Production and anti-inflammatory and analgesic effects of sphaeralgin from transformed and non-transformed cells in suspension cultures of Sphaeralcea angustifolia (Cav.) G. Don

Rogelio Reyes-Pérez
Centro de Investigacion Biomedica del Sur
Pilar Nicasio-Torres
Centro de Investigacion Biomedica del Sur
Manáses González-Cortazar
Centro de Investigacion Biomedica del Sur
José De Jesús Arellano-García
Universidad Autonoma del Estado de Morelos
Rosa Mariana Montiel-Ruiz
Centro de Investigacion Biomedica del Sur
Rodrigo Vargas-Ruiz
Universidad Autonoma de Tamaulipas
Ma. Dolores Pérez-García
Centro de Investigacion Biomedica del Sur
Juanita Perez-Hernandez
kika_juanita@yahoo.com.mx

Centro de Investigacion Biomedica del Sur https://orcid.org/0000-0003-4547-9820

Research Article

Keywords: Agrobacterium rhizogenes, anti-inflammatory, cells in suspension, benzopyrone, dicoumarin, Sphaeralcea angustifolia

Posted Date: March 26th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4087197/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on August 27th, 2024. See the published version at https://doi.org/10.1007/s11240-024-02839-w.

Abstract

Sphaeralcea angustifolia is a plant with confirmed anti-inflammatory, immunomodulatory and gastroprotective effects. These properties can be attributed to scopoletin, tomentin, sphaeralcic acid, iso-sphaeralcic acid and 8-methyl-iso-sphaeralcic acid compounds isolated from cells in suspension and hairy root cultures. Genetic transformation with *Agrobacterium rhizogenes* can be used in *S. angustifolia* cell cultures to increase the production of active secondary metabolites and stimulate the production of other compounds. We observed that non-transformed cells in a suspension of *S. angustifolia* had a higher growth index after two and three weeks of culture (9.29 and 11.84, respectively) compared with cells in suspension transformed with *A. rhizogenes*. Both cultures produced sphaeralcic acid, and boosted production was detected in the transformed cells (0.19 and 0.16 mg/g, respectively). In addition, transformed and non-transformed cells produced a new compound identified as sphaeralgin (dicumarine); a higher yield of sphaeralgin was detected in the transformed cells (2.21 mg/g dry biomass). The anti-inflammatory effects of sphaeralgin in edema models induced with 12-O-tetradecanoylphorbol-13-acetate and □-carrageenan inhibited edema formation in a dose-dependent manner, with a mean effective dose (ED_{50}) of 0.25 mg/ear and 64.56 mg/kg, respectively. During the late phase of the formaline test, sphaeralgin had an antinociceptive effect, with an ED_{50} of 1.35 mg/kg. Statistically, a 1 mg/kg dose of sphaeralgin (49%) had a similar effect to that of 10 mg/kg indomethacin (52%).

Introduction

Herbal medicine has been recognized by the World Health Organization (WHO) as an effective tool for treating diseases; such medicine can either be used alone or in tandem with other medical remedies. Plants that are used for medicinal purposes contain active ingredients (secondary metabolites) that have been used in the development of commercial drugs (Wawrosch and Zotchev 2021).

Sphaeralcea angustifolia (Cav) G. Don (Malvaceae) is known in Mexico as vara de San José or hierba del negro. This plant is used to treat fractures, inflammation, and gastric diseases (Mekes and Nicasio 2023). The aerial tissue extracts of this plant have exhibited anti-inflammatory activity, and researchers have identified scopoletin as this plant's primary active compound (Aguilar et al. 1994; Argueta et al. 1994; Meckes et al. 2004; Juárez-Ciriaco et al. 2008; García-Rodríguez et al. 2012). The topical administration of a gel formulation prepared with an aerial tissue dichloromethane extract of *S. angustifolia* at 1% standardized in scopoletin revealed therapeutic efficacy and tolerability in patients with osteoarthritis who were capable of reducing associated symptoms such as pain, inflammation and joint stiffness (Romero-Cerecero et al. 2013). Scopoletin has been isolated from many plants and shown to possess anti-inflammatory (Perez et al. 2014), antioxidant (Jain et al. 2002; Shaw et al. 2003; Moon et al. 2007; Ding et al. 2008; Gwak et al. 2011), nuclear transcription factor (NF-κβ) and regulatory effects on the production of pro- and anti-inflammatory cytokines and antiangiogenic mediators in a mouse model of arthritis (Pan et al. 2009). Additionally, scopoletin has been reported to have antiproliferative (Thani et al. 2010), antithyroid, antihypertensive (Aldi et al. 2015), antihyperuricemic (Zeng et al. 2020)

and antidiabetic (Jang et al. 2020) effects. The yield of scopoletin is low (0.0020–0.0067 g/g of dry weight, DW) in *S. angustifolia* (Pérez-Hernández et al. 2014; Reyes-Pérez et al. 2021). However, using cells in a suspension culture of this species has proven to be viable for improving the production of scopoletin (Nicasio-Torres et al. 2016). Reports have shown that the production of scopoletin in a cellular suspension cultivated under nitrate restriction conditions was 60-fold greater than that reported for wild plants (Pérez-Hernández et al. 2014). In addition, tomentin and sphaeralcic acid were identified in this culture as having powerful anti-inflammatory effects in acute and chronic mouse models (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2017). Both compounds can modulate the production of pro- and anti-inflammatory cytokines (Nicasio-Torres et al. 2017; Serrano-Román 2021). The production of sphaeralcic acid in the cellular suspension was extended to an agitated tank bioreactor, and yields of 3.47 mg/g dry biomass were obtained (Pérez-Hernández et al. 2019). The cultivation of plant cells is considered to be one route for producing compounds; however, only a few large-scale commercial systems have been established to date (Caili and Meizhe 2021).

Another way of producing active molecules involves genetic transformation mediated by *Agrobacterium rhizogenes*; this technique has been used to establish cultures of transformed roots he partial or complete insertion of the RI plasmid (Root Inducing plasmid) into the plant genome during the infection process can result in the production of rhizogenic calluses or calluses due to the overexpression of Activation-Induced cytosine Deaminase (AID) (Mallol et al. 2001; Bulgakov et al. 2002; Shkryl et al. 2008).

Sphaeralcea angustifolia is susceptible to transformation by the strain *A. rhizogenes* ATCC 15834, and the establishment of five lines of transformed roots has been reported: four lines that produce the compounds sphaeralcic acid and scopoletin and one line that overproduces sphaeralcic acid at higher yields than the wild plant (Reyes-Pérez et al. 2021). During the transformation of *S. angustifolia* leaf explants, a callus was produced that was able to grow constantly without the addition of growth regulators. We accordingly set about establishing transformed and non-transformed cells in suspension cultures to evaluate the growth rate and production of scopoletin and sphaeralcic acid; we also sought to determine the production of new active molecules and evaluate their anti-inflammatory and analgesic effects.

Materials and Methods

Plant material

The calli and transformed calli were provided by the research group of Dr. Nicasio-Torres at Centro de Investigación Biomédica del Sur in Xochitepec, Morelos, México.

1) Callus cultures of *S. angustifolia* were established from leaf explants in Murashige and Skoog (MS) media supplemented with 30 g/L sucrose and 1 mg/L naphthaleneacetic acid (NAA, Sigma-Aldrich, Steinheim, Germany) in combination with 0.1 mg/L kinetin (Kin, Sigma-Aldrich, Steinheim, Germany), 2.5 g/L phytagel and a pH of 5.7 (Nicasio-Torres et al. 2016).

2) The transformed callus culture from *S. angustifolia* was previously generated from leaf explants infected with the *A. rhizogenes* ATCC 15834/pTDT strain (Reyes-Pérez et al. 2021). Callus free of *A. rhizogenes* was cultivated in glass bottles with MS media supplemented with 30 g/L sucrose and 2.5 g/L phytagel at pH 5.7.

Both callus lines were incubated at 25 ± 2°C with a photoperiod of 16-hour light/8-hour darkness under 50 µmol m^2 s^{-1} warm white fluorescent light. The medium was refreshed every 4 weeks to achieve proliferation.

Transformation confirmation

Total DNA from the transformed calli and non-transformed *Sphaeralcea angustifolia* plantlets (2.5 months after in vitro germination) was extracted using the cetyl-trimethyl ammonium bromide (CTAB) protocol (Murray and Thompson 1980; Porebski et al. 1997; Kale Soman et al. 2020) and used as a template for PCR analysis to determine the presence of the *rolC* gene (Bonhomme et al. 2000; Reyes-Pérez et al. 2021) and the absence of the virD2 gene (Haas et al. 1995; Reyes-Pérez et al. 2021) in *Agrobacterium rhizogenes*-mediated transformation. The forward primer 5' TGTGACAAGCAGCGATGAGC 3' and reverse primer 5' GATTGCAAACTTGCACTCGC 3' were used to amplify a 490 bp fragment of the *rol*C gene, and the second primer forward 5' ATGCCCGATCGAGCTCAAGT 3' and reverse forward 5' ACCGTCGGCTCTACAAACCCAGTCC 3' were used to amplify a 338 bp fragment of the *virD2* gene. The *virD2* gene was used as a control since it was not transferred to the plant cell during the transformation process. We used a DNA amplification kit (Vivantis, Selangor Dural Ehsan, Malaysia) and followed the manufacturer's directions. The DNA samples were prepared in PCR tubes (200.0 µL) on ice comprising 1 µL of DNA template, 5.0 µL of 10.0X Taq DNA polymerase reaction buffer, 2.0 µL of 50.0 mM $MgCl_2$, 1.0 µL of each primer (10.0 µM), 2.0 µL of dNTP mixture (2.0 mM), 0.5 µL of recombinant Taq DNA polymerase (5.0 U/µL) and 37.5 µL of nuclease-free water; the total reaction volume was 50.0 µL. PCR amplification of both genes was carried out using a Mastercycler Gradient device (Eppendorf, Hamburg, Germany) under the following conditions: 1 five-min cycle at 95°C, 35 one-min cycles of denaturation at 95°C, 1 min of annealing at 50°C and 1 min of extension at 72°C. As a final step, we conducted one five-min cycle of final extension at 72°C (Moreno-Anzúrez et al. 2017; Reyes et al. 2021).

The products generated by PCR were separated by electrophoresis in a 1% agarose gel at 100 volts for 60 min and visualized using a UV transilluminator (BioDoc-It™ Imaging System, Upland, CA, USA) using ethidium bromide staining for detection.

Cells in suspension cultures

Sphaeralcea angustifolia cells in suspension in batch cultures were started with 3 g of friable callus and/or transformed callus transferred to 250 ml Erlenmeyer flasks with 80 mL of culture medium.

1) Non-transformed cells in suspension were cultivated in MS media supplemented with 30 g/L sucrose, 1.0 mg/L NAA and 0.1 mg/L Kin at pH 5.7 (Perez-Hernandez et al. 2014; Nicasio-Torres et al. 2016).

2) Transformed cells in suspension were cultivated in MS liquid media without growth regulators supplemented with 30 g/L sucrose at pH 5.7.

Flasks with calli were placed in an orbital shaker at 110 rpm (New Brunswick Scientific Co., Inc., United States) and incubated at 25 ± 2°C with a photoperiod of 16-hour light/8-hour darkness under 50 µmol m^2 s^{-1} warm white fluorescent light.

To promote cellular proliferation, flasks with cells in suspension or transformed cells in suspension were vacuum filtered on a Buchner funnel (Whatman filter paper No. 1, 9 cm in diameter) every three weeks, and the biomasses were renewed every fifteen days with the same inoculum and incubation conditions.

Growth and production kinetics

To assess the growth of the transformed and non-transformed cells in suspension, both suspensions were cultivated in batches under the same conditions described above. Next, three flasks of each suspension were collected upon culture and 1, 2 and 3 weeks later. Each flask was vacuum filtered using a Buchner funnel (Whatman filter paper No. 1, 9 cm diameter), the retained cellular biomass was washed with sterile distilled water and the biomass was dried in an oven (Thelco 160 DM) at 65°C for 48 h. The growth index (GI) at 1, 2 and 3 weeks of growth was determined in DW using the following formula:

$$GI = \frac{(final\ dry\ weight\ -\ initial\ dry\ weight\ )}{initial\ dry\ weight}$$

Statistical analysis

The GI data obtained after 1, 2 and 3 weeks of culture from 1) non-transformed cells cultivated in MS media supplemented with 1.0 mg/L NAA and 0.1 mg/L Kin and 2) transformed cells cultivated in MS media without growth regulators were compared using the Student t-test. We used SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA); *p* values less than 0.05 were considered to be statistically significant.

Chemical analysis

Extract preparation

Independently, Dry biomasses of three flaks after 0, 1, 2 and 3 weeks of culture for the non-transformed and transformed cells in suspension extracted three times via maceration (24 h for each procedure) at room temperature using a mixture of reactive-grade solvents CH_2Cl_2:CH_3OH 9:1 (Merck, Darmstadt, Germany) in a ratio of 1:50 (w/v). The extracts from each biomass were filtered, pooled and concentrated to dryness under reduced pressure. The contents of sphaeralcic acid and scopoletin in the CH_2Cl_2:CH_3OH extracts were determined using high-performance liquid chromatography (HPLC) following the previously reported procedures (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016, 2017; Pérez-Hernández et al. 2019; Reyes-Pérez et al. 2021).

Statistical analysis

Data pertaining to sphaeralcic acid production after 1, 2 and 3 weeks of culture using 1) non-transformed cells and 2) transformed cells were compared using the Student t-test. We used SAS version 9.1 software; *p* values less than 0.05 were considered to be statistically significant.

High-performance liquid chromatography

The HPLC analyses were performed using Waters equipment (2695 Separation Module) with a diode array detector (2996, 190–600 nm) linked using Manager Millennium software (Empower 1; Waters Corp., Boston, MA, USA). Compound separation in the extracts (20 µL) was performed in a Spherisorb RP-18 column (250.0 × 4.6 mm, 5.0 µm; Waters Corp.) incubated at 25°C. A high-purity mobile phase gradient of (A) H_2O with trifluoroacetic acid (TFA) (0.5%, Sigma-Aldrich, Steinheim, Germany) and (B) high-purity CH_3CN (Merck, Darmstadt, Germany) were eluted at a flow rate of 1.0 mL/min. The compounds were detected by monitoring the absorbances of scopoletin at λ = 344 nm and sphaeralcic acid at λ = 357 nm. The identification of scopoletin (99% purity; Sigma-Aldrich, Steinheim, Germany), sphaeralcic acid (95%, purified previously by our group) and sphaeralgin (98% purity, isolated from transformed and non-transformed cell cultures) was carried out by comparing their absorption spectra and retention times (10.3 min for scopoletin, 22.8 min for sphaeralcic acid and 9.9 min for sphaeralgin). The entire experiment ran for 30 min.

Extraction and sphaeralgin purification

Chemical fractionation of dichloromethane-methanol extracts

The HPLC analyses of the CH_2Cl_2:CH_3OH extracts of the non-transformed and transformed cells in suspensions revealed a signal corresponding to a compound with a retention time of 9.89 min and absorption peaks at λ_{max} = 215.7 and 376.6 nm, respectively (Fig. 1). The data pertaining to the scopoletin, sphaeralcic acid and sphaeralgin contents in the cells in suspensions extracted from 1) non-transformed cells and 2) transformed cells were compared using the student t-test. We used SAS version 9.1 software; *p* values less than 0.05 were considered to be statistically significant.

The extracts of the transformed (5.50 g, 4.23% yield dry biomass) and non-transformed (4.43 g, 2.21% yield dry biomass) cells in suspension were independently fractioned following the same methodology.

The extract was adsorbed and fractionated on glass columns packed with silica gel (4.5 × 61.0 cm, 70–230 mesh, Merck, Darmstadt, Germany) using an *n*-hexane:ethyl acetate:methanol gradient system with 10% polarity increments. Twenty-mL aliquots (47) obtained from each extract were combined into 11 fractions (SaC1F1-11).

Isolation of the SaC1F9 fraction

The SaC1F9 fraction (1.10 g) selected for isolation and purification of the compound was absorbed and chromatographed over silica gel RP-18 (2 × 43 cm, 40–63 µm; Merck, Darmstadt, Germany) and in a packed open column (62 × 3 cm) with 10 g of silica RP-18. Ten-mL aliquots were collected using a H_2O:CH_3CN system with polarity increments of 5%; the column was eluted with 100% methanol at the conclusion of the experiment.

Aliquots ranging from 35–55 (SaC1F9-5) through a compound with a similar chromatographic profile were combined and purified using column chromatography with RP-18 silica with an elution system of H_2O:CH_3CN and an increasing polarity characterized by jumps of 2.5% (10 mL aliquots). Aliquots of SaC2F9-5-3 with one compound were combined and analyzed using Thin Layer Chromatography (TLC) and HPLC. The pure compound, with a purity of 98%, was identified by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR).

Acetylation of SaC2F9-5-3

The SaC2F9-5-3 fractions (280 mg) were acetylated via the addition of 3 mL of acetic anhydride (Merck, Darmstadt, Germany) and allowed to react for 15 minutes; next, 1 mL of pyridine (Sigma-Aldrich, Steinheim, Germany) was added, and the mixture was constantly stirred for 1 h. To separate the acetylated compounds, the samples were diluted with water (50 mL) and extracted with ethyl acetate (50:50, v/v) in quadrature. The collected ethyl acetate phases were combined and concentrated under reduced pressure on a rotary evaporator Buchi R-124 (Buchi Labortechnik AG, Flawil, Suiza).

Isolation of the acetylated SaC2F9-5-3ACE

The acetylated compounds were purified in open columns (52.0 × 1.5 cm) packed with silica gel (10 g), and 10 mL aliquots were obtained using a elution system (*n*-hexane: ethyl acetate:methanol) characterized by 10% increases in polarity.

Seventy aliquots were grouped into 12 fractions, which were in turn pooled and dried under reduced pressure on a rotary evaporator Buchi R-124. The purity of the acetylated compound (19 mg, SaC2F9-5-3ACE) was analyzed using TLC and HPLC and identified with one- and two-dimensional NMR.

General experimental procedures

One- and two-dimensional NMR spectra of the natural compounds and acetylated compounds were recorded on an Agilent DD2-600 at 600 MHz using $CDCl_3$. Chemical changes are reported in ppm relative to tetramethylsilane (TMS).

Ultra-Performance Liquid Chromatography (UPLC) conditions

The molecular weights of the isolated compounds were determined using an Acquity UPLC kit (Waters Corp., Milford, MA, USA). The separation system included a quaternary pump, a column furnace and an autosampler with a photodiode array detector coupled to a Xevo triple quadruple mass spectrometer at

150°C. The solvation temperature was 500°C, and the solvation gas flow rate was 700 L/h of nitrogen. Argon was withdrawn as the impingement gas at a flow rate of 0.10 mL/min (Thermo Fisher Scientific, Bremen, Germany). The compound (5 µL) was analyzed on an Acquity UPLC BEH RP-18 column (2.1 × 50.0 mm, 1.7 µm; Waters Corp.) with a mobile phase gradient of high purity: (A) H_2O with TFA (0.5%, Sigma-Aldrich, Steinheim, Germany) and (B) CH_3CN of high purity (Merck, Darmstadt, Germany) were eluted at a flow rate of 0.3 mL/min. The entire experiment was run for 20 min.

Anti-inflammatory and analgesic effects of sphaeralgin

Experimental animals

Female ICR strain mice (weight 28g) were subjected to anti-inflammatory and nociceptive assessments. The rodents were allowed to acclimate to the conditions of Bioterium for one week; they were exposed to a temperature of 25 ± 2°C with a 12-hour light/dark cycle; food and water were provided *ad libitum*. The procedures involving animals were conducted in accordance with the federal regulations for animal experimentation care (SAGARPA, NOM-062-ZOO-1999, Mexico). The research protocol was approved by the Local Committee for Research in Health (CLIS-1702) and the Ethical Committee (CONBIOETICA 17 CEI 00120190121) of the Instituto Mexicano del Seguro Social (IMSS) on 18 December 2018 (registration number: 2018-1702-16).

Carrageenan-induced mouse paw edema

The ICR mice were divided into six groups (with seven mice in each group). Animals were administered orally 1 hour before to λ-carrageenan injection, the animals were group 1 received the vehicle control (water-Tween 20 at 2%), and animals in group 2 received indomethacin at 5 mg/kg. The animals in groups 3–6 received previously purified sphaeralgin in doses of 10, 20, 40 and 80 mg/kg, respectively. Inflammation was induced in the mice by injecting 0.02 mL of 1% λ-carrageenan into the subplantar region of the right paw of each mouse.

Paw volume was measured 0, 1, 3 and 5 h after carrageenan injection using a digital micrometer (Mitutoyo-MDC-1” SB, Mitutoyo America Corporation, United States). The percentage of inhibition was obtained using the following formula: Inhibition % = [(control edema-treatment edema/control edema) × 100] (Winter et al. 1962).

TPA -induced mouse ear edema

The ICR mice were then divided into five groups, with seven animals in each group. 12-O-tetradecanoylphorbol-13-acetate (TPA) (2.5 µg dissolved in 20 µL of acetone) was applied to the internal and external surfaces of each mouse’s right ear to trigger edema. Doses of 0.062, 0.125, 0.250, and 0.500 mg/ear of the previously purified sphaeralgin (98% purity) or indomethacin (2 mg/ear) were dissolved in acetone and applied topically to both ears immediately after TPA administration. Four hours after administration of the inflammatory agent, the animals were sacrificed by cervical dislocation, and circular sections 6 mm in diameter were taken from both the right (treated) and left (non-treated) ears,

which were weighed to determine inflammation. The percentage of inhibition of edema was calculated using the following formula: Inhibition % = [(control edema-treatment edema/control edema) × 100] (De Young et al. 1989; Paya et al. 1992; Pérez et al. 2014; Nicasio-Torres et al. 2016).

Statistical analysis

The percentages of edema inhibited by the bioassays were analyzed using one-way analysis of variance (ANOVA); *p* values less than 0.05 were considered to indicate statistical significance. Significant differences among the treatment means were determined using Tukey's test. After the data pertaining to dose-versus-edema-inhibition in the ear edema and inhibition in paw edema were linearly regressed, we calculated the square of the Pearson correlation coefficient (SAS, ver. 9.1; SAS Institute, Inc.).

Formalin test

The mice were divided into six groups, with five mice in each group. The mice were placed in an open acrylic observation chamber (30 cm in diameter) for 30 min to allow them to acclimate to their surroundings. Twenty microliters of formalin (2.5%) was administered subcutaneously (s.c.) into the dorsal area of the right hind paw of each mouse. Nociceptive behavior was quantified as the amount of time that each mouse spent licking their injected paw over a 40-min observation period. Formalin-induced licking was biphasic: in the early phase (0–5 min), direct chemical stimulation of nociceptors occurred; in the late phase (15–40 min), inflammatory mediators were released. The animals received an oral administration of the vehicle (Tween 20, 3%), dichloromethane-methanol extract from cellular cultures of *Sphaeralcea angustifolia* or sphaeralgin (0.5, 1.0 or 2.0 mg/kg) 30 min prior to formalin injection. A positive control (indomethacin) was administered (10 mg/kg) 15 min prior to the formalin injection (Hunskaar and Hole 1987; Shibata et al. 1989).

Statistical analysis

The data are presented as means ± standard error of the mean (SEM); statistical significance was analyzed using one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered to indicate statistical significance. Significant differences among the treatment means were calculated using the Tukey test. After the data pertaining to dose-versus-edema-inhibition in the ear edema and inhibition in paw edema were linearly regressed, we calculated the square of the Pearson correlation coefficient. The data were analyzed using the statistical program SAS, ver. 9.1

Results and Discussion

Growth index

The cells in the suspension of *S. angustifolia* were established in MS media supplemented with vegetal growth regulators, and the transformed cell line was established in MS media free of phyto-regulators. We confirmed the transformation of this cell line via PCR by amplifying a 490-bp band of a fragment of the *rolC* gene (Fig. 2).

The GI in the culture of non-transformed cells was greater than that of the transformed cell suspensions after 2 and 3 weeks (Table 1). The GI of non-transformed cells was similar to that reported previously for a *S. angustifolia* cell suspension (7.91 ± 0.65) cultivated in MS media supplemented with nitrate (2.74 mM) after 16 days in culture (Nicasio-Torres et al. 2016). According to the literature, *rolC* gene expression does not negatively affect the growth of transformed calli (Shkryl et al. 2008).

The production of scopoletin, sphaeralcic acid and sphaeralgin in cells in suspension cultures

The presence of scopoletin and sphaeralcic acid in the suspensions of the extracts of both the non-transformed and transformed cell lines after 1, 2 and 3 weeks in culture was confirmed by HPLC. We used those data to compare the retention times and absorption spectra of the extracts with those of standards.

Scopoletin was not detected after 1, 2 or 3 weeks in any batch culture. According to the literature, this compound is found in low concentrations, and its production is linked to biotic or abiotic stress. The production of scopoletin occurs between 24 and 48 hours after its stimulation (Whitehead and Threlfall, 1992; Nicasio-Torres et al. 2016).

On the other hand, sphaeralcic acid was detected in both the non-transformed and transformed cells in suspensions. The highest yield of sphaeralcic acid was observed in the transformed cells after 2 and 3 weeks in culture; those levels were two orders of magnitude greater than those observed in the non-transformed cells (Table 1). This sphaeralcic acid yield was 10.5-fold greater than that reported for non-transformed cell suspensions cultivated in MS media supplemented with 2.74 mM nitrate (Nicasio-Torres et al. 2016); this nutritional restriction could accordingly be applied to improve sphaeralcic acid production in transformed cellular suspensions. A sphaeralcic acid yield of 3.47 mg/g DW was reported in a non-transformed cell suspension grown in MS media supplemented with 2.74 mM nitrate in a mechanical agitation bioreactor (Pérez-Hernández et al. 2019).

The extracts from 100 g of dried biomass of non-transformed and transformed cells in suspension were analyzed using HPLC. The transformed cell cultures exhibited higher yields of scopoletin (0.012 mg/g), sphaeralcic acid (0.124 mg/g) and sphaeralgin (2.210 mg/g) than the non-transformed cell cultures of scopoletin (0.003 mg/g), sphaeralcic acid (0.011 mg/g) and sphaeralgin (0.030 mg/g).

After 10 years in culture, the non-transformed cell suspension line did not produce scopoletin or sphaeralcic acid; this finding is possibly due to somaclonal variations caused by the addition of NAA to the MS media. Primary events, which are controlled by exogenously applied plant growth regulators and can trigger morphogenesis via cell cycle disruption, can induce genetic variability and alter the production of secondary metabolites (Peschke and Phillips 1992; Ashokhan et al. 2020). The use of auxins in cells in suspension cultures has been shown to increase genetic variation by increasing the rate of DNA methylation (Duta-Cornescu et al. 2023).

Identification of sphaeralgin

The same compound (SaC2F9-5-3, 10 mg) was obtained as an amorphous yellow precipitate soluble in methanol from the CH_2Cl_2:CH_3OH extracts of both the transformed and non-transformed cell biomasses. The compound exhibited an absorption band with λ_{max} at 215 and 376 nm in the ultraviolet (UV) part of the spectrum. The mass spectrum (MS) revealed a corresponding molecular peak at *m/z* 605.21, corresponding to the molecular formula $C_{28}O_{15}H_{28}^{+}$. The ^{1}H NMR spectrum exhibited two aromatic ring singlet signals at δ 6.84 (1H, s) and 6.82 (1H, s) and two characteristic double-bond singlet signals at δ 8.61 and 8.58 corresponding to H-5, H-5', H-4 and H-4', respectively, for a dicoumarin skeleton of the benzopyrone type. The first coumarin skeleton (A) has two methoxyl substituents at δ 3.88 (3H, s) and 4.05 (3H, s), which are assigned to C-6 and C-8, respectively, and an additional hydroxyl group (OH) at C-7, which is acetylated. The other coumarin skeleton (B) contains two methoxylated groups at δ 3.96 (3H, s) and 4.02 (3H, s) at positions C-7' and C-8', respectively. Analysis of the proton spectrum revealed an oxygen base doublet signal at δ 5.28 (J = 7.6 Hz, H-1") for a hemiacetal group according to ^{13}C NMR at 101.13 (C-1"), which is characteristic of an anomeric carbon of a sugar (Table 2). The Correlation Spectroscopy (COSY) data and evaluations of spin-spin coupling and chemical changes allowed us to identify the hexose-type sugar known as β-D-glucopyranose. The position of glucopyranose on the coumarin backbone (B) was unambiguously defined at C-6" due to the long-range correlation (HMBC) between C-6' (δ 142.25) of the aglycone and H-1" (δ 5.28) of the glucose unit (Fig. 3). Additionally, the connectivity of coumarins A and B was established by the correlation of H-4 (δ 8.61) with the carbon signal at three ligatures at δ 119.02 (C-3') and the H-4' signal (δ 8.58) with signal carbon (HMBC) at δ 119.61 (C-3); this compound corresponds to a glycosylated dicumarin 6,8-dimethoxy-7acetoxy-2-oxo-2H-chromen-7',8'-dimethoxy-6 '-glucopyranosyl-2-oxo-2H chromen as a natural product that is termed sphaeralgin (**1**) and per-acetylated sphaeralgin (**1a**) (Fig. 4).

Anti-inflammatory activity of sphaeralgin

Carrageenan-induced mouse paw edema

Sphaeralgin inhibited the formation of subplantar edema 5 hours after damage was induced (Table 3 and Fig. 5); at a dose of 40 mg/kg (40.35%), it had a similar effect to that of indomethacin (43.90%). It had a decreased effect compared with 45 mg/kg dose of tomentin (68%) and sphaeralcic acid (66%) (Pérez-Hernández et al. 2014). Dicoumarin also had a dose-dependent effect and a mean effective dose (ED_{50}) of 64.56 mg/kg.

TPA-induced mouse ear edema

In the auricular inflammation model, sphaeralgin inhibited edema formation by 83% at a dose of 0.5 mg/ear. Additionally, a dose-response effect was observed at an ED_{50} of 0.25 mg/ear (Table 4 and Fig. 6). Furthermore, significant differences were noted in the treatment inhibition percentages ($F_{0.05}$= 69.63, p = 0.0001; Tukey test$_{0.05}$=30.57). According to Garcia-Rodriguez et al. (2012) and Pérez-Hernández (2014), the topical effect of sphaeralgin at a dose of 0.5 mg/ear (83%) was greater than that

of other compounds identified in *S. angustifolia* plants (i.e., tomentin 47% and scopoletin 52%). Also, the topical effect of sphaeralgin was similar to that of sphaeralcic acid (86%) and indomethacin (82%, 2 mg/ear) at higher doses.

Table 4
Inhibitory activity of sphaeralgin and the drug indomethacin on ear edema model induced with TPA (12-*O*-tetradecanoylphorbol-13-acetate)

Sphaeralgin is a bicoumarin produced in transformed and non-transformed cell suspension cultures whose structure and biological activity have not been described. As a result, we suggest that this molecule is new to the species *S. angustifolia*.

Bicoumarins exhibit antibacterial, anticoagulant, anti-inflammatory, antiviral, antiparasitic and antitumoral activities (Venugopala et al. 2013; Detsi et al. 2017; Ren et al. 2018). Dicuarolum and dicumarol (anticoagulants) have been approved for therapeutic purposes in clinical practice (Vane et al. 1998; Vekariya et al. 2014; Lucas 2016; Detsi et al. 2017). The CH_2Cl_2:CH_3OH extract of the transformed and non-transformed cell cultures was shown to contain sphaeralcic acid, which has been proven to be anti-inflammatory (Pérez-Hernández et al. 2014). Similarly, scopoletin, tomentin and sphaeralcic acid have been reported to have immunomodulatory effects on the regulation of pro- and anti-inflammatory cytokines, and scopoletin has been shown to have an angiogenic effect by inhibiting the formation of new blood vessels (Pan et al. 2010; Nicasio-Torres et al. 2017; Serrano-Román 2020). Clinically, standardized gels of *Sphaeralcea angustifolia* extract have been used to treat patients with osteoarthritis (Romero-Cerecero et al. 2013). Recent studies have shown that a standardized fraction of tomentin, scopoletin and sphaeralcic acid strengthens its pharmacological use in osteoarthritic treatment (Serrano-Román et al. 2021). Similarly, the gastroprotective effects of sphaeralcic acid (74% TNF-α, 49% IL-1β, 43% IL-6) and tomentin (53% TNF-α, 48% IL-1β, 33% IL-6) are due to their ability to inhibit inflammatory mediators of the gastric mucosa (Serrano-Román et al. 2023). The presence of sphaeralgin might be responsible for the biological effect of the complete extract of the suspension cell cultures of *S. angustifolia*.

The formalin test

This investigation represents the first time that the antinociceptive effects of *S. angustifolia* cells in suspension have been evaluated. The dichloromethane-methanol extract of the transformed cells in suspension had a significantly boosted analgesic effect (i.e., increased by 40%) compared with that of the control group. The effect of the extract might be due to the synergistic interaction of sphaeralgin with scopoletin and sphaeralcic acid (Pérez-Hernández et al. 2014; Meckes-Fisher and Nicasio-Torres 2023). During the formalin test, sphaeralgin at a dose of 2 mg/kg exhibited a 59% analgesic effect during the

late phase; it had a dose-dependent effect and an ED_{50} of 1.35 mg/kg. Statistically analgesic effect, a 1 mg/kg dose of sphaeralgin (49%) similar to that observed for 10 mg/kg indomethacin (52%).

The analgesic response exerted by sphaeralgin during the late phase was associated with an anti-inflammatory effect inhibited the synthesis of cyclooxygenases, prostaglandins, and other mediating molecules that act during inflammatory processes, such as histamine, serotonin and bradykinin (Hunskaar and Hole 1987).

Abbreviations

MS Murashige and Skoog

NAA Naphthaleneacetic acid

Kin Kinetin

TFA Trifluoroacetic acid

TPA 12-*O*-tetradecanoylphorbol-13-acetate

CTAB cetyl-trimethylammonium bromide

AID Activation-Induced Cytosine Deaminase

NF-κβ Nuclear factor-kappa B

WHO World Health Organization

DW: dry weight

GI growth index

Declarations

The authors declare no conflict of interest.

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

The authors have no relevant financial or non-financial interests to disclose

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juanita Perez Hernandez and Rogelio Reyes Perez. The first draft of the manuscript was written by Juanita Perez Hernandez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

The research protocol was approved by the Local Committee for Research in Health (CLIS-1702) and the Ethical Committee (CONBIOETICA 17 CEI 00120190121) of the Instituto Mexicano del Seguro Social (IMSS) on 18 December 2018 (registration number: 2018-1702-16)

Acknowledgments

This work was supported by Basic Grant 662122 from Consejo Nacional de Ciencia y Tecnología, México (CONAHCyT México) for the doctoral studies of Rogelio Reyes-Pérez at the Natural Science Doctoral Program of Centro de Investigación en Biotecnología (CeIB), Universidad Autónoma del Estado de Morelos, México and Estancias Postdoctorales Por México 2022 CONAHCYT of Ph.D. Juanita Pérez Hernández.

Authors Contributions Statement

All authors contributed to the study conception and design.

R.R.P. established the transformed and non-transformed cell cultures, obtained the extract from the transformed cell cultures and isolated, purified and identified the new sphaeralgin compound; he also wrote the scientific manuscript.

P.N.T. proposed this study and participated in the review and editing of the scientific manuscript.

M.G.C. directed the isolation and structural identification of the new sphaeralgin compound.

D.P.G. used HPLC and UPLC to help identify the new sphaeralgin compound.

M.M.R. and R.V.R. helped conduct the antinociceptive biological tests.

J.d-J.A.G. helped review the scientific manuscript and the *Agrobacterium rhizogenes* strains for the transformation of the cell cultures and their establishment.

J.P.H. participated in the entire project and helped conduct the anti-inflammatory biological tests. She also helped isolate, purify and structurally identify the new sphaeralgin compound; she also contributed to writing and editing the scientific manuscript.

Material preparation, data collection and analysis were performed by J.P.H

The first draft of the manuscript was written by J.P.H and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Ashokhan S, Othaman R, Abd Rahim MH, Anuar Karsani S, Syafawati Yaacob. (2020) Effect of plant growth regulators on coloured callus formation and accumulation of azadirachtin, an essential biopesticide in Azadirachta indica. Plants 9, 1-17. doi: 10.3390/plants9030352

2. Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux MA, Jacquin-Dubreuil A (2000) Tropane alkaloid production by hairy roots of Atropa belladonna obtained after transformation with Agrobacterium rhizogenes 15834 and Agrobacterium tumefaciens containing rol A, B, C genes only. J Biotechnol 81(2–3):151–158. doi:10.1016/S0168-1656(00)00287-X
3. Bulgakov VP, Tchernoded G K, Mischenko N P, Khodakovskaya MV, Glazunov VP, Radchenko SV, Zhuravlev YN (2002) Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on 1518 anthraquinone production by Rubia cordifolia callus cultures transformed with the rolB and rolC genes. J biotechnol, 97(3), 213-221.
4. Caili L, Meizhe W (2021). Application of hairy root culture for bioactive compounds production in medicinal plants. Curr Pharm Biotechnol 22 (5): 592-608. doi:10.2174/1389201021666200516155146
5. Detsi A, Kontogiorgis C, and Hadjipavlou-Litina D (2017) Coumarin derivatives: an updated patent review 2015-2016). Expert Opin Ther Pat 27(11), 1201–1226. doi:10.1080/13543776.2017.1360284.
6. De Young LM, Kheifets JB, Ballaron SJ, Young JM (1989) Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions 26 (3-4): 335-341. doi: 10.1007/BF01967298
7. Ding Z, Dai Y, Hao H, Pan R, Yao X, and Wang Z (2008) Anti-inflammatory effects of scopoletin and underlying mechanisms. Pharmaceutical Biology 46(12), 854–860. https://doi.org/10.1080/13880200802367155
8. Duta-Cornescu G, Constantin N, Pojoga DM, Nicuta D and Simon-Gruita A (2023) Somaclonal variation—Advantage or disadvantage in micropropagation of the medicinal plants. Int J Mol Sci 24(1), 838. https://doi.org/10.3390/ijms2401083
9. García-Rodríguez RV, Chamorro Cevallos G, Siordia G, Jiménez-Arellanes MA, Chávez-Soto MA, Meckes-Fischer M (2012) *Sphaeralcea angustifolia* (Cav.) G. Don extract, a potential phytomedicine to treat chronic inflammation. Bol Latinoam Caribe Plantas Med Aromat 11, 454–463
10. Gwak MK, Choi HS, Manochai B, Hwa Hong J (2011) Extraction Procedures for Free Radical Scavenging Activity from Noni Fruit (*Morinda citrifolia*). Korean J Med Crop Sci 19(1), 38–46. https://doi.org/10.7783/KJMCS.2011.19.1.038
11. Haas JH, Moore LW, Ream W, Manulis S (1995) Universal PCR primers for detection of phytopathogenic *Agrobacterium strains*. Appl Environ Microbiol 61(8):2879–2884. doi: 10.1128/aem.61.8.2879-2884.1995
12. Hunskaar S, Hole K (1987) The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. Pain 30(1):103–114. doi.org/10.1016/0304-3959(87)90088-1
13. Jain DC, Pant N, Gupta MM, Bhakuni RS, Verma RK, Tandon S, Kumar S (2002) U.S. Patent No. 6,337,095. Washington, DC: U.S. Patent and Trademark Office.
14. Juárez-Ciriaco M, Román-Ramos R, González-Márquez H, Meckes-Fischer M (2008) Efecto de *Sphaeralcea angustifolia* sobre la expresión de citocinas pro y antiinflamatorias. LabCiencia con Noticias Técnicas del Laboratorio 2: 21-23

15. Lucas S (2016) The pharmacology of indomethacin. Headache. J Headache Pain 56 (2), 436-446. doi.org/10.1111/head.12769.
16. Mallol A, Cusidó RM, Palazón J, Bonfill M, Morales C, Piñol MT (2001) Ginsenoside production in different phenotypes of *Panax ginseng* transformed roots. Phytochemistry 57(3):365-371.
17. Meckes M, David-Rivera AD, Nava-Aguilar V, Jiménez A (2004) Activity of some Mexican medicinal plant extracts on carrageenan- induced rat paw edema. Phytomedicine 11(5):446–45. doi: 10.1016/j.phymed.2003.06.002
18. Meckes M, Nicasio-Torres P (2023) The Journey of a Medicinal Plant throughout Science: *Sphaeralcea angustifolia* (Cav.) G. Don (Malvaceae). Plants 12: 321. doi: https://doi.org/10.3390/plants12020321
19. Moon PD, Lee B H, Jeong HJ, An HJ, Park SJ, Kim HR, Ko SG, Um JY, Hong SH, Kim H (2007) Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the 1634 IκB/NF-κB signal cascade in the human mast cell line HMC-1. Eur J Pharmacol 555(2–3):218–225. https://doi.org/10.1016/j.ejphar.2006.10.021
20. Moreno-Anzúrez NE, Marquina S, Alvarez L, Zamilpa A, Castillo-España P, Perea-Arango I, Torres PN, Herrera-Ruiz M, Díaz García ER, García JT, Arellano-García J (2017) A cytotoxic and antiinfammatory campesterol derivative from genetically transformed hairy roots of Lopezia racemosa Cav. (Onagraceae). Molecules 22 (1)118. doi: 10.3390/molecules22010118
21. Nicasio-Torres MP, Perez-Hernandez J, Gonzalez-Cortazar M, Meckes-Fisher M, Tortoriello J, Cruz-Sosa F (2016) Production of potential anti-inflammatory compounds in cell suspensión cultures of *Sphaeralcea angustifolia* (Cav.) G. Don. Acta Phyiol Plant 38: 209. doi: 10.1007/s11738-016-2211-x
22. Nicasio-Torres MP, Serrano-Román J, Pérez-Hernández J, Jiménez-Ferrer E, Herrera-Ruiz M (2017) Effect of Dichloromethane-Methanol Extract and Tomentin
Obtained from Sphaeralcea angustifolia Cell Suspensions in a Model of Kaolin/ Carrageenan-Induced Arthritis. Planta Medica International Open 4: e35-e42. DOI: https://doi.org/10.1055/s-0043-108760
23. Pan R, Dai Y, Yang J, Li Y, Yao X, Xia Y (2009) Anti-angiogenic potential of scopoletin is associated with 1676 the inhibition of ERK1/2 activation. Drug Dev Res 70(3), 214–219. https://doi.org/10.1002/ddr.20297
24. Pan R, Gao XH, Li Y, Xia YF, Dai (2010) Anti-arthritic effect of scopoletin, a coumarin compound occurring in Erycibe obtusifolia Benth stems, is associated with decreased angiogenesis in synovium. Fundam Clin Pharmacol 24: 477–490. doi: 10.1111/j.1472-8206.2009. 00784.x
25. Payá M, Halliwell B, Hoult J (1992) Interactions of a series of coumarins with reactive oxygen species: scavenging of superoxide, hypochlorous acid and hydroxyl radicals. *Biochem Pharmacol 44*(2):205-14. doi: 10.1016/0006-2952(92)90002-z
26. Pérez-Hernández J, González-Cortazar M, Marquina S, Herrera-Ruiz M, Meckes-Fischer M, Tortoriello J, Cruz-Sosa F, María del Pilar Nicasio-Torres (2014) Sphaeralcic Acid and Tomentin, Anti-

inflammatory Compounds Produced in Cell Suspension Cultures of *Sphaeralcea angustifolia*. Planta Med 80 (2-3): 209-14. doi: 10.1055/s-0033-1360302

27. Pérez-Hernández J, Martínez-Trujillo A, Nicasio-Torres P (2019) Optimization of active compounds production by interaction between nitrate and copper in *Sphaeralcea angustifolia* cell suspension using Response Surface Methodology. Plant Cell Tissue Organ Cult136:407–413. doi: 10.1007/s11240-018-1516-4
28. Ren Q, Gao C, Xu Z, Xu Y, Liu ML, Wu X, Guan JG, Feng L (2018) Bis-coumarin Derivatives and Their Biological Activities. Curr Top Med Chem. 18(2):101-113. doi: 10.2174/1568026618666180221114515
29. Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. Adv genet 30:41-75. doi: 10.1016/S0065-2660(08)60318-1
30. Reyes-Pérez R, Herrera-Ruiz M, Perea-Arango I, Martínez-Morales F De Jesús, Arellano García J, Nicasio-Torres P (2022) Anti-inflammatory compounds produced in hairy roots culture of *Sphaeralcea angustifolia*. Plant Cell. Tissue Organ Cult 149: 351–361. doi: 10.1007/s11240-021-02162-8
31. Romero-Cerecero, Mariana Meckes-Fischer, Alejandro Zamilpa, Jesús Enrique Jiménez-Ferrer, Pilar Nicasio-Torres, Dolores Pérez-García, Jaime Tortoriello (2013) Clinical trial for evaluating the effectiveness and tolerability of topical *Sphaeralcea angustifolia* treatment in hand osteoarthritis. J Ethnopharmacol 147(2): 467–473. doi: 10.1016/j.jep.2013.03.040
32. Serrano-Roman JL. (2020) Pharmacokinetic evaluation of a standardized fraction (scopoletin, tomentin and sphaeralcic acid) obtained from cell cultures of Sphaeralcea angustifolia, administered orally in mice. Doctoral Thesis in Biological and Health Sciences. Metropolitan Autonomous University. Iztapalapa Unit. Mexico City, July, 110 p.
33. Serrano-Román J, Nicasio-Torres P, Hernández-Pérez E, Jiménez-Ferrer E (2021) Pharmacokinetic Study of Anti-osteoarthritic Compounds of a Standardized Fracction from *Sphaeralcea angustifolia*. Pharmaceutical 14(7):610. doi: 10.3390/ph14070610
34. Serrano-Roman J, Herrera-Ruiz M, Gonzalez-Cortazar M, Nicasio-Torres P (2023) Anti-ulcerogenic proprieties of *Sphaeralcea angustifolia* on gastric ulcer in mice. Rev Bras Farmacogn 33:863-869. doi: 10.1007/s43450-023-00393-8
35. Shaw CY, Chen CH, Hsu CC, Chen CC, Tsai Y C (2003) Antioxidant properties of scopoletin isolated from Sinomonium acutum. Phytother Res 17(7):823–825. https://doi.org/10.1002/ptr.1170
36. Shibata M, Ohkubo T, Takahashi H, Inoki R (1989) Modified formalin test: Characteristic biphasic pain response. Pain 1989, 38, 347–352
37. Shkryl Y N, Veremeichik G N, Bulgakov V P, Tchernoded G K, Mischenko N P, Fedoreyev S A, Zhuravlev Y N (2008) Individual and combined effects of the rolA, B, and C genes on anthraquinone production in Rubia cordifolia transformed calli. Biotechnol Bioeng 100 (1): 118-125. doi: 10.1002/bit.21727

38. Thani W, Vallisuta O, Siripong P, Ruangwises N (2010) Anti-proliferative and antioxidative activities of Thai 1774 noni/Yor (Morinda citrifolia Linn.) leaf extract. Southeast Asian J Trop Med Public Health 41(2), 482–489
39. Vane J, FRS, Botting R (1998) Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med 104 (3):2S-8S. doi: 10.1016/S0002-9343(97)00203-9
40. Vekariya RH, Patel HD (2014) Recent Advances in the Synthesis of Coumarin Derivatives via Knoevenagel Condensation: A Review. Synth Commun 44(19), 2756–2788. doi:10.1080/00397911.2014.926374
41. Venugopala KN, Rashmi V, Odhav B (2013) Review on natural coumarin lead compounds for their pharmacological activity. Biomed Res Int 1-14. doi: 10.1155/2013/963248
42. Wawrosch C, Zotchev S (2021) Production of bioactive plant secondary metabolites through in vitro technologies-status and Outlook. Appl Microbiol Biotechnol 105:6649-6668. doi:10.1007/s00253-021-11539-w
43. Whitehead IM and Threlfall DR (1992) Production of phytoalexins by plant tissue cultures. J Biotechnol 26:63-81 doi: https://doi.org/10.1016/0168-1656(92)90069-L
44. Winter CA, Risley EA, Nuss GW (1962) Carrageenin-Induced Edema in Hind Paw of the Rat as an Assay for Antiinflammatory Drugs. Exp Biol Med 111(3), 544–547. doi:10.3181/00379727-111-27849
45. Zeng Y, Ma Y, Yang Z, Mao J, Zheng Y (2020) Antihyperuricemic efficacy of Scopoletin-loaded Soluplus micelles in yeast extract/potassium oxonate-induced hyperuricemic mice. Drug Development and Industrial. Pharmacy 46(9):1550–1557. https://doi.org/10.1080/03639045.2020.1811302

Tables

Table 1 Growth index and production of scopoletin and sphaeralcic acid in transformed and non-transformed cells in suspensions of *Sphaeralcea angustifolia*

Treatment	Concentration (mg/ear)	Edema (mg)	Edema inhibition (%)
Control	-	13±0.01	-
Sphaeralgin	0.0625	11±0.01	12±4.75^{d}
	0.125	8±0.01	34±5.95^{c}
	0.25	6±0.02	50±3.82^{b}
	0.5	2±0.01	83±0.56^{a}
Indomethacin	2.0	1±0.02	82±2.87^{a}

Mean ± standard error of the mean (SEM), *n*=8.

Values with the same letter are statistically the same, Tukey test $F_{0.05}$= 69.63.

Table 2 ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectral data for Sphaeralgin compound per-acetylated (1a) in $CDCl_3$

Time	Sphaeralgin Edema inhibition (%)				Indomethacin	$F_{0.05}$
(h)	10 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg	5 mg/kg	
1	12.41±1.76	13.70±1.63	18.36±4.82	18.28±4.22	22.60±2.29	2.39
3	18.61±4.77	26.53±1.78	27.15±4.72	26.88±1.02	28.69±2.60	2.02
5	23.20±1.63^{c}	32.02±1.51^{c}	40.35±4.87^{b}	55.20±2.07^{a}	43.90±2.98^{b}	30.57

Mean ± standard error of the mean (SEM) (*n* = 7).

Values with the same letter are statistically similar $p \leq 0.05$, to the Tukey test (for inhibition % at 5 h).

Table 3 Inhibitory activity of sphaeralgin and the drug indomethacin on λ-carrageenan footpad edema (CFE) administered intraperitoneally (i.p.)

Position	δ_H (δ, J in Hz)	δ_C (δ)
2		159.45
3		119.61
4	8.61, s	143.73
5	6.84, s	104.37
6		149.36
7		136.72
8		140.99
2’		159.64
3’		119.02
4’	8.58, s	143.72
5’	6.81, s	104.81
6’		141.58
7’		149.94
8’		141.58
8a		142.25
8b		115.81
8a’		144.73
8b’		117.23
1”	5.28, d (7.6)	101.13
2”	5.33,dd (7.6, 6.91)	71.92
3”	5.27, dd (6.9, 6.9)	72.98
4”	5.26, dd (6.9, 6.9)	68.33
5”	3.88, ddd (2.7, 7.6, 5.5)	72.37
6α	4.24, dd (5.52, 12.43)	62.07
6β	4.11, dd (7.6, 6.91)	
OMe-6	3.89, s	56.59
OMe-8	4.05, s	62.02
OMe-7’	3.96, s	56.63

OMe-8’	4.02, s	62.17
AcO-6’	2.40, s	168.45
AcO-6’	2.07, s	169.50
AcO-6’	2.04, s	169.58
AcO-6’	2.04, s	170.47
AcO-6’	2.05, s	170.72

Table 4 Inhibitory activity of sphaeralgin and the drug indomethacin on ear edema model induced with TPA (12-*O*-tetradecanoylphorbol-13-acetate)

Time of culture (weeks)	**Transformed**			**Non-transformed**		
	GI	Scopoletin (mg/g)	Sphaeralcic acid (mg/g)	GI	Scopoletin (mg/g)	Sphaeralcic acid (mg/g)
1	4.31±0.4	ND	ND	4.54±0.37	ND	0.007±0.0
2	7.26±0.8	ND	0.19±0.01**	9.29±0.54*	ND	0.007±0.0
3	6.69±0.7	ND	0.16±0.05 **	11.84±0.29**	ND	0.009±0.0

The values are the mean ± standard deviation (n=3). ND: not detected.

According to t-student test **p< 0.001, * p< 0.05 were different significantly. Growth rate (GI) at two and three weeks, t-test 16.39 and 11.57; sphaeralcic acid contentat two and three week, t-test 2.99 and 5.66.

Figures

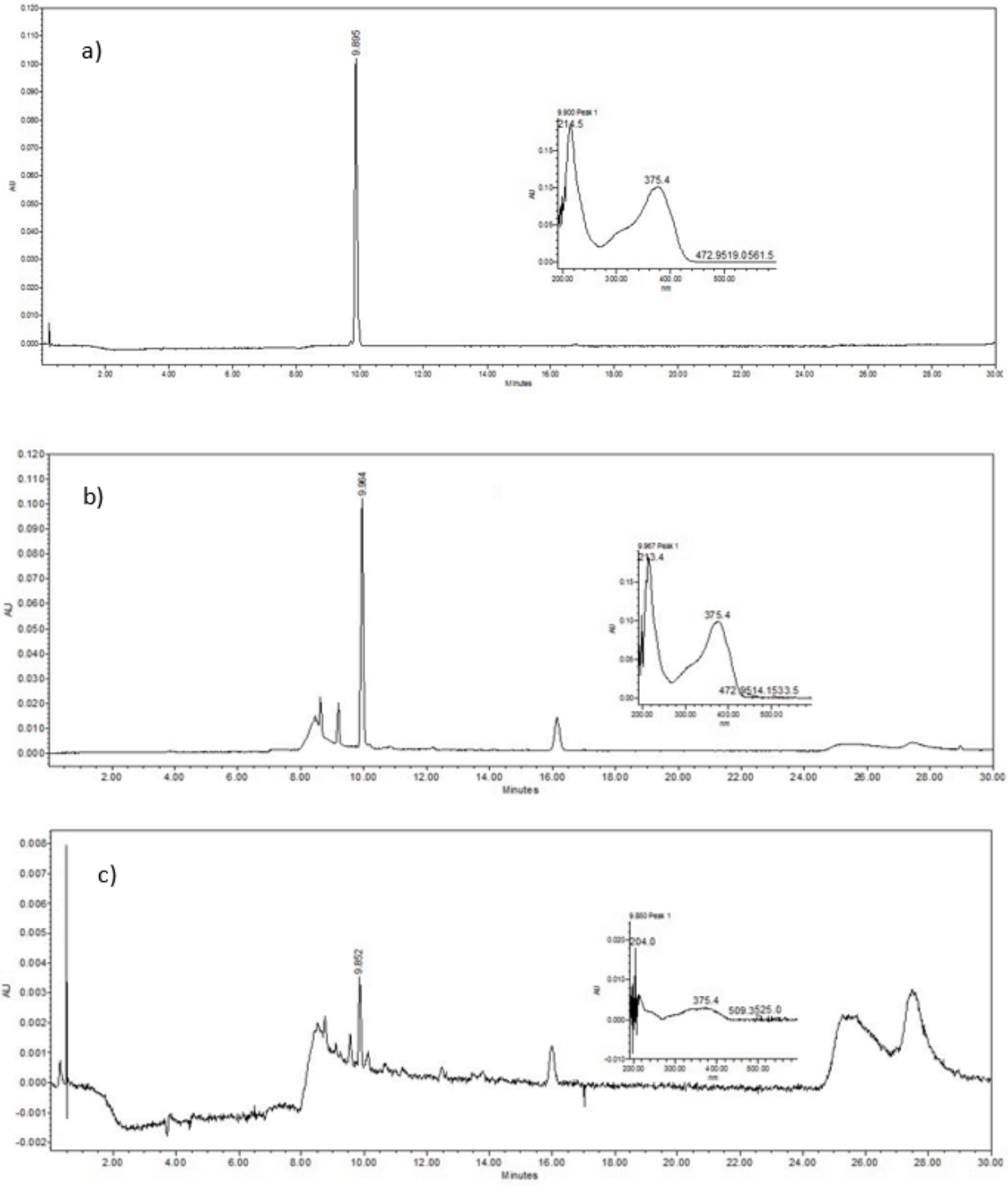


Figure 1

Chromatograms and absortion spectrum of sphaeralgin (a), and dichloromethane-methanol extracts from the transformed cell cultures (b) and non-transformed cell cultures (c)

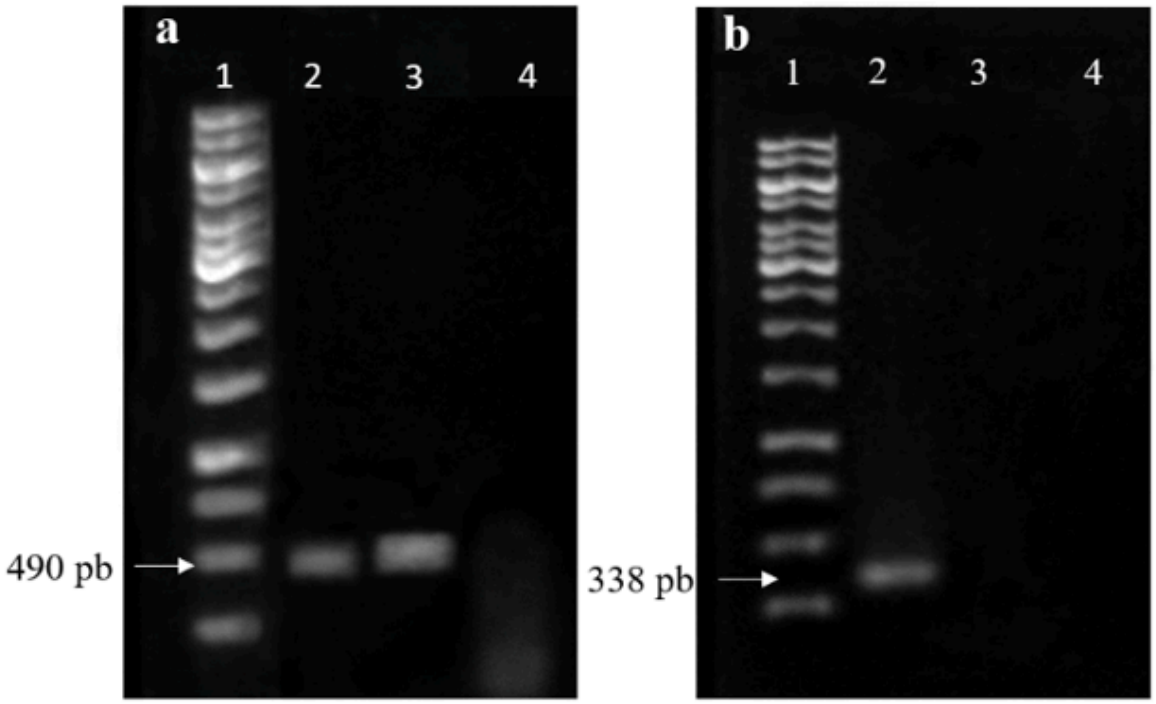


Figure 2

PCR products of SaL3.1transformed cell line of *Sphaeralcea angustifolia*: a) lane 1: Kb DNA marker, lane 2: amplified band of *rolC* from DNA of *Agrobacterium rhizogenes* (positive control), lane 3: amplified band of *rolC* from the DNA of SaL3.1 cell line, lane 4: DNA of *S. angustifolia* wild plantlets (negative control); b) Lane 1: 1 Kb DNA marker, lane 2: amplified band of *VirD2* from DNA of *Agrobacterium rhizogenes* (positive control), lane 3 DNA of SaL3.1 cell line, lane 4: DNA of *S. angustifolia*wild plantlets (negative control)

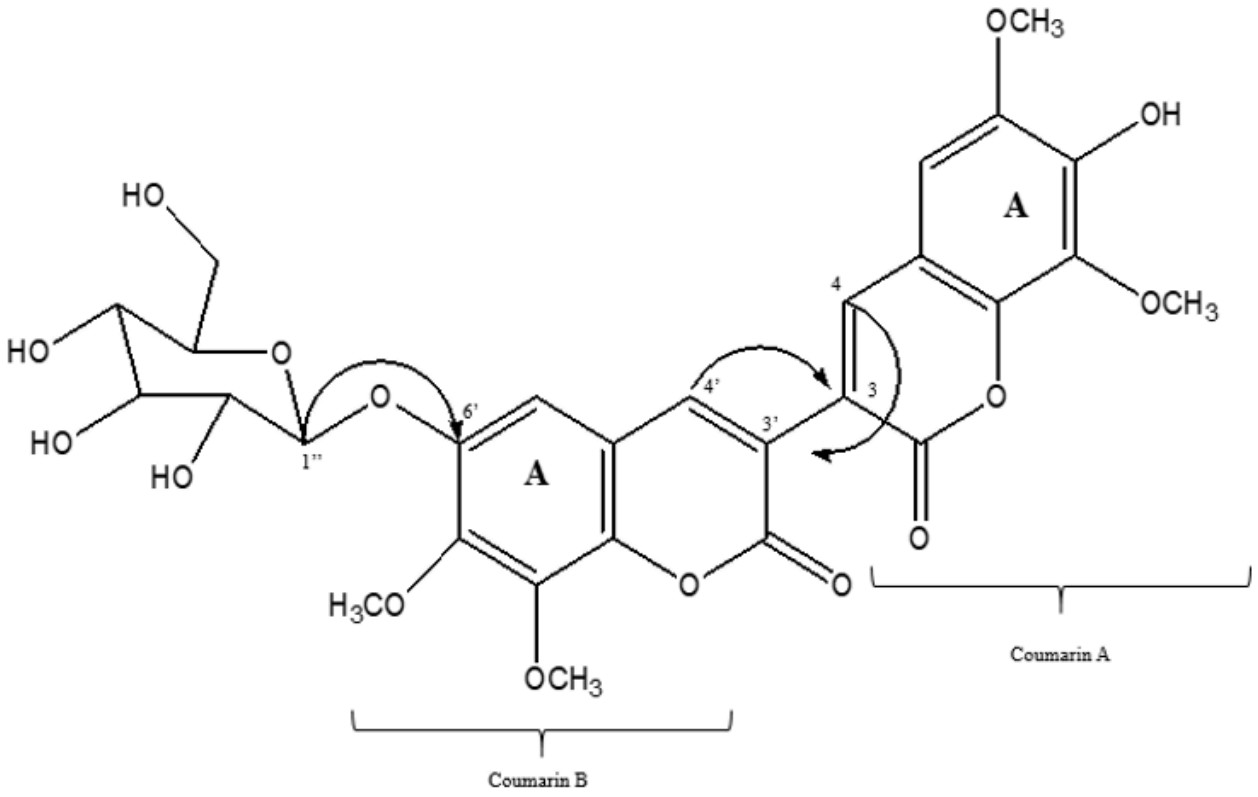
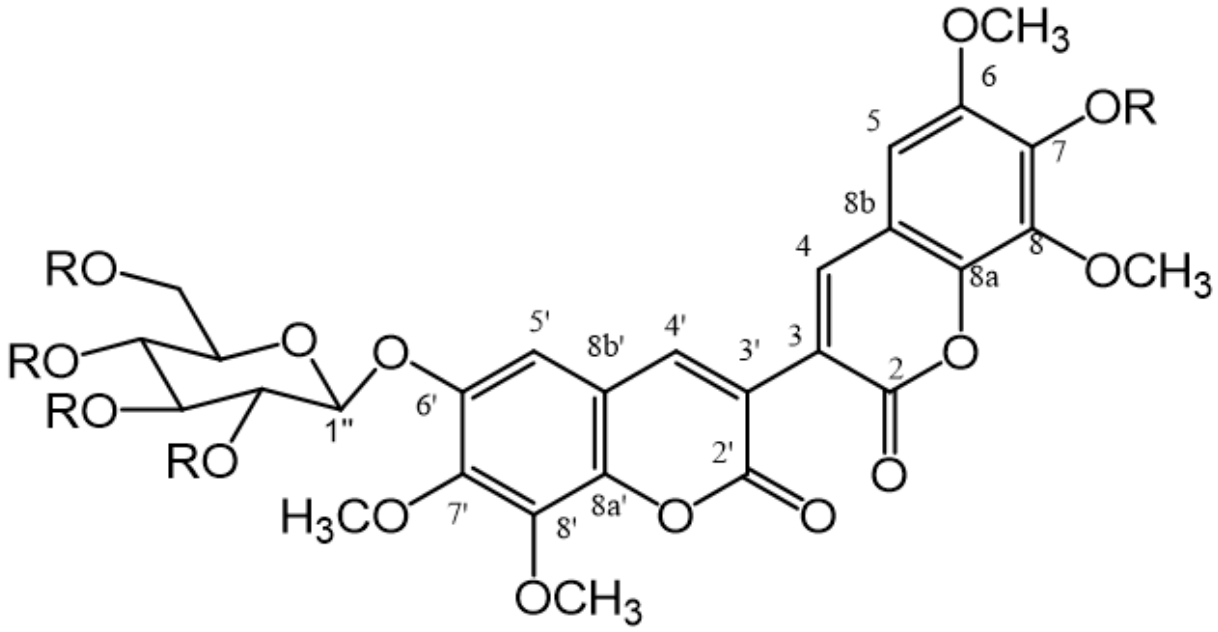


Figure 3

HMBC correlations for C-6' and HMBC correlations for C-4 of Sphaeralgin obtained from the dichloromethane-methanol extract of transformed and non-transformed cells in suspension of *Sphaeralcea angustifolia*



(1) R = H

(1a) R = OAc

Figure 4

Chemical structure of dicoumarin natural product (**1**) named Sphaeralgin and its acetylated derivate (**1a**) isolated from the dichloromethane-methanol extract of transformed and non-transformed cells in suspension of *Sphaeralcea angustifolia*

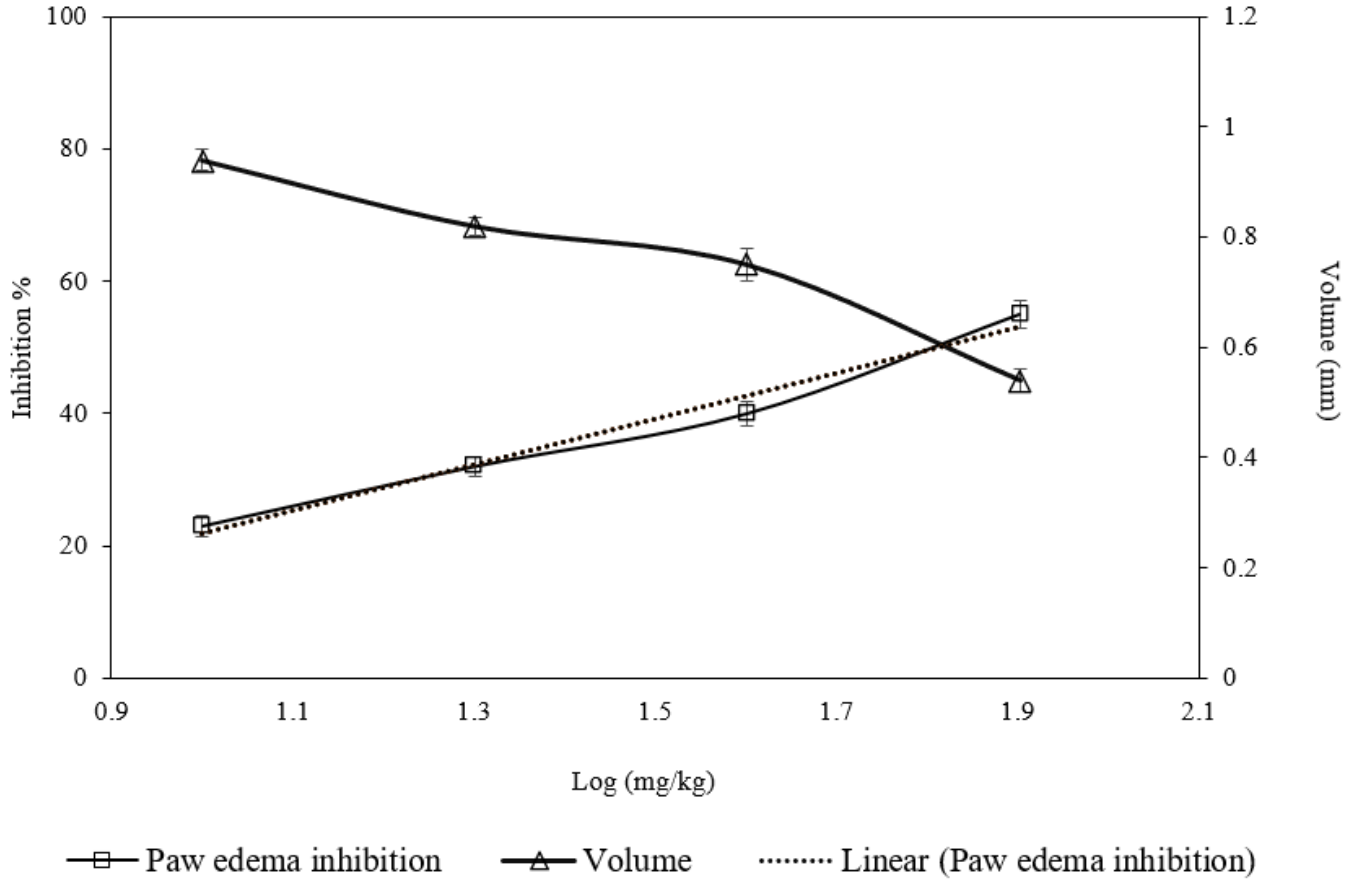


Figure 5

Relationship between the dose of sphaeralgin and the inhibition percentage of subplantar edema, as well as between the dose and the volume of the mouse paw induced with λ–carrageenan. The “best-fit” line was generated by linear regression of the data. Mean±EEM (*n*=7)

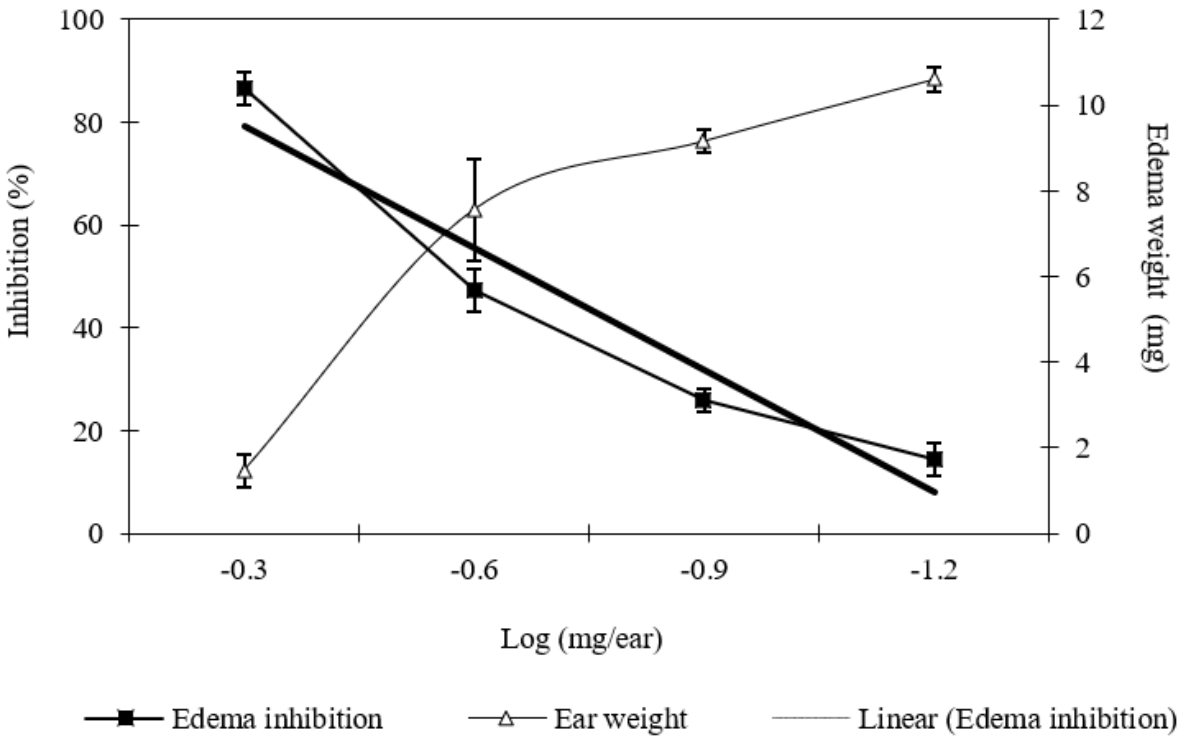


Figure 6

Relationship between the dose of sphaeralgin and inhibition percentage, as well as between dose and the ear weight of the mouse auricular edema induced with12-*O*-tetradecanoylphorbol-13-acetate (TPA). The “best-fit” line shown was generated by linear regression of the data; squares of correlation coefficient (R^2) regression equations are reported. Vertical bars represent the standard error of the means (SEM) (n = 7)

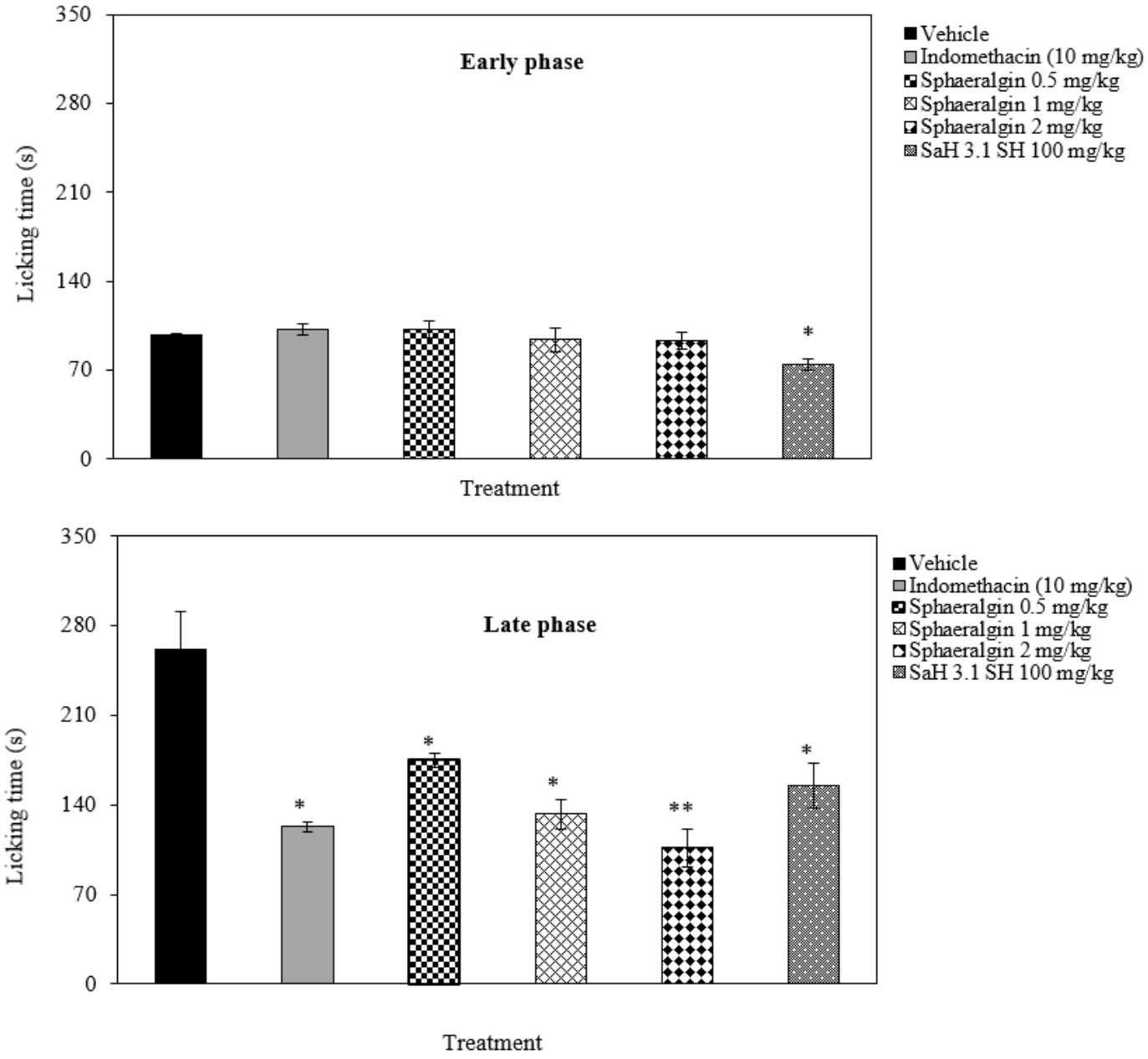


Figure 7

Effect of the sphaeralgin (0.5, 1 and 2 mg/kg) and dichloromethane-methanol extracto of *Sphaeralcea angustifolia* cell cultures in the formalin test in mice. The date are expreses whith the mean ± standard error of the mean (SEM) (n = 5). Significant differences among treatment means were calculated according by the Tukey test, *$p \leq 0.05$, **$p \leq 0.01$