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RESEARCH ARTICLE

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Production of anti-inflammatory compounds in *Sphaeralcea angustifolia* cell suspension cultivated in stirred tank bioreactor

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Sphaeralcea angustifolia is a plant used for the treatment of inflammatory processes. Scopoletin, tomentin, and sphaeralcic acid were identified as the compounds with anti-inflammatory and immunomodulatory effects. Successful establishment of the cell culture in Erlenmeyer flasks has been reported previously. The aim of this study was to evaluate the ability of cells in suspension from *S. angustifolia* grown in a stirred tank bioreactor and demonstrate their capacity to produce bioactive compounds. Cells in suspension grown at 200 rpm reached a maximal cell biomass in dry weight at 19.11 g/L and produced 3.47 mg/g of sphaeralcic acid. The mixture of scopoletin and tomentin was only detected at the beginning of the culture (12.13 μg/g). Considering that the profile of dissolved oxygen during the cultures was lesser than 15%, it is possible that the low growth at 100 rpm could be due to oxygen limitations or to cell sedimentation. At 400 rpm, a negative effect on cell viability could be caused by the increase in the hydrodynamic stress, including the impeller tip, average shear rate, and Reynolds number. The sphaeralcic acid content in the cell suspension of *S. angustifolia* obtained in the bioreactor was two orders of magnitude greater than that reported for the culture grown in Erlenmeyer flasks.

KEYWORDS
coumarins, scopoletin, *Sphaeralcea angustifolia*, sphaeralcic acid, tomentin

1 | INTRODUCTION

Sphaeralcea angustifolia (Cav.) G. Don (Malvaceae) is a medicinal plant found in Mexico that is employed to treat inflammatory processes [1]. The dichloromethane extract from the aerial tissues of this species was active in acute inflammation models and induced-arthritis models in mice [2]. Administration of dichloromethane extract modulates the response of proinflammatory interleukins (ILs), such as IL-1β and IL-6 and tumor necrosis factor alpha (TNF-α), and anti-inflammatory IL-10 in rat-induced arthritis with complete Freund adjuvant [3,4]. Scopoletin (Figure 1) was the active compound detected in the extract [4]. Administration of a gel formulation elaborated with a 1% dichloromethane extract of *S. angustifolia* standardized in scopoletin and showed therapeutic effectiveness and tolerability when administered to patients with osteoarthritis, a chronic disease of the joints and the most frequent form of arthritis [5]. *S. angustifolia* possesses medical potentials; however, collecting these plants from their natural habitat is restricted by the Mexican Ministry of the Environment and Natural Resources [6]. Plant cell culture is a useful method for the production of valuable plant-derived

Abbreviations: DO, dissolved oxygen; DW, dry weight; IL, interleukins; MS medium, Murashige and Skoog medium; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; vvm, volumes of air/medium volume/min

compounds that could be a viable alternative for harvesting wild populations of endangered plants. The suspension cell cultures of this species were established to produce the bioactive compounds. Cell suspensions developed in Murashige and Skoog (MS) medium [7] and with total nitrate concentration reduced to 2.74 mM produced anti-inflammatory compounds (Figure 1), such as scopoletin, tomentin, and sphaeralcic acid; the production of sphaeralcic acid is associated with the cell suspension growth and its greater level was reached at day 16 when the maximal cell biomass was also obtained [8,9]. Dichloromethane/methanol extracts from the medium and the biomass of cell suspensions arrested at day 16 of culture, demonstrated similar anti-inflammatory effects on the Carrageenin-induced rat Footpad Edema; the biomass extract exerted a dose-dependent inhibitory effect. The biomass extract also exhibited a protective effect against 12-*O*-Tetradecanoyl Phorbol-13-Acetate (TPA)-induced mouse-ear irritation [8,9]. Tomentin and sphaeralcic acids were active in both acute inflammatory models, and the effect of sphaeralcic acid in the TPA model was dose dependent [9].

Dichloromethane:methanol extract from biomass of cells in suspension of *S. angustifolia*, as well as tomentin, sphaeralcic acid, and scopoletin, were active as anti-inflammatories and reduced the mean body weight lost in Freund adjuvant- and kaolin/carrageenan-induced arthritis, respectively. In addition, all of them modulate the response of proinflammatory IL-1β, IL-6, and TNF-α and anti-inflammatory IL-4 and IL-10, and scopoletin possess an antiangiogenic activity [10,11].

Commercial production of active compounds such as the bioactive compounds of *S. angustifolia* cultures, only became possible when large-scale cultivation in bioreactors was developed. Bioreactors are devices in which biological or biochemical processes are performed in an environment of monitoring and controlled operating conditions (pH, temperature, pressure, nutrient supply). The possibility of large-scale application is due to its high level of reproducibility, control, and process automation. Georgiev and Weber [12], in their review work on the use of bioreactors for plant cell culture, recognized that stirred tank bioreactors are those most widely used for culturing plant cells. There several advantages include easy scale-up, good fluid mixing, and O_2 transfer capacity, the availability of numerous impeller types, and easy compliance with current good manufacturing practices requirements. Nevertheless, agitation and aeration conditions are operating variables that must be defined to prevent cellular sedimentation and damage to cells by means of a hydrodynamic stress condition. It was reported that, for some plant cell cultures, growth and secondary metabolite production improved to a greater degree when they were grown in agitated tank-type bioreactors than in Erlenmeyer shaken flasks. Such is the case of the culture of *Beta vulgaris* cells to produce betalains [13] and of *Rubia tinctorum* to produce anthraquinones [14]. In both cases, it is reported that the hydrodynamic stress of bioreactors improves the production of the metabolites of interest. In contrast, for *Azaridachta indica* cell suspension, it was reported that when these were cultivated in a stirred tank-type bioreactor, an agitation speed of 800 rpm was detrimental for cell growth and the production of limonoids, whereas the cells reached a maximal growth of 16 g of dry weight (DW)/L with an agitation speed of 400 rpm. The harmful effect at 800 rpm was evidenced by the decreasing cell viability associated with the presence of small eddies [15].

It is therefore important to establish the appropriate agitation and aeration conditions in the system to ensure a continuous transfer of nutrients from the culture medium to the cells. It is also important to avoid problems of hydrodynamic stress that affect cell growth, the production of interest compounds, and to maintain an optimal concentration of dissolved oxygen (DO) in the culture medium without generating limitations during the culture periods. For this process, design and scale-up, extensive knowledge of growth and compound-production kinetics is essential. The objective of this study was to cultivate *S. angustifolia* cell suspension in bioreactors for the production of scopoletin, tomentin, and sphaeralcic acid; to our knowledge, there are no reports to date on this development.

PRACTICAL APPLICATION

Sphaeralcea angustifolia is a medicinal plant from Mexico that is employed to treat inflammatory processes. Tomentin, scopoletin, and sphaeralcic acid were identified as the compounds with anti-inflammatory activity in this species. Considering that collecting these plants from their natural habitat is restricted, biotechnological systems provides advantages for the massive growth of cells in bioreactors and their possible solution in obtaining compounds that are difficult to obtain from wild grown plants. In this work, an effort was made to establish the operating condition in a stirred tank bioreactor more suitable for the development of *S. angustifolia* cell culture and the production of its active compounds. The results show that it is possible for the sphaeralcic acid content obtained in the bioreactor to reach two orders of magnitude greater than the one reported for the culture developed in Erlenmeyer flasks. The hydrodynamic conditions established in the laboratory bioreactor can be used to scale-up the plant cell culture of *S. angustifolia*.

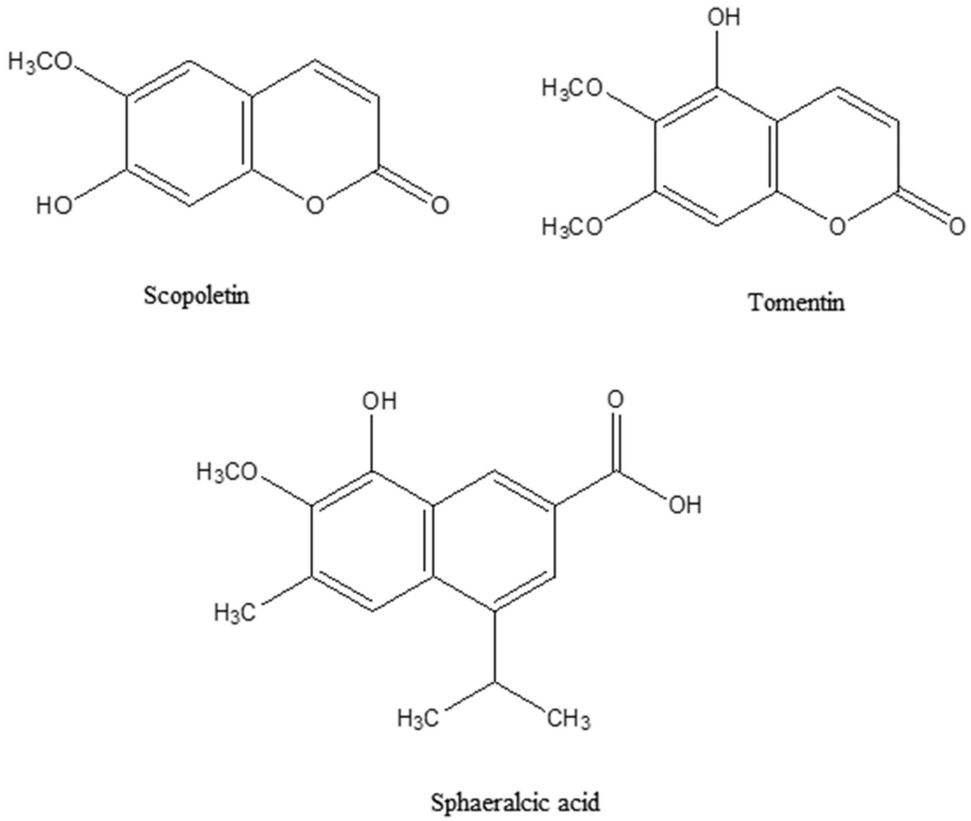

FIGURE 1 Anti-inflammatory compounds, such as tomentin, scopoletin, and sphaeralcic acid, produced in suspension cell cultures of *Sphaeralcea angustifolia*

2 | MATERIALS AND METHODS

2.1 | Calli and cells in suspension cultures

Calli cultures were developed from leaf explants of *S. angustifolia* plants grown in full MS medium [7] supplied with 1.0 mg/L of α-naphtalenacetic acid (NAA), 0.1 mg/L of kinetin (Kin), and 30.0 g/L of sucrose, adjusted to pH 5.7, 3.0 g/L of PhytaGel (Sigma), and autoclaved to 1 kg/cm^2 for 18 min at 120°C. Calli in glass containers were incubated at 26 ± 2°C during a light/dark (16 h:8 h) photoperiod under 50 μM/m/s warm white fluorescent light intensity.

The cells in suspension in batch culture of *S. angustifolia* were established with an inoculum of 4% of calli fresh biomass in 80 mL of liquid MS medium [7] with 2.74 mM of total nitrate (NH_4NO_3, 1.59 mM and KNO_3, 1.15 mM) supplied with 1.0 mg/L of naphthaleneacetic acid, 0.1 mg/L of kinetin (Kin), and 30.0 g/L of sucrose, adjusted to pH 5.7 and autoclaved as it was previously described. Flasks of cell suspensions were placed in an orbital shaker at 110 rpm (New Brunswick Scientific Co., shaking amplitude was 25 mm) and incubated at 26 ± 2°C during a light:dark (16 h:8 h) photoperiod under 50 μM/m/s warm white fluorescent light intensity. The successful *S. angustifolia* cell suspension was transferred into a new medium under sterile conditions every 16 days, utilizing the same inoculum of 4% w/v for a period of 2 years [9,11].

2.2 | Mode of operation and characteristics of the bioreactor

Sphaeralcea angustifolia cell suspension was cultivated in a 2-L stirred tank bioreactor (Applikon, Schiedam, Netherlands) with a 1.4-L working volume of MS medium with 2.74 mM of total nitrate, supplied with 1.0 mg/L of NAA, 0.1 mg/L of Kin, and 30.0 g/L of sucrose, adjusted to pH 5.7, autoclaved as it was previously described. To characterize the growth of the cell suspension, the bioreactor was inoculated with the sediment of fresh biomass 20% w/v of cells in suspension in growth phase cultivated in flasks under the nutritional conditions described previously. The bioreactor was maintained at 26 ± 2°C and the pH was controlled at 5.7 by the addition of HCl (0.1 N) or NaOH (0.5 M). The bioreactor was operated at 100, 200, and 400 rpm using a Rushton impeller type (impeller to tank diameter of 0.384) with four blades (50 mm), and the aeration system was set at 0.1 volumes of air/medium volume/min.(vvm) utilizing sterile gas from an air pump added through a flow meter and an air filter (0.2 μm). pH and DO tension were monitored online. Oxygen tension with a polarographic electrode (Applisens Dissolved Oxygen

Sensor Model Z010032520; Applikon) connected to a biocontroller (Model ADDI 1030). Also, every other day, the volumetric oxygen transfer coefficient (k_La) was evaluated by the dynamic method [16,17].

2.3 | Growth kinetics

Growth curves under each bioreactor work condition were obtained by registration of the DW of filtered biomass from a 25-mL sample of cell suspension at the beginning of cultures and every 2–3 days during a period of 2 weeks. An aliquot of each supernatant biomass was filtered using a Buchner vacuum filter (Whatman No. 1), the biomass was dried at 50°C for 24 h to determine DW, and the medium was stored for its later extraction. The Growth Index was calculated considering maximal biomass obtained with reduction of the inoculum and divided by the inoculum, while the maximal growth rate (μ_{max}) was calculated by means of a semilog calculation of exponential phase and time (graph not shown); doubling time of the equation was $Dt = \ln 2/\mu$max [8]. Active compound curves were obtained by quantification of the mixture of coumarins (scopoletin and tomentin) and sphaeralcic acid in the extracts from the biomasses and the culture medium of these batch cultures.

2.4 | Cell viability

Cell viability was determined considering membrane integrity by means of the Evans blue dye (0.25%, w/v) exclusion test [18].

2.5 | Hydrodynamic stress parameters

The following hydrodynamic parameters were considered to determine the effect on growth of the *S. angustifolia* cell culture and the production of compounds scopoletin, tomentin, and sphaeralcic acid in the bioreactor:

Impeller tip speed (v_i) expressed in m/s was calculated according to Equation (1) described by Raposo and Lima-Costa [19]:

$$v_i = \pi \times N \times Di, \tag{1}$$

where N is the rotational speed of the impeller (1/s) and Di is the impeller diameter (0.05 m). The average energy dissipation rate (ε), expressed in kW/kg is the specific power input (kW/m^3) (P/V) divided by the density of the culture, that is, ρ (1.018 kg/m^3), according to Equation (2) [20]:

$$\varepsilon = P/\rho V = Np\, N^3 D_i^5/V, \tag{2}$$

where N is the rotational speed of the impeller (1/s), Di is the impeller diameter (0.05 m), Np is the power number for the relevant impeller at the prevailing value of the Reynolds number ($[ND_i{}^2p]\ /\mu_{app}$), and v is the dissipation of medium volume.

The average shear rate (Y_{av}), expressed in 1/s was estimated for the Rushton turbine using Equation (3) [19], with the proportionality constant (k) having a value of 10 for this propeller.

$$Y_{av} = k \times N. \tag{3}$$

Reynolds number for pseudoplastic fluids (Re) was calculated as follows:

$$\mathrm{Re} = \left(\left(Di^2 N^{2-n}\delta\right)/0.1K\right)(n/6n+2)^n, \tag{4}$$

where δ is the density (1.018 Kg/m^3), n is the flow behavior index (0.6 dimensionless), and K is the fluid consistency index (0.5 Pas seg^n) of the Ostwald de Weale model (power law, $\tau = K\ Y^n$) [13].

2.6 | Production of coumarins (scopoletin and tomentin) and sphaeralcic acid

The dry biomass (200 mg) of each sampling point during growth kinetics was extracted at room temperature three times by maceration (24 h for each procedure) with a mixture of reagent-grade dichloromethane:methanol (9:1, v/v) (Merck, México). The dichloromethane:methanol extracts obtained for each sample were filtered through filter paper (Whatman No. 1), pooled, and concentrated to dryness under reduced pressure. The extracts obtained were dissolved in high purity methanol (Merck) for their high-performance liquid chromatography (HPLC) analyses [8,9].

The culture media of each kinetic sampling point (25 mL) was partitioned (v/v) three times with dichloromethane; extracts from each medium were pooled and concentrated to dryness under reduced pressure. The extracts obtained were dissolved in high purity methanol (Merck) for their HPLC analysis [8,9].

Analyses of HPLC were carried out in a Waters system (2695 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 ver. 1; Waters Corporation, México). Separations were performed in a Spherisorb RP-18 column (250 × 4.6 mm, 5 μm; Waters Corporation) employing a constant temperature of 25°C during the analyses. Samples (20 μL) were eluted at a 1.0 mL/min flow rate with a gradient of mobile phases (Table 1) of (A) high-purity H_2O with trifluoroacetic acid (TFA) (0.5%) and (B) high-purity CH_3CN (Merck), and the compounds were detected by monitoring absorbance for coumarins at λ = 340 nm and for sphaeralcic acid at λ = 357 nm. The chromatographic method had a 25 min run time. Identification of scopoletin (99%; Sigma–Aldrich Química, México) and tomentin (95%), as well as sphaeralcic acid (98%), was

TABLE 1 Gradient of mobile phases used in the HPLC analyses of biomass and culture medium extracts of cells in suspensions of *Sphaeralcea angustifolia* cultivated in stirred tank bioreactor

Time (min)	(A) H_2O–TFA 0.5%	(B) CH_3CN
0	100	0
1	100	0
3	95	5
7	70	30
13	50	50
17	0	100
22	0	100
25	100	0

performed by comparing their retention times (tomentin: 10.969 min, scopoletin: 11.51 min, coumarin mixure: 11.2 min, and sphaeralcic acid: 23.489 min) and absorbance spectra. The tomentin and sphaeralcic-acid compounds were isolated and purified at our laboratory from the *S. angustifolia* cell suspension, according to the procedure already reported [9]. Calibration curves were constructed with standard solutions of 0.5, 1, 2, 4, 8, 12, and 16 μg/mL; scopoletin presented a regression equation of $Y = 118442X + 130042$ and $R^2 = 0.9784$ and, for sphaeralcic acid, these were $Y = 7435.9X + 1806.3$ and $R^2 = 0.9968$.

2.7 | Statistical analysis

Growth parameters (growth index, μ_{max}, *Dt*, and maximal biomass), and the production of sphaeralcic acid obtained in each experimental condition were compared by means of Analysis of Variance; factors with a $p \leq 0.05$ were considered significant. Significant differences in the experiments were calculated by $Tukey_{0.05}$ test (SAS, see 9.1; SAS Institute, Inc.).

3 | RESULTS AND DISCUSSION

The culture of cells in a suspension of *S. angustifolia* grown in a bioreactor under different agitation conditions did not have lag phase; the exponential phase was initiated directly (Figure 2). Cultures grown at 200 rpm reached a maximal cell growth after 11 days in culture with a maximal cell biomass in DW of 19.11 ± 2.25 g/L and a μ_{max} of 0.14 ± 0.008 L/days. Maximal biomass obtained in the cell suspension cultivated at 200 rpm was statistically higher than those of cell suspensions grown at 100 and 400 rpm (Table 2). However, growth rates determined in the cell suspensions cultivated with 100 and 200 rpm did not present significant differences and were superior to those obtained in the cultures at 400 rpm. The pH of the culture medium was controlled throughout the culture at a value of 5.7 ± 0.1.

At the beginning of kinetics, the cultures had 100% cell viability under all stirring conditions. However, at the end, cell viability in the cultures was different: at 200 rpm, cellular viability decreased to 85%, while the viability of 100 and 400 rpm decreased to 50–60%, respectively. Considering these results, it is possible that the low growth obtained at 100 rpm was due to the sedimentation problems of the cells, while at 400 rpm, a negative effect could be caused by the increased hydrodynamic stress conditions. In both cases, these problems affected the cellular viability in the cultures. At the highest stirring rate, foam formation and cell adhesion were also observed on the wall of the bioreactor and these could limit the absorption of nutrients from the culture medium by the cells, altering the cellular metabolic processes and the production of secondary metabolites [15,21].

Maximal biomass of cell suspension in culture from *S. angustifolia* obtained in the bioreactor was 1.42-fold superior to that generated in the cultures developed in Erlenmeyer flasks [11]; nevertheless, the μ_{max} was lowest. This result is consistent with that described for other species, such as *B. vulgaris* [13], *A. indica* [15], and *Burcera linanoe* [22]; maximal biomasses were obtained in the stirred tank-type bioreactor rather than in Erlenmeyer flasks. The stirred bioreactor tank is considered a good option for plant cell culture.

The chromatograms of biomasses and media extracts obtained by HPLC analysis from cells in suspensions of *S. angustifolia* in the bioreactor reveal compounds with a retention time and absorption spectrum similar to those of the mixture of coumarins and sphaeralcic acid pure compounds (Figure 3). Sphaeralcic acid is the main compound accumulated extracellularly and intracellularly in the cells in suspensions of *S. angustifolia*. Figure 4 shows the intracellular accumulation of sphaeralcic acid, and that it reaches values up to two orders of magnitude greater than that detected in extracellular form. In the cellular suspension cultivated at 200 rpm, the intracellular sphaeralcic acid production was associated to the growth, the highest intracellular accumulation was obtained at days 7 (52.15 mg/L) and 9 (43.19 mg/L). As reported in the cell cultures of *S. angustifolia* grown in Erlenmeyer flasks [8], at the beginning of the culture (days 2 and 4), sphaeralcic acid was excreted and concentrations were similar in the three stirring speeds. It was also observed that at the end of the culture (day 11), the greatest extracellular accumulation of sphaeralcic acid occurs especially in the cell suspension cultivated at a stirring speed of 100 rpm, corresponding to the condition under which the culture lost the greatest viability. This may suggest that during this period, excretion of sphaeralcic acid is due to a rupture of the cells. The sphaeralcic acid content of *S. angustifolia* obtained in the bioreactor is two orders of magnitude greater than that reported for the culture grown (0.25 mg/L) in Erlenmeyer flasks [8,9]. Sphaeralcic acid is a de novo compound isolated from cells in suspensions of *S. angustifolia*; besides, it is the most

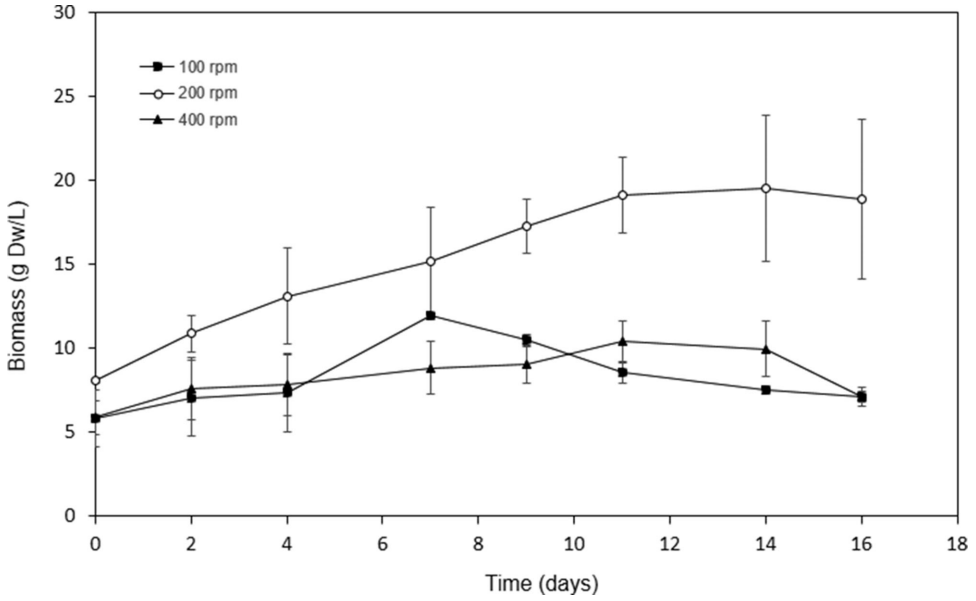

FIGURE 2 Growth curves in batch of cell suspension cultures from *Sphaeralcea angustifolia* developed in different conditions of agitation (100, 200, and 400 rpm) with 0.1 vvm in stirred tank bioreactor. Mean ± standard error of the mean

TABLE 2 Growth parameters of *Sphaerealcea angustifolia* cell suspensions grown under different conditions of agitation and 0.1 vvm in stirred tank bioreactor

Stirring rate (rpm)	μ_{max}(1/days)	Duplication time (days)	Maximal biomass (g/L DW)	Maximal biomass time	Growth index (GI)
100	0.10 ± 0.016^a	7.14 ± 0.96^a	11.93 ± 0.16^b	7	1.04 ± 0.53
200	0.14 ± 0.008^a	4.95 ± 0.28^a	19.11 ± 2.25^a	11	1.36 ± 0.20
400	0.04 ± 0.007^b	16.11 ± 3.45^b	10.42 ± 0.84^b	11	0.77 ± 0.09

Mean ± standard error of the mean (SEM) ($n = 3$). According to the Tukey multiple range test, each growth parameters are significantly different when these are followed by a different letter ($p \leq 0.05$).

abundant compound found in the dichloromethane:methanol extract. The dichloromethane:methanol extract and phaeralcic acid are active as anti-inflammatories, they modulate the response of proinflammatory IL-1β, IL-6, and TNF-α and anti-inflammatory IL-4 and IL-10, they reduce the mean body weight lost in chronic inflammation model in rats, and no toxic effects were identified.

The mixture of scopoletin and tomentin was only detected intracellularly in the cell suspension cultivated at 100 and 200 rpm (Figure 3). In the stirred suspensions at 200 rpm, the peaks emitted of coumarin mixture that could be quantified on the calibration curve were at the beginning of culture (day 2: 133.89 μg/L, and day 4: 36.04 μg/L), and these levels were one order of magnitude lower than those extracellularly detected in the cell cultures grown in Erlenmeyer flasks [8]. In contrast, in the stirred suspensions at 100 rpm, the highest quantified intracellular content was detected at day 9 of culture (213.03 μg/L), when the cell growth and cell viability in the suspension culture were reduced may be due to low oxygen availability in the culture medium.

It is well-known that phytoalexins, such as the coumarins, are solely accumulated in plants under abiotic stress conditions or by pathogen attack (biotic stress) as a first mechanism of defense [23]. Production of these types of compounds in cell suspension cultures in some cases is null or achieved at low yields, but there are some existing reports in which scopoletin production has been achieved by in vitro cultures of different plant species, such as *Ammi majus* [24], with the use of elicitors, such as 1, 2, 3-benzothiadiazole-7-carbothioic acid, S-methyl ester (BION) and with a lysate of *Enterobacter sakazaki*. Scopoletin was identified in the callus culture derived from hypocotyls of *A. majus* seedlings; the most effective elicitor for increasing its production was the *E. sakazaki* lysate. Siatka and Reichling [25] reported the stimulation of and the 12-fold increase in the accumulation of scopoletin in cell suspension cultures of *Archangelica officinalis* elicited with *Fusarium oxysporum* in comparison with the control (0.2 mg/mL). There are also other reports on the production of secondary metabolites in cells in suspension cultures of plants stimulated with copper utilized as abiotic elicitor. Modification of copper content in *Angelica archangelica* cell suspension cultures stimulated the production of scopoletin, with a dose-dependent effect both in the biomass and in the culture medium, reaching highest concentrations of scopoletin on employing 5–50 μM of copper [26].

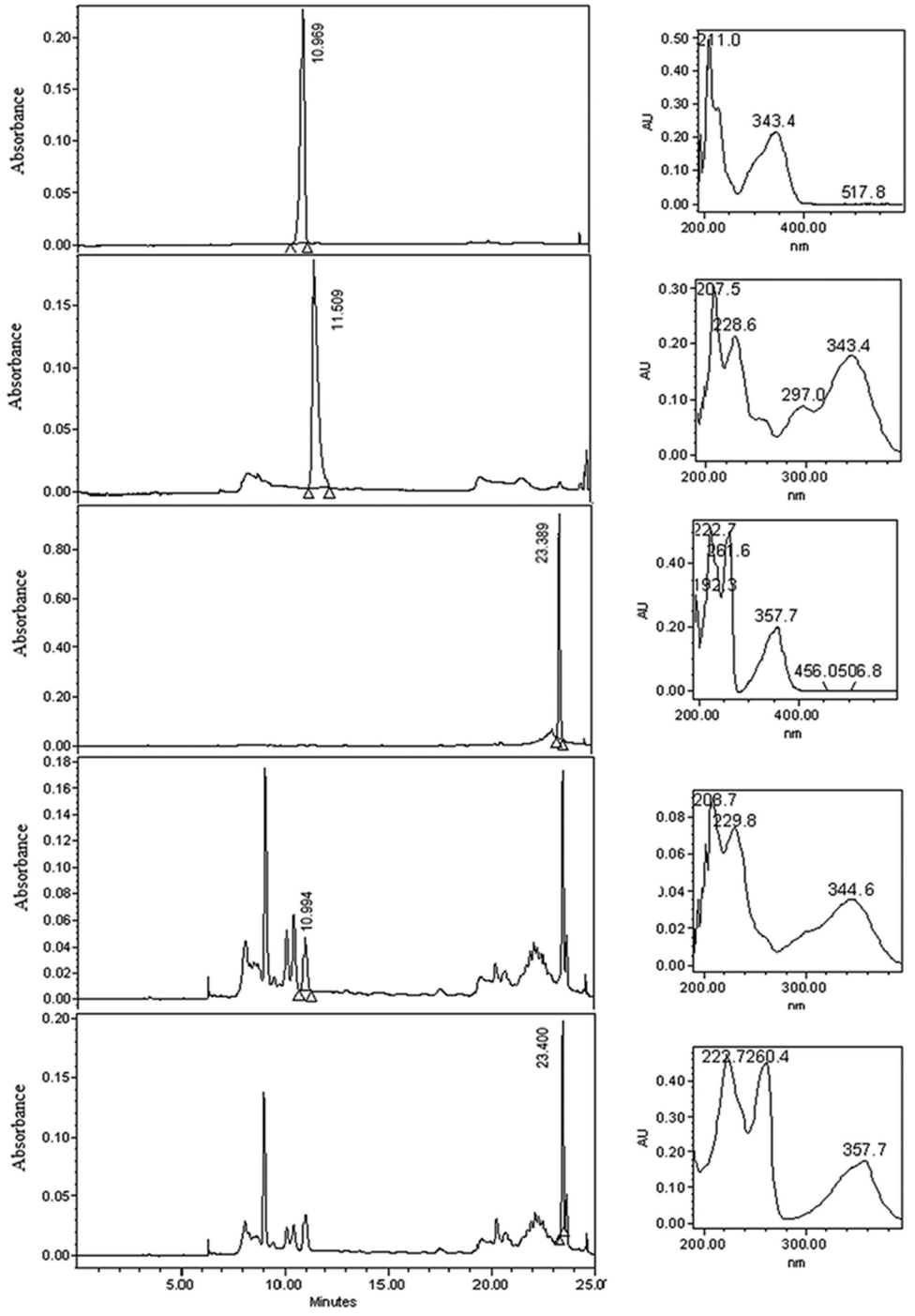

FIGURE 3 Chromatograms and absorption spectrum of tomentin (λ = 343 nm, rt = 10.96 min), scopoletin (λ = 343 nm, rt = 11.5 min), sphaeralcic acid (λ = 357 nm, rt = 23.3) identified in the dichloromethane:methanol extracts from biomass of *Sphaeralcea angustifolia* cell suspension at day 9 in culture, as a mixture of tomentin and scopoletin (λ = 343 nm, rt = 10.99 min) and sphaeralcic acid (λ = 357 nm, rt = 23.3)

Excess of copper ions causes an oxidative stress in the cells, they can react with H_2O_2 and O_2, causing at the same time, an increase in the activity of antioxidant enzymes and inducing processes that generate OH, triggering the activation of signaling pathways for the gene expression of antioxidant enzymes and synthesis of phenolic compounds [26]. These strategies could be applied to the cell cultures in suspensions of *S. angustifolia* to stimulate the production of scopoletin, tomentin, and sphalercic acid.

These results suggest that agitation speed in the bioreactor is a determining parameter for the growth of *S. angustifolia* cell cultures and the production of coumarins, such as scopoletin and tomentin, as well as for sphaeralcic acid; stirring speeds above 200 rpm reduce the growth. Studies carried

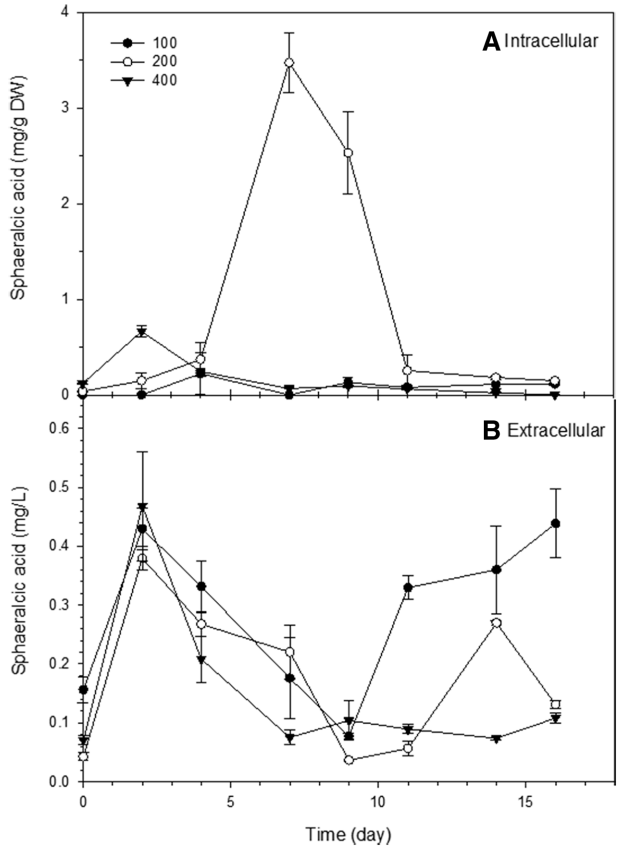

FIGURE 4 Accumulation (A) and excretion (B) of sphaeralcic acid in cultures of *S. angustifolia* suspension cells grown under different agitation conditions (100, 200, and 400 rpm) with 0.1 vvm in stirred tank bioreactor. Mean ± standard error of the mean

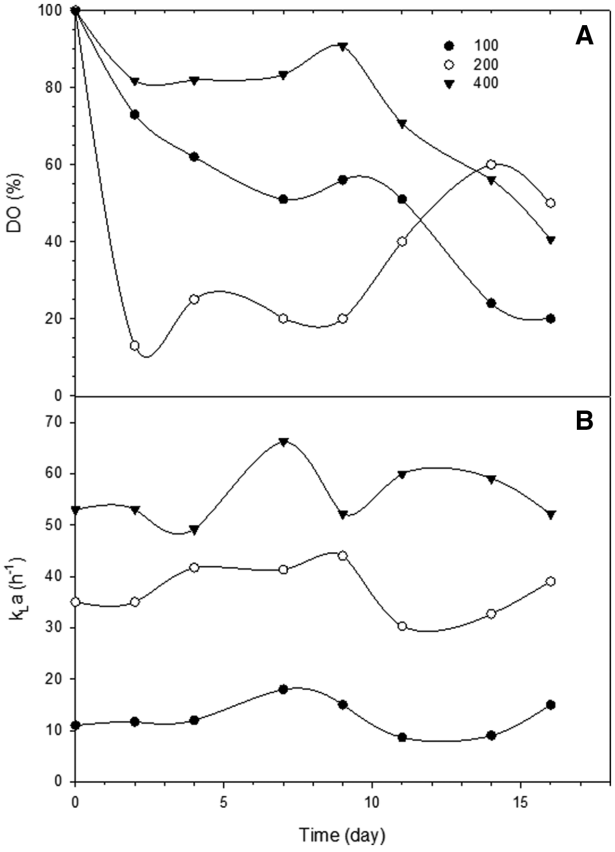

FIGURE 5 Dissolved oxygen (DO) and volumetric oxygen-transfer coefficient (k_La), during batch cell suspension cultures of *Sphaeralcea angustifolia* developed in different conditions of agitation (100, 200, and 400 rpm) with 0.1 vvm in stirred tank bioreactor

out in *Azadirachta indica, Borojoa patinoi*, and *Thevetia peruviana* show that agitation rate of 200 rpm was also favorable for the growth and production of active compounds [27].

The agitation speed used in the cell cultures of *S. angustifolia* was directly related to the content of DO in the culture medium. k_La values at 400 rpm were of 50–65/h, the availability of DO was greater than 80% until day 10; while at 200 rpm k_La values were of 30–45/h with the lowest DO during the phase of cell growth; finally at 100 rpm the amount of oxygen decreased up to 60% at 10 days of culture, with k_La values of 10–15/h (Figure 5). It is possible that limitations in cell growth and the production of sphaeralcic acid observed at 100 rpm could be a consequence of oxygen limitation.

As a consequence of the increase in agitation speed from 100 to 400 rpm, it was observed that hydrodynamic stress indicators were also modified: a four-fold increase in the speed of the impeller tip (v_i) and in average shear rate (Y_{av}) were observed, and one of seven times more in Reynolds number (Table 3). The Reynolds number values calculated for the cell culture exposed to 100 and 200 rpm were 361 and 958, respectively, indicating that the cell culture was in a laminar flow regime, while at 400 rpm, the Reynolds value was 2645,

TABLE 3 Hydrodynamic stress parameters determined for *Sphaeralcea angustifolia* cell suspensions cultivated in a 2-L stirred tank bioreactor

Stirring rate (rpm)	*Vi* (m/s)	$\mathcal{E}$ (W/kg)	Y_{av} (1/s)	Re (–)
100	0.34	10.85×10^{-4}	16.66	361.80
200	0.67	271×10^{-4}	33.33	958.85
400	1.34	6	66.66	2645.45

indicating that the cells were in a transitional flow regime. The culture of cells of *S. angustifolia* at 400 rpm exhibited a decrease in its growth, in its viability, and showed lowest production of sphaeralcic acid; these negative effects can be attributed to the increase in the hydrodynamic stress condition present in the tank at the agitation speed of 400 rpm, mainly in Reynolds stress and in turbulent eddy length, as has been suggested for the cultivation of *A. indica* cells [15,23]. Busto et al. [14] indicated that cultures of *R. tinctorum* cells exposed in a stirred tank bioreactor operated at an agitation speed of 450 rpm underwent a viability loss and lower cell multiplication than those grown in Erlenmeyer flasks. The authors suggest that this may be due to the fact that the cells in the bioreactor are exposed to eddies of 0.03 mm, which is one

half of size of an eddy produced in an Erlenmeyer flask; these eddies could damage the cells or aggregates in the *R. tinctorum* cultures. Rodríguez-Monroy and Galindo [13] indicated that *B. vulgaris* cells have the ability to grow in a stirred tank under an impeller tip speed as high as 0.95 m/s. Comparing the results obtained in stirred tank bioreactor with those of cultures performed in shake flasks, a decrease in cell concentration, betalain production, and a growth rate was observed. In that study, it was observed that the rheological behavior of bioreactor broths was pseudoplastic, while in the Erlenmeyer medium, this was Newtonian. The authors indicated that the change in the rheology can play important role in the reduction of hydrodynamic stress during cultivation and that it allows the growth of cells in the bioreactor.

4 | CONCLUDING REMARKS

The cell suspension of *S. angustifolia* was successfully cultured in a 2-L stirred tank bioreactor, operated at 200 rpm using a Rushton-type impeller and MS medium with 2.74 mM of total nitrate. The cultures reached a maximal growth of 19.11 g/L and mainly produced sphaeralcic acid with an intracellular production up to two orders of magnitude greater than that determined in the extracellular form. Further, the sphaeralcic acid content (52.15 mg/L) was two orders of magnitude greater than that reported for the culture grown in Erlenmeyer flasks at the same nutritional conditions. It will be mandatory to explore in the *S. angustifolia* cells in suspension cultivated in the stirred tank bioreactor, under operation conditions now established, other abiotic elicitors, such as metals (copper, zinc, or cobalt), as well as biotic elicitors (lysates of bacteria or fungi) to improve coumarins and sphaeralcic acid production.

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CONFLICTS Of INTEREST

The authors have declared no conflict of interest.

REFERENCES

1. Martínez, M., Catálogo De Nombres Vulgares Y Científicos De Plantas Mexicanas, Fondo de Cultura Económica, México; 1979.
2. Meckes, M., David-Rivera, A. D., Nava-Aguilar, V., Jimenez, A., Activity of some Mexican medicinal plant extracts on carrageenan-induced rat paw edema. *Phytomedicine*. 2004, *11*, 446–451.
3. Júarez-Ciriaco, M., Román-Ramos, R., González-Márquez, H., Meckes-Fischer, M., Efecto de *Sphaeralcea angustifolia* sobre la expresión de citocinas pro y antiinflamatorias. *Lab. Ciencia. Not Tec. Lab*. 2008, *2*, 21–24.
4. García-Rodríguez, R. et al., *Sphaeralcea angustifolia* (Cav.) G. Don extract, a potential phytomedicine to treat chronic inflammation. *Bol. Latinoam. Caribe Plant Med. Aromat*. 2012, *11*, 468–477.
5. Romero-Cerecero, O. et al., Clinical trial for evaluating the effectiveness and tolerability of topical *Sphaeralcea angustifolia* treatment in hand osteoarthritis. *J. Ethnopharmacol*. 2013, *147*, 467–473.
6. SEMARNAT, Norma Oficial Mexicana NOM-059-ECOL-2001, (2001).
7. Murashige, T., Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*. 1962, *15*, 473–497.
8. Nicasio-Torres, P.M. et al., Production of potential anti-inflammatory compounds in cell suspension cultures of *Sphaeralcea angustifolia* (Cav.) G. Don. *Acta. Physiol. Plant*. 2016, *38*, 209–220.
9. Pérez-Hernández, J. et al., Sphaeralcic acid and tomentin anti-inflammatory compounds produced in cell suspension cultures of *Sphaeralcea angustifolia*. *Planta Med*. 2014, *80*, 209–214.
10. Pan, R., Gao, X. H., Li, Y., Xia, Y. F., Dai, Y., Anti-arthritic effect of scopoletin, a coumarin compound occurring in *Erycibe obtusifolia* Benth stems, is associated with decreased angiogenesis in synovium. *Fundam. Clin. Pharmacol*. 2010, *24*, 477–490.
11. Nicasio-Torres, M. P. et al., Effect of dichloromethane-methanol extract and tomentin obtained from *Sphaeralcea angustifolia* cell suspensions in a model of kaolin/carrageenan-induced arthritis. *Planta Med*. 2017, *4*, e35–e42.
12. Georgiev, M. I., Weber, J., Bioreactors for plant cells: hardware configuration and internal environment optimization as tools for wider commercialization. *Biotechnol. Lett*. 2014, *36*, 1359–1367.
13. Rodriguez-Monroy, M., Galindo, E., Broth rheology, growth and metabolite production of Beta vulgaris suspension culture: a comparative study between cultures grown in shake flasks and in a stirred tank. *Enzyme Microb. Technol*. 1999, *24*, 687–693.
14. Busto, V. D., Rodríguez-Talou, J., Giulietti, J. C. Merchuk, J. C., Effect of shear stress on anthraquinones production by *Rubia tinctorum* suspension cultures. *Biotechnol. Prog*. 2008, *24*, 175–181.
15. Villegas-Velásquez, S. et al., Hydrodynamic stress and limonoid production in *Azadirachta indica* cell culture. *Biochem. Eng. J*. 2017, *122*, 75–84.
16. Doran, P. M., *Bioprocess Engineering Principles*, Second Edition, Academic Press, London; 2013.
17. Bedoya Pérez, J., Hoyos Sánchez, R., Agitation-aeration relation effects on cell culture of *Azadirachta indica A*. Juss neem on azadirachtin production in a stirred tank bioreactor. *Rev. Fac. Nac. Agron. Medellín*. 2010, *63*, 5293–5305.
18. Orozco-Sanchez, F., et al., Oxygen limitations to grow *Azadirachta indica* cell culture in shake flasks. *Rev. Mex. Ing. Quim.* 2011, *10*, 343–352.
19. Raposo, S. and Lima-Costa, M. E., Effects of the hydrodynamic environment and oxygen mass transfer on plant cell growth and

milk-clotting protease production in a stirred-tank reactor. *Eng Life Sci*. 2012, *12*, 441–449.

20. Kieran, P. M., Malone, D. M., MacLoughlin, P. F., *Effects of Hydrodynamic and Interfacial Forces on Plant Cell Suspension Systems, Influence of Stress on Cell Growth and Product Formation*, Springer Berlin Heidelberg, Berlin, Heidelberg; 2000, 139–177.
21. Trejo-Tapia, G., Rodriguez-Monroy, M., Cellular aggregation in secondary metabolite production in in vitro plant cell cultures. *Interciencia*. 2007, *32*, 669–674.
22. Pavon-Reyes, L. et al., Cell culture of *Bursera linanoe* in a stirred tank bioreactor for production of linalool and linalyl acetate. *Nat. Prod. Commun.* 2017, *12*, 319–322.
23. Kai K., Mizutani M., Watanabe K., Sakata K., Accumulation of coumarins in *Arabidopsis thaliana*. *Phytochemistry*. 2006, *67*, 379–386.
24. Staniszewska I., Królicka A., Maliński E., Łojkowska E., Szafranek J., Elicitation of secondary metabolites in in vitro cultures of *Ammi majus* L. *Enzyme Microb. Technol*. 2003, *33*, 565–568.
25. Siatka T., Reichling J., Stimulation of scopoletin accumulation in *Archangelica officinalis* Hoffm, cell suspension cultures by fungal elicitors. *Herba Polonica*. 2000, *46*, 12–17.
26. Siatka T., Chlebek J., Host'alkova A., Copper (II) sulfate stimulates scopoletin production in cell suspension cultures of *Angelica archangelica*. *Nat. Prod. Commun*. 2017, *125*, 1779–1780.
27. Alvarez-Yela, A. C., Chiquiza-Montaño, L. N., Hoyos, R., Orozco-Sánchez, F., Rheology and mixing analysis of plant cell cultures (*Azadirachta indica*, *Borojoa patinoi* and *Thevetia peruviana*) in shake flasks. *Biochem. Eng. J*. 2016, *114*, 18–25.

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