT

Cell suspension cultures from *Sphaeralcea angustifolia* produce compounds with anti-inflammatory activity, including scopoletin, tomentin, and sphaeralcic acid. Antiarthritic activity of scopoletin isolated from *S. angustifolia* and *Erycibe obtusifolia* wild plants was demonstrated in a complete Freud's adjuvant-induced arthritis model. In this project, the dichloromethane-methanol extract and tomentin isolated from *S. angustifolia* cell suspension were evaluated in mice using a kaolin/carrageenan-induced arthritis model. After 9 days of treatment, the anti-inflammatory effect of *S. angustifolia* dichloromethane-methanol extract was 72 % at a dose of 100 mg/kg, a recovery of 55 % was observed with methotrexate (5.0 mg/kg), and the anti-inflammatory effect for tomentin was dose-dependent with a median effective dose of 10.32 mg/kg. The concentration of pro-inflammatory cytokines interleukin-1 β and tumor necrosis factor alpha in the left joint of groups treated with *S. angustifolia* dichloromethane-methanol extract and tomentin at different doses was significantly lower than that detected in the group damaged with kaolin/carrageenan, and the concentration of interleukin-10 and interleukin-4 (anti-inflammatory cytokines) was significantly higher. The drop in mean body weight of mice treated with methotrexate (-4.34 ± 0.47 g) was higher in comparison with the kaolin/carrageenan group (-2.40 ± 0.29 g). On the other hand, the mean body weight of mice treated with *S. angustifolia* dichloromethane-methanol extract (-1.19 ± 0.17 g) was similar, and mice treated with tomentin (0.19 ± 0.11 g) was lower. The dichloromethane-methanol extract and tomentin isolated from *S. angustifolia* diminished joint edema induced by kaolin/carrageenan, possibly by acting as an immunomodulatory of the inflammatory response.

Abbreviations

BW	body weight
CFE	λ -carrageenan footpad edema
CFA	complete Freud's adjuvant
ED ₅₀	median effective dose
IL	interleukin
K/C	kaolin/carrageenan
MS	Murashige and Skoog
PSS	physiological saline solution

SaDM	<i>Sphaeralcea angustifolia</i> dichloromethane-methanol
TPA	12-O-tetradecanoyl phorbol-13-acetate

Introduction

Rheumatoid arthritis is a progressive and chronic autoimmune disease that causes systemic damage and that may occur in all ages, although it entertains a high prevalence in middle age. The joints

(synovial) are the most affected system, but the joints are also capable of disturbing other organs, such as the kidneys and lungs, among others. The disease develops as a painful condition, with loss of function and mobility. Later, the damage dimension is considerable since the quality of life of patients is diminished and enormous disability is caused [1].

Treatment of patients with this illness is based on the use of disease-modifying antirheumatic drugs and entertains the goal of diminishing the symptoms and preventing structural damage and disability; examples of these groups include etanercept, adalimumab, and methotrexate. Also, nonsteroidal anti-inflammatory drugs and glucocorticoids are employed. However, all of these drugs present several toxicity effects such as hepatotoxicity, myelosuppression, and an elevated risk of infections (bacterial and viral) [2]. There is a need for new treatments with the possibility of decreasing the collateral toxic effects of chronic therapy for rheumatoid arthritis, thereby improving the life quality of patients.

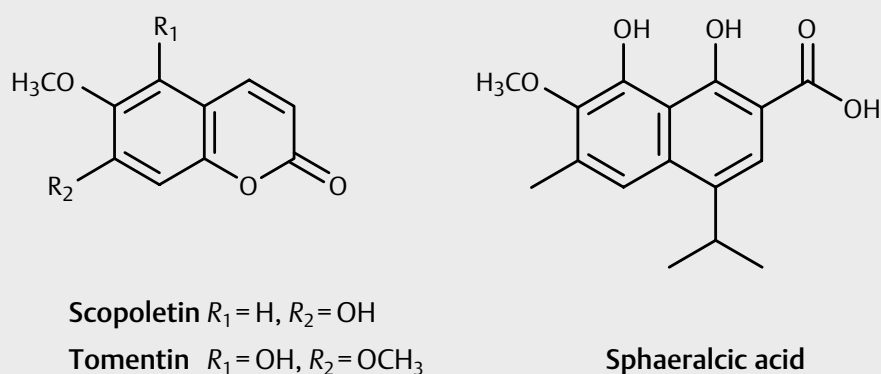
Fresh aerial tissues of *Sphaeralcea angustifolia* (Cav.) G. Don (Malvaceae) are employed to treat inflammatory processes [3–5]. The dichloromethane extract from the aerial tissues of this species was active in TPA-induced mouse ear edema, CFE in mice, and CFA-induced arthritis in rats. Scopoletin (► Fig. 1) was the active compound detected in the extract [6–8]. Since the collection of *S. angustifolia* plants from their natural habitat is restricted by the Mexican ministry of the environment and natural resources (SEMARNAT, NOM-059-ECOL-2001), the cell suspension culture of this species was established to produce the bioactive compound. Cell suspension developed in MS medium, with the total nitrate concentration reduced to 2.74 mM, produces the anti-inflammatory compounds (► Fig. 1) scopoletin, tomentin, and sphaeralcic acid [9]. Intraperitoneal administration (100 mg/kg) of the dichloromethane extract from the medium ($42 \pm 3\%$) and the dichloromethane-methanol extract from the biomass ($39 \pm 9\%$) of the *S. angustifolia* cell suspension showed a similar anti-inflammatory effect on CFE. The dichloromethane-methanol (9:1, v/v) extract (SaDM) from the biomass exerted a dose-dependent inhibitory effect with an ED_{50} of 137.63 mg per kg on CFE [10]. The SaDM extract from the biomass (2.0 mg per ear) was also inhibited by TPA-induced mouse ear edema by 78 % [9]. Tomentin and sphaeralcic acid were active in both acute inflammatory models: the anti-inflammatory effects on

CFE at a dose of 45.0 mg/kg via the intraperitoneal route was 67 % for sphaeralcic acid and 62 % for tomentin. TPA-induced mouse ear edema was inhibited by sphaeralcic acid ($87 \pm 3\%$) and tomentin ($48 \pm 7\%$) to 1.0 mg per ear. The effect of sphaeralcic acid was dose-dependent with an ED_{50} of 93 mM [9]. The anti-inflammatory (52 %, 0.50 mg/ear) and antiarthritic effects of scopoletin isolated from *S. angustifolia* and *Erycibe obtusifolia* Benth. (Convolvulaceae) wild plants have already been reported [8, 11]. The cell suspension comprises a controlled culture with a high-quality production of scopoletin, tomentin, and sphaeralcic acid and is useful as an alternative for obtaining standardized extracts and for isolating compounds, with the purpose to carry out their evaluation in a K/C-induced arthritis animal model and to advance in the knowledge of its beneficial effects.

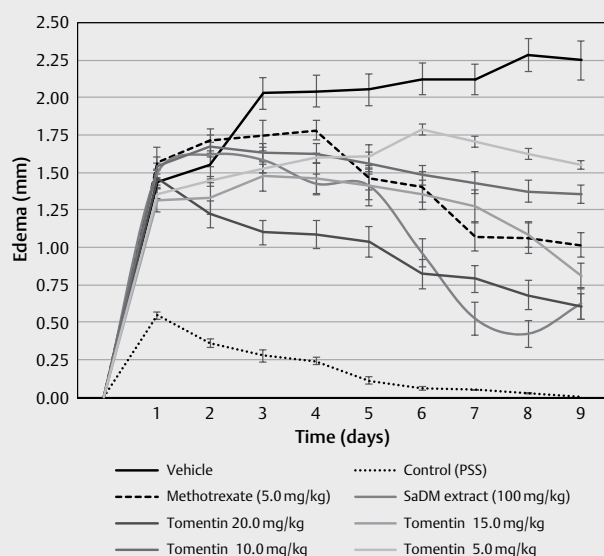
Results

The antiarthritic activity of SaDM extract and tomentin were evaluated in an arthritis model in mice induced with K/C for a period of 10 days. In this arthritis model, oral administration of vehicle 1 % Tween 20 (negative control) presented an increasing development of joint edema from the application of immunogens. Maximal inflammation was obtained on day 3 and this edema remained until day 9 (► Fig. 2). The animals were lethargic and had difficulty walking; the legs of the majority of these were fissured and secreted a thick yellowish liquid. Tissue inflammation was evaluated as a percentage with respect to baseline size. On day 9 after the induced damage, the joint increased its size by 2.25 mm; this parameter was considered 100 % of inflammation (► Table 1). In the PSS group, mice not receiving K/C, develop edema until day 1, which was lower (0.55 ± 0.08) than that developed by the negative control. Additionally, inflammation decreased with time and the animals recovered the initial size of their joints (► Fig. 2).

In the mouse group treated with 5.0 mg/kg per day of methotrexate, maximal inflammation developed at day 2 and continued to be evident at day 4. After this time, edema was continuously reduced until day 9 of treatment (► Fig. 2 and ► Table 1). The mice demonstrated no difficulty in supporting their legs and walking. In the group treated with 100.0 mg/kg of SaDM extract daily, inflammation remained constant for 3 days, and from day 4, the inflam-



► Fig. 1 Chemical structures scopoletin, 7-hydroxy, 6-methoxycoumarin; tomentin, 5-hydroxy-6,7-dimethoxycoumarin; and sphaeralcic acid, 2-(1,8-dihydroxy-4-isopropyl-6-methyl-7-methoxy) naphthoic acid.



► **Fig. 2** Effect of oral administration of methotrexate (5.0 mg/kg), SaDM extract, and tomentin (5.0, 10.0, 15.0, and 20.0 mg/kg) on left joint size in mouse arthritis model induced with K/C.

► **Table 1** Effect of SaDM extract from the biomass of *S. angustifolia* cell suspension and tomentin administered via the oral route on the left joint after 10 days of K/C-induced edema.

Treatment	Dose (mg/kg)	Edema (mm)	Edema inhibition (%)
Negative control (vehicle)	–	2.25 ± 0.13	–
Physiological saline solution (PSS)	–	0	–
Methotrexate	5.0	1.01 ± 0.08 **	55
SaDM extract	100.0	0.63 ± 0.10 **	72
Tomentin	5.0	1.55 ± 0.03 **	31
	10.0	1.36 ± 0.06 **	40
	15.0	0.81 ± 0.09 **	64
	20.0	0.61 ± 0.08 **	73
Mean ± SEM (n = 12). ** Edema volumes significantly different (p < 0.0001) according to the Dunnett test			

► **Table 2** Effect of SaDM extract from the biomass of *S. angustifolia* cell suspension and tomentin administered via the oral route on BW after 10 days of K/C-induced edema.

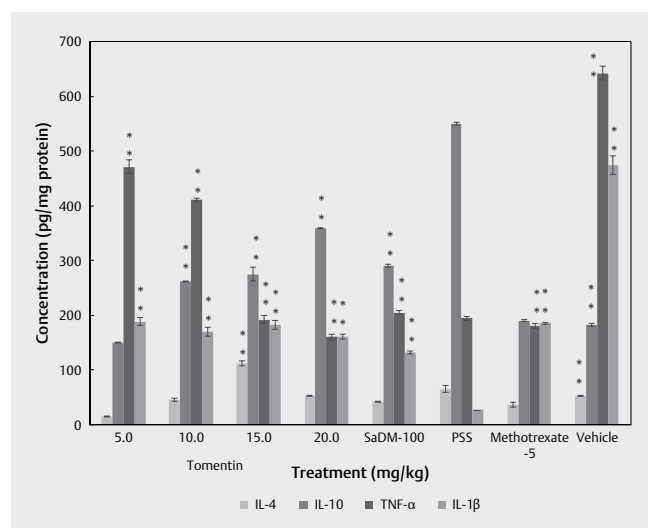
Treatment	Dose (mg/kg)	Body weight (day 0)	Body weight (day 9)	Weight differences (g)
Negative control (vehicle)	–	30.52 ± 2.58	29.02 ± 1.78	– 2.40 ± 0.29
Physiological saline solution (PSS)	–	28.06 ± 2.87	30.34 ± 2.50	2.28 ± 0.16
Methotrexate	5.0	30.18 ± 2.85	25.83 ± 2.81	– 4.35 ± 0.47 **
SaDM extract	100.0	31.29 ± 1.39	30.10 ± 1.24	– 1.19 ± 0.17
Tomentin	5.0	28.90 ± 1.57	27.59 ± 1.83	– 1.31 ± 0.19 **
	10.0	27.60 ± 2.16	27.78 ± 2.23	0.18 ± 0.03 **
	15.0	27.28 ± 1.74	27.70 ± 1.22	0.42 ± 0.08 **
	20.0	29.74 ± 2.26	30.65 ± 1.88	0.91 ± 0.06 **
Mean ± SE (n = 12). ** BW values significantly different (p < 0.0001) according to Student's t-test				

mation continuously decreased until day 9 (► **Table 1**). Redness of the leg was minimal, and its appearance was similar to that prior to the damage being caused.

In mice treated with pure tomentin compound, at 5.0 mg/kg, joint edema increased during the first 6 days (1.79 ± 0.03 mm), without reaching the edema volume of the negative control group. In mice treated with 10.0 and 15.0 mg/kg, the initial edema that formed remained until day 5 (1.68 ± 0.06 and 1.47 ± 0.10 mm, respectively), after which it declined (► **Table 1**). Only at the 20.0 mg/kg dose did the joint edema in the mice decrease from day 1 of treatment until day 9 (► **Fig. 2** and ► **Table 1**). Administration of methotrexate, SaDM extract, and tomentin caused a significant decrease of articular edema when treatments were compared with the negative control group ($p < 0.0001$). Tomentin exhibited dose-dependent inhibition of joint edema induced with K/C (► **Table 1**), with a median ED₅₀ of 10.32 mg/kg (**Fig. 1S**, Supporting Information). All groups of mice treated with tomentin showed no cracks in the legs, and no yellowish fluid secretion was observed.

The mean BW of mice in the PSS group increased during the study period, with a weight gain at day 9 (► **Table 2**). Articular administration of K/C caused a decrease in the BW of mice gradually, until day 9. Methotrexate-administered animals also tended to lose weight in a similar manner to that of the negative control group. According to the Student's t-test, the average weight loss at day 9 was superior ($p < 0.0001$) to that of the negative control. In contrast, in mice treated with the SaDM extract at 100.0 mg/kg, a lost weight was observed during the first 2 days (-1.92 ± 0.17 g), with a tendency to recover this from day 3. After 9 days, the weight loss was not statistically different from the negative control group. Mice treated with 5.0, 10.0, 15.0, and 20.0 mg/kg of tomentin showed a weight reduction only on day 1 of the induced damage, with a tendency to recover it. Weight loss in the group treated with 5.0 mg/kg was statistically lower than that registered in the negative control group (Student's t-test, $p < 0.0001$). In the groups treated with 10.0, 15.0 and 20.0 mg/kg, there was a slight increase in the BW. Nevertheless, the weight gain was lower than that of the PSS group (► **Table 2**).

To understand how the SaDM extract and tomentin exert their effects on arthritis, immunoanalyses were performed to examine the participation of inflammatory (TNF- α and IL-1 β) and anti-inflammatory (IL-4 and IL-10) cytokines in joint tissue derived from K/C-induced arthritic mice with or without treatment. Carrageen-



► **Fig. 3** Effect of SaDM and tomentin from *S. angustifolia* cell suspensions on tumor necrosis factor alpha (TNF- α), interleukin-1beta (IL-1 β), IL-4, and IL-10 in joint edema in mice induced with K/C. Each bar represents the mean \pm SEM, n = 4. ** Means represent differences of the treatment groups compared with the negative control group according to Student's t-test ($p < 0.0001$) and differences of the negative control group compared with the PSS group according to the Student's t-test ($p < 0.0001$).

an promoted a significant increase of TNF- α and IL-1 β concentration in comparison to the healthy (SSE) mice. On the other hand, the content of anti-inflammatory cytokines IL-4 and IL-10 (► **Fig. 3**) decreased significantly. In animals treated with 5 mg/kg methotrexate daily, levels of pro-inflammatory cytokines TNF- α and IL-1 β decreased in joints when compared with the negative control group. The content of anti-inflammatory cytokines IL-4 and IL-10 (► **Fig. 3**) did not improve.

In the group treated with the SaDM extract at a dose of 100 mg/kg, TNF- α and IL-1 β levels were statistically lower than that presented in the negative control. Furthermore, TNF- α levels were similar to those detected in the healthy mice. The SaDM extract did not give rise to significant changes to the K/C group in IL-4 joint levels. However, the SaDM extract was able to increase IL-10 levels relative to the negative control (► **Fig. 3**).

In mice treated with tomentin, pro-inflammatory TNF- α levels were lower than those of the negative control group. This effect depended on the dose administered. At the 20-mg/kg dose, the content of this cytokine was statistically similar than that of mice treated with methotrexate (► **Fig. 3**). In all groups receiving tomentin, the IL-1 β levels were statistically lower than that of the negative control, and no dose-dependent effect was observed. In addition, the cytokine levels were similar to those of mice treated with methotrexate. The results showed that cytokine IL-4 and IL-10 levels increase with increasing doses of tomentin (► **Fig. 3**).

Discussion

Cell suspension culture is an excellent technique to produce scopoletin, the active compound identified in the wild plant of *S. angustifolia* as well as tomentin and sphaeralcic acid anti-inflam-

matory compounds. The SaDM extract from *S. angustifolia* cell suspension is capable of diminishing the edema development induced by diverse irritating agents, such as TPA or λ -carrageenan [9, 10]. However, the SaDM extract and tomentin in the rheumatoid-related diseases treatment remains unknown. With this purpose in mind, the present study was designed based on the disease course of K/C-induced arthritis.

Rheumatoid arthritis is a symmetric polyarticular arthritis that primarily affects small diarthrodial joints of the hands and feet [11]. K/C-induced arthritis is used as an animal model of human rheumatoid arthritis, which is difficult to assess in human systems [12]. The arthritis-like signs of K/C-induced arthritis in mice resemble several histopathological features of human rheumatoid arthritis, including mononuclear cell infiltration and synoviocyte hyperplasia, resulting in pannus formation followed by bone and cartilage destruction [13, 14]. Joint inflammation in experimental arthritis induced with K/C in mice exhibited maximal joint edema in the first 24 h accompanied by an exudate [15]. Methotrexate has long been used in the treatment of rheumatoid arthritis and it has significantly inhibited joint edema, but the mean BW of these mice dropped, which may be one of the side effects of methotrexate. The SaDM extract also inhibited joint edema in K/C-induced mouse arthritis, demonstrating only redness in the left joint. This effect was higher than that reported for the dichloromethane extract of the wild plant administered via the intraperitoneal route at the same dose in the model of chronic inflammation with CFA [8]. The BW lost in mice treated with the SaDM extract was similar than that of the K/C group.

The SaDM extract to 100 mg/kg and tomentin to 20 mg/kg had the highest anti-inflammatory activity. During the whole period of tomentin administration, the joint edema size decreased progressively. This phenomenon was only observed during the first 5 days of SaDM extract administration, while at this time, the extract effect was lower than that observed by tomentin. Subsequently, there was a rapid decrease in the joint edema (biphasic effect) and at the last day of treatment with the SaDM extract had a similar effect to that of tomentin. Each 100 mg of SaDM extract contains 0.10 mg of scopoletin, 0.10 mg of tomentin, and 0.19 mg sphaeralcic acid (**Fig. 2S**, Supporting Information). The amount of tomentin administered in the SaDM extract (100 mg/kg) was much lower than when given only tomentin (20 mg/kg). This phenomenon can be seen as a dose-dependent effect. In addition, the chemical content of the SaDM extract is complex and it could exert anti-inflammatory activity by different mechanisms of action associated with the compounds already identified and the presence of others. Scopoletin, for example, possesses a wide range of pharmacological activities [16–19]. Scopoletin was isolated from *S. angustifolia* and *E. obtusifolia*, and the anti-inflammatory effects have already been evaluated in TPA-induced mouse ear edema [8, 19]. In rat CFA-induced arthritis, intraperitoneal administration of scopoletin dose-dependently reduced both inoculated and non-inoculated joint edema, and elevated the mean BW [20]. Scopoletin was capable of ameliorating synovial hyperplasia, reduced the presence of inflammatory cells in the synovium, and diminished erosive changes in cartilage and bone in addition to exhibiting angiogenesis inhibition. Also, scopoletin selectively inhibited the overexpression of IL-6 rather than TNF- α or IL-1 β in synovial tissues of rats with CFA-induced arthritis [20].

Tomentin also inhibited both inoculated and non-inoculated joint edema in K/C-induced mouse arthritis and the BW loss was similar to that of the K/C-induced arthritis group. The anti-inflammatory effect of tomentin is dependent on its coumaric origin. It has been reported that various compounds of this group, natural or synthetic, act as inhibitors of the cyclooxygenase and lipoxygenase enzymes, which modulate the biosynthesis of prostaglandins and leukotrienes, respectively, with the latter participating in the process of chronic inflammation [21].

Injection of 2% carrageenan into the knee joint in rats promotes the activation of macrophages, dendritic cells, and lymphocytes. These cells in turn produce TNF- α , IL-1 β and IL-6, and other cytokines that are responsible for the inflammatory response. Once the focus of damage is localized, the production of pro-inflammatory cytokines ceases and the production and release of anti-inflammatory cytokines such as IL-4 and IL-10 begins, with the latter in turn disabling the effector cells [14, 15]. In the results presented here, it was observed that intra-articular administration of K/C caused widespread damage in mice due to the rise in the pro-inflammatory cytokine (TNF- α and IL-1 β) levels and a decrease in the levels of anti-inflammatory cytokines (IL-4 and IL-10).

It has been mentioned that carrageenan-induced arthritis models possess many features of human rheumatoid arthritis. The administration of this substance resulted in localized inflammation associated with the production of a number of mediators such as TNF- α and IL-1 β [22]. In a study in our laboratory, it has been shown that the intra-articular administration of carrageenan, as an experimental procedure, induced an important local increment of TNF- α and IL-1 β [23]. It has been demonstrated that TNF- α induces cartilage and bone degradation, and the penetration of lymphocytes, macrophages, and neutrophils to the joint cavity. IL-1 β in vitro induces cytokine production by synovial mononuclear cells, and when it is blocking IL-1 β and components of the receptor for IL-1 β , it has been shown to be effective in reducing inflammation and, particularly, articular damage in several rodent models of rheumatoid arthritis. These pro-inflammatory cytokines and interleukins are involved in osteoclast differentiation, inflammation, and bone erosion [24], enhance the synthesis of metalloproteinases and proliferation of synovial cells resulting in cartilage degradation [25], and are capable of stimulating the production and secretion of other cytokines such as IL-6, which also promotes cartilage degradation. In the K/C-induced arthritis animal model, this cytokine was also elevated in the articulation [22]. While IL-4 and IL-10 are regulators that control the progression of rheumatoid arthritis, the level of these interleukins may lead to protection in order to prevent cartilage destruction and bone erosion [26].

The SaDM extract and tomentin were also capable of modulating cytokine concentrations. IL-1 β and TNF- α levels decrease, accompanied by an increase of interleukins IL-4 and IL-10, activity that can prevent the damage induced by K/C controlling the inflammation and progression of rheumatoid arthritis. The reduction of TNF- α and increase of IL-10 was tomentin doses-dependent. In rats with CFA-induced arthritis and treated with the dichloromethane extract of the aerial parts from the *S. angustifolia* wild plant, the levels of pro-inflammatory interleukins IL-1 β and IL-6 were also reduced. Furthermore, the anti-inflammatory cytokine IL-10 level was increased [7]. It is proposed that IL-10 has a down-

regulation activity on the levels of the pro-inflammatory cytokines like TNF- α .

The histopathology analyses for the dichloromethane:methanol extract and tomentin are a perspective of this experimental project. It will also be important to isolate and identify other components with anti-inflammatory activity in the SaDM extract, as well as to evaluate the effect of sphaeralcic acid and the interaction of the active compounds identified in the extract to determine if there is synergy in their effects in the K/C-induced arthritis animal model. These advances are important for the development of a phytomedication with the standardized SaDM extract in the active components for a clinical trial.

Materials and Methods

Plant material

Plants and fruits of Vara de San Jose plants were collected in Hidalgo State, Mexico. Plant samples were authenticated by Abigail Aguilar, M.Sc., Head of the Herbarium at the Instituto Mexicano del Seguro Social in Mexico City [IMSSM], as *S. angustifolia*, and vouchers were stored for reference under #14294.

Plant cell culture

Suspension-cultured cells in batches of *S. angustifolia* were grown in 250 mL flasks with 80 mL of whole MS liquid medium [27], with 27.4 mmol of total nitrates (NH₄NO₃ 15.9 mmol, and KNO₃ 11.5 mmol) supplied with 1 mg/L of α -naphthalene acetic acid in combination with kinetin (0.1 mg/L) and supplemented with 30 g/L of sucrose. The flasks were placed in an orbital shaker at 110 rpm (New Brunswick Scientific Co., Inc.) and incubated at 26 \pm 2 °C during a light:dark (16 h:8 h) photoperiod under 50 μ M/m²/sec warm white fluorescent light intensity (9–10). The biomasses were changed to fresh medium under sterile conditions every 16 days, utilizing an inoculum of 4% (w/v) of fresh biomass.

Stimulation of scopoletin, tomentin, and sphaeralcic acid production

Utilizing the same inoculum, cell suspensions cultivated in whole MS medium were transferred into 1 L flasks with 400 mL of MS medium, with the total nitrate concentration reduced to 2.74 mmol (NH₄NO₃ 1.59 mmol, and KNO₃ 1.15 mmol) and were incubated under previously described conditions. Cultures were arrested on day 16 of the culture to obtain the biomass [9, 10].

Extraction and tomentin isolation

Suspension-cultured cells from flasks were filtered and the biomasses were pooled and dried at room temperature. Then, the dry biomass was extracted 3 times by maceration at room temperature with a mixture of grade-reactive solvent (CH₂Cl₂:CH₃OH 9:1; Merck) at a ratio of 1:20 (w/v) at 24 h for each. The dichloromethane-methanol extracts were filtered, pooled, and concentrated to dryness under reduced pressure. The scopoletin, tomentin, and sphaeralcic acid content in the SaDM extract was determined by HPLC [9, 10].

To purify the tomentin compound, the SaDM extract was fractionated by silica gel column chromatography (9 \times 28 cm, 70–230 mesh; Merck) using an n-hexane-ethyl acetate-methanol gradient system

(grade-reactive solvents; Merck) with 5 % polarity increments. Aliquots of 500 mL were collected, and samples with a similar TLC profile were integrated into 10 fractions (1–10). The fraction with tomentin was through an open silica gel RP-18 column (1.5 × 28 cm, 40–63 mesh; Merck) with an H₂O:CH₃CN elution system (grade-reactive solvents; Merck) with an increasing polarity of 10 %, and the compound was isolated from pooled subfractions (70:30, H₂O:CH₃CN) [9]. The structure of tomentin was confirmed by comparison of spectroscopic data (Table 1S, Supporting Information) ¹H NMR [9].

HPLC conditions

HPLC analyses were carried out in a Waters system (2 695 Separation Module) coupled to a diode array detector (2996) with a 190–600 nm detection range and operated by the Manager Millennium software system (Empower 1; Waters Corp.). Separations were performed in a Spherisorb® RP-18 column (250 × 4.6 mm, 5 µm; Waters) employing a constant temperature of 25 °C during analyses. Samples (20 µL) were eluted at a 1.2 mL/min flow rate with (A) high-purity H₂O (TFA-1.0 %) and (B) high-purity CH₃CN gradient mobile phases (Merck), and were detected by monitoring absorbance at 340 nm. The mobile phase was started with water (100 %) and was maintained for 1 min. Then, the concentration of solvent B was gradually increased to 15 % (at 1 min), 37 % (at 10 min), and 85 % (at 2 min). During the next 2 min, solvent B was increased to 100 % and this proportion was maintained for 3 min. Finally, the next 3 min were utilized to return the mobile phase to the initial condition. The chromatographic method had a 22-min run time. Calibration curves were constructed with a standard solution of 2, 4, 8, 12 and 16 µg/mL. Scopoletin presented a regression equation of $Y = 21\,985(X) - 117\,889$ and $R^2 = 0.9997$, while for tomentin these were $Y = 46\,018(X) - 394\,683$ and $R^2 = 0.9992$ and for sphaeralcic acid, these were $Y = 2\,576(X) - 23\,696$ and $R^2 = 0.9996$. The retention times (rt) of compounds tomentin at $\lambda = 343$ nm (≥ 93 %), scopoletin at $\lambda = 343$ nm (99 %; Sigma-Aldrich Química México), and sphaeralcic acid (≥ 98 %) at $\lambda = 357$ nm were 13.37 min, 13.65 min and 18.00 min, respectively. Sphaeralcic acid was isolated and purified at our Laboratory from *S. angustifolia* cell suspensions according to the procedure already reported [9]. Compound identifications were performed by comparing their rt and absorbance spectra [9, 10].

Animals

We used ICR outbred-strain female mice weighting 25.0–30.0 g each. The experiments were performed according to Mexican Official Norm 062-ZOO-1999 (Technical Specifications for the Production, Care and Use of Laboratory Animals) and international ethical guidelines for the care and use of laboratory animals. The animals were maintained at a temperature of 22 ± 3 °C, 70 ± 5 % humidity, and 12-h/12-h light/dark cycles with water and food ad libitum.

The experimental protocol was evaluated by the committee CLIEIS-1701 (Comité Local de Investigación y Ética en Investigación y Salud) from the Instituto Mexicano del Seguro Social (IMSS, Mexico), and approved January 1, 2011, with the register number 2011-1701-3.

Arthritis model induced by kaolin/carrageenan

This experimental design lasted 10 days. There were 8 groups of 12 mice each as follows: initially, measurements were taken from

the baseline size of the left joint with a Mitutoyo-brand digital micrometer (Micrometric Digimatic Calibration MDC-1™SB; Mitutoyo Products). Then, the animals were anesthetized with pentobarbital sodium administered i.p. at a dose of 55 mg/kg. Later, these mice were slowly injected with a kaolin (4 %, 40 µL) solution in the joint cavity of the left knee. Consecutively, flexions and extensions were performed during 15 min in the joint administered with the drug. Immediately, the mice were injected in the same left knee joint cavity with 40 µL of carrageenan at 2 %. Similarly, flexions were carried out for an additional 5 min [28].

Administration of the treatments was conducted each day by oral route, and was initiated 1 day after damage induction until day 10 (9 days of treatment). The negative control group (group 1) received oral administration of the vehicle without any drugs (negative control); the positive control group (group 2) was administered with 5.0 mg/kg of methotrexate (≥ 98 % purity; Sigma-Aldrich Química México). In group 3, mice were treated with 100 mg/kg of SaDM extract standardized in its content of scopoletin, tomentin, and sphaeralcic acid, according to previous reports of dichloromethane-methanol extract from cell suspension biomasses evaluated in acute inflammation models and DE₅₀ determined in a sub-plantar edema model in mice. In groups 4, 5, 6, and 7, mice were treated with 5.0, 10.0, 15.0, and 20.0 mg/kg, respectively, of tomentin (93 % purity). A baseline group of healthy animals was also employed. These animals were injected with PSS into the left joint (group 8) as a substitute of K/C.

Each day, BW and left joint size (mm) in the controls and treated groups were determined with respect to the baseline size of the left knees, and the percentage of inhibition of edema development was calculated utilizing the following formula:

$$\text{Inhibition \%} = \left[\left(\frac{\text{negative control edema} - \text{treatment edema}}{\text{negative control edema}} \right) \times 100 \right].$$

Homogenization of joint tissue

The left joint was liquefied to a temperature of 4 °C and, under this condition, the samples were manipulated for analysis. The bone tissue was placed into a mortar and covered with dry ice. Then, the tissue was pulverized and completely disintegrated until the dry ice was sublimated. The disintegrated tissue was placed in a vial with 2 mL of PBS (pH 7.4) with phenylmethylsulfonyl fluoride at 0.01 % dissolved in isopropyl alcohol (Merck, México). The joint was completely homogenized with a T-10 Ultra Turrax Homogenizer during 15 s for its disintegration. It was allowed to rest for 30 s, and the procedure was repeated 5 additional times [29]. Afterward, the samples were placed into a centrifuge at 14 000 rpm for 5 min. We obtained 300 µL aliquots in centrifuged microtubes, which were immediately stored at -70 °C for cytokine analyses.

Cytokine analyses

Quantification of cytokines IL-1 β , TNF- α , IL-10, and IL-4 was carried out by the ELISA method employing a kit (OptEIA™ ELISA sets; BD Biosciences) and following the manufacturer's instructions. On 96-well plates, 100 µL/well of the antibody were added and incubated for 12 h at 4 °C. Once this time had elapsed, the plate was washed 3 times with 300 µL/well of PBS buffer. Subsequently, 100 µL of PBS with FBS at 10 % and pH 7.0 were added and preserved

during 1 h at room temperature. The contents were discarded and the plate was washed 3 times with 300 µL/well of PBS buffer. Finally, 100 µL of the standard, target (PBS with FBS), and test samples were added to the corresponding wells. The plate was incubated for 2 h at room temperature. The contents were discarded and the plate was washed 3 times with 300 µL/well of PBS buffer. For TNF- α , IL-4, and IL-10, 100 µL/well of antibody detection were added plus a streptavidin-horseradish peroxidase enzyme, which was incubated for 1 h. After that, the plate was washed 7 times with 300 µL/well of PBS buffer.

For IL-1 β , 100 µL/well of antibody detection were added, which was incubated for 1 h and washed with PBS 0.05 % of Tween 20, 300 µL/well 5 times, followed by the streptavidin-horseradish peroxidase enzyme (100 µL/well), which was incubated at room temperature and washed with PBS 0.05 % of Tween 20, 300 µL/well 7 times.

For all cytokines, 100 µL with O-phenyldiamine substrate (1 OPD tablet with 1 of urea dissolved in 20 mL of distilled water) were added to the wells. These ELISA plaques were incubated for 30 min at room temperature, under total darkness, and then a stop solution (2 N H₂SO₄) was added. Each plaque was read at a 450 nm wavelength in a Stat Fact Awareness Technologies 2100 spectrophotometer.

Statistical analysis

Data obtained from joint edema (inhibition %) in the left joints and BW were analyzed by one-way analysis of variance (ANOVA), and *p* values of ≤ 0.05 were considered significant. For joint edema and BW, significant differences among treatment means were calculated by the Dunnett_{0.05} test. For cytokines (pro- and anti-inflammatory) levels, Student's *t*-test was applied to compare each treatment vs. the negative control.

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Conflicts of Interest

The authors declare no conflict of interest.

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