

Article

Establishment of a Cell Suspension Culture of *Ageratina pichinchensis* for the Improved Production of Anti-inflammatory Compounds

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Abstract: Many species of the Asteraceae family are used in traditional Mexican medicine for possessing healing properties. *Ageratina pichinchensis* (Asteraceae) is a plant used for the treatment of gastric ulcers, deep wounds and for its antifungal effects. The aim of this study was to establish a cell suspension culture of *A. pichinchensis*, quantify the anti-inflammatory constituents 2,3-dihydrobenzofuran and 3-epilupeol, to evaluate the anti-inflammatory potential of its extracts and perform a phytochemical analysis. Cell suspension cultures were established in MS culture medium supplemented with 30 g L⁻¹ sucrose and 1.0 g L⁻¹ α -naphthaleneacetic acid (NAA) plus 0.1 mg L⁻¹ 6-furfurylaminopurine (KIN). The ethyl acetate extracts of cell suspension cultures analyzed by GC revealed that the maximum production of compounds. The anti-inflammatory activity of these extracts exhibited significant inhibition of NO production. Furthermore, the phytochemical study of EtOAc and MeOH extracts of the biomass on day 20 led to the identification of 17 known compounds. The structures of compounds were assigned by analysis of 1D and 2D NMR data and the remainder by GC-MS. This is the first report of the production of the (-)-Artemesinol, (-)-Artemesinol glucoside, Encecalin and 3,5-diprenyl-acetophenone compounds by a cell suspension cultures of *A. pichinchensis*.

Keywords: Cell suspension culture; anti-inflammatory activity; phytochemical analysis

1. Introduction

The genus *Ageratina* (Asteraceae) consist of about 1200 species and it is distributed in temperate and subtropical regions of America, Europe, Africa and Asia [1-4]; in Mexico, about 164 species of *Ageratina* are registered [5-6]. Several species of this genus have been studied and these studies have demonstrated bactericidal, antifungal, antiviral, analgesic, cytotoxic, anti-inflammatory and to treat gastric ulcers effects [7-12]. In the state of Morelos, Mexico, *A. pichinchensis* is traditionally used to treat gastric ulcers and heal deep wounds mainly. Phytochemically the aerial parts of *A. pichinchensis* are characterized by containing sterols, triterpenes, benzochromenes and benzofurans [13-14]. The benzochromenes isolated from *A. pichinchensis* showed insecticidal [12], antifungal [15-16] and gastroprotective activities [17]. However, the production of these compounds can be unsustainable

through the large-scale planting of *A. pichinchensis* and be affected because many secondary metabolites tend to vary their production according to the season of the year and climatic fluctuations as has happened with other species [18]. Alternatively, numerous studies show that plant cell cultures could play an appropriate role for the constant and controlled production of bioactive compounds [19-23], for example, the production of the compound shikonin that has shown an important effect on the inhibition of the growth of cancer cells, tumor invasion, anti-inflammatory and healing, which is obtained from the roots of the Asian plant *Lithospermum erythrorhizon*, has a yield of 1 to 2% and depends on the geographic distribution and climate, in addition the wild plant requires 5 to 7 years of growth for its production, while cultivated plant cells can contain 12 to 20%, which is an advantage over the wild plant [24-26]. Examples like this support the use of plant cells for the production of value-added compounds, recently our research group reported the isolation and anti-inflammatory effect of (2S,3R)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2) and 3-epilupeol (3), from the ethyl acetate extract of a callus culture of *A. pichinchensis* [27].

In this study we established a cell suspension culture of *A. pichinchensis* and improved the production of compounds 2 and 3. Furthermore, the extracts were evaluated for anti-inflammatory activity. The phytochemical analysis of the EtOAc and MeOH extracts of this culture, allowed the isolation and identification of seventeen known compounds accumulated including the anti-inflammatory compounds 2 and 3. Some of the identified compounds have been described with anti-inflammatory and antimicrobial activity.

2. Results and Discussion

2.1. Cell Suspension Culture and Growth Kinetics

It has been shown that friable callus is most suitable for establishing cell suspension cultures of any plant species. *A. pichinchensis* cells transferred to MS liquid culture medium with the same growth regulators as in callus cultures (1.0 mg L^{-1} NAA and 0.1 mg L^{-1} KIN) grew easily, which showed abundant biomass and slightly yellowish appearance (Fig. 1a, b, c). Similar results were observed in suspension cell cultures of *Stevia rebaudiana* which were disintegrated in a period of 7 days and cells also acquired a yellowish appearance [28]. In this study, cell growth of *A. pichinchensis* was faster (22 days) in liquid culture medium MS than in callus cultures, in contrast, callus reached their maximum growth between and 30 - 40 days [27]. This can be caused by the facilitated absorption of nutrients in liquid medium [29].

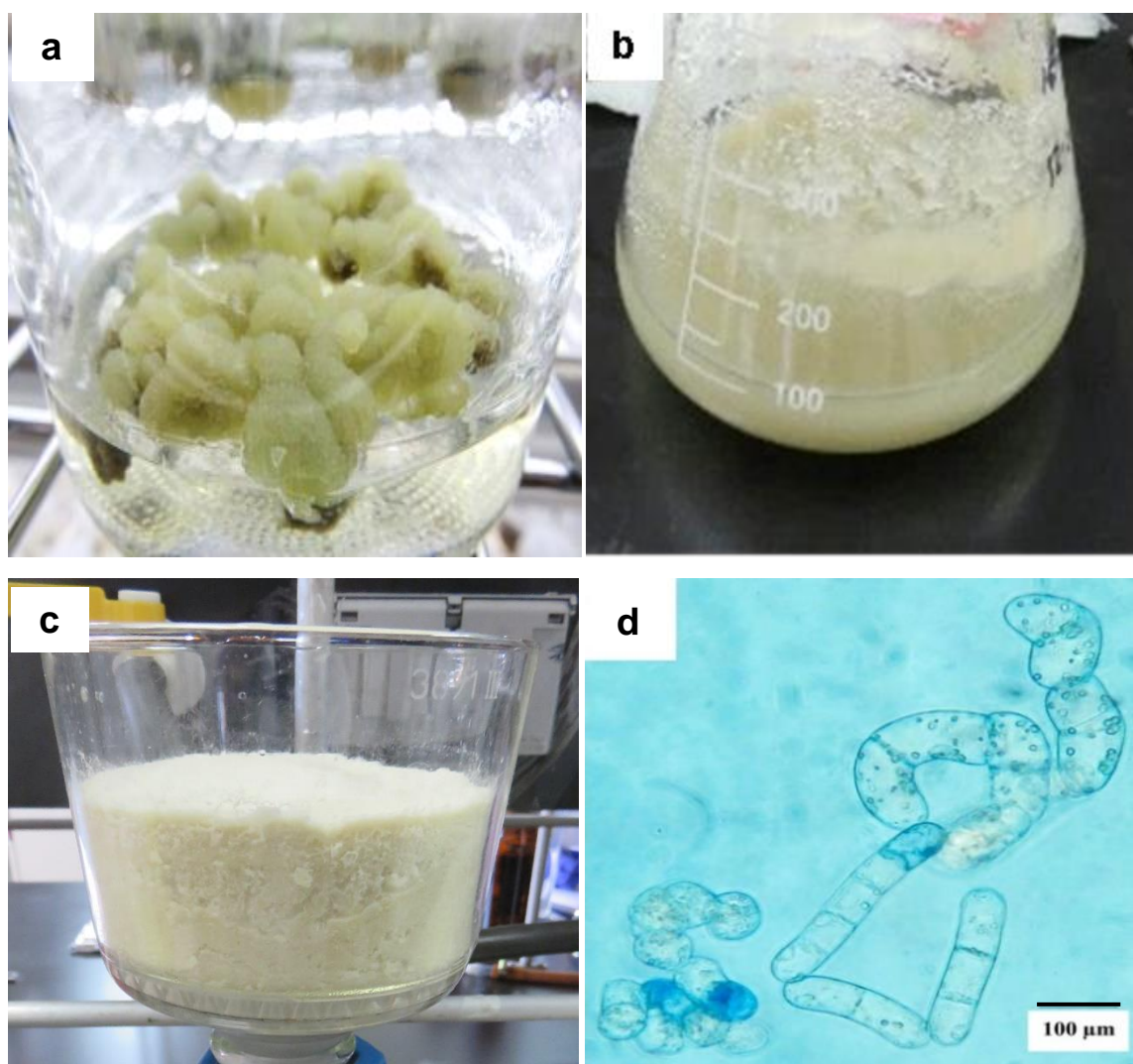


Figure 1. Cell culture from *A. pichinchensis* leaves explants. a) friable callus at 20 days of culture; b) cell suspension culture with abundant biomass at 16 days of culture; c) filtered biomass from “b”; d) microscopic image of cell suspension culture (100x).

The growth kinetic of *A. pichinchensis* cell suspension cultures was maintained until 22 days, over which it showed a typical growth curve (Fig. 2). The growth kinetic was characterized by a lag phase of 4 days, during which the biomass reached 2.37 g L⁻¹ DW; subsequently, the cells entered the exponential phase and lasted until day 16. During this time, the maximum biomass accumulation was observed (13.28 g L⁻¹) which was about 5.6-fold over initial dry weight. The specific growth rate (μ) was 0.20 days⁻¹ and doubling time (Dt) was 3.01 days⁻¹ until reaching a stationary phase, in which, the cell culture showed a brown appearance and decreased growth (Fig. 2). In terms of the consumption of total sugars, an abrupt decrease of the sugar content was observed until day 6, but the biomass increased; the remainder sugar was stable after day 14 (Fig. 2), which may be due to consumption of the nutrients and the lack of oxygen in the medium [30, 31].

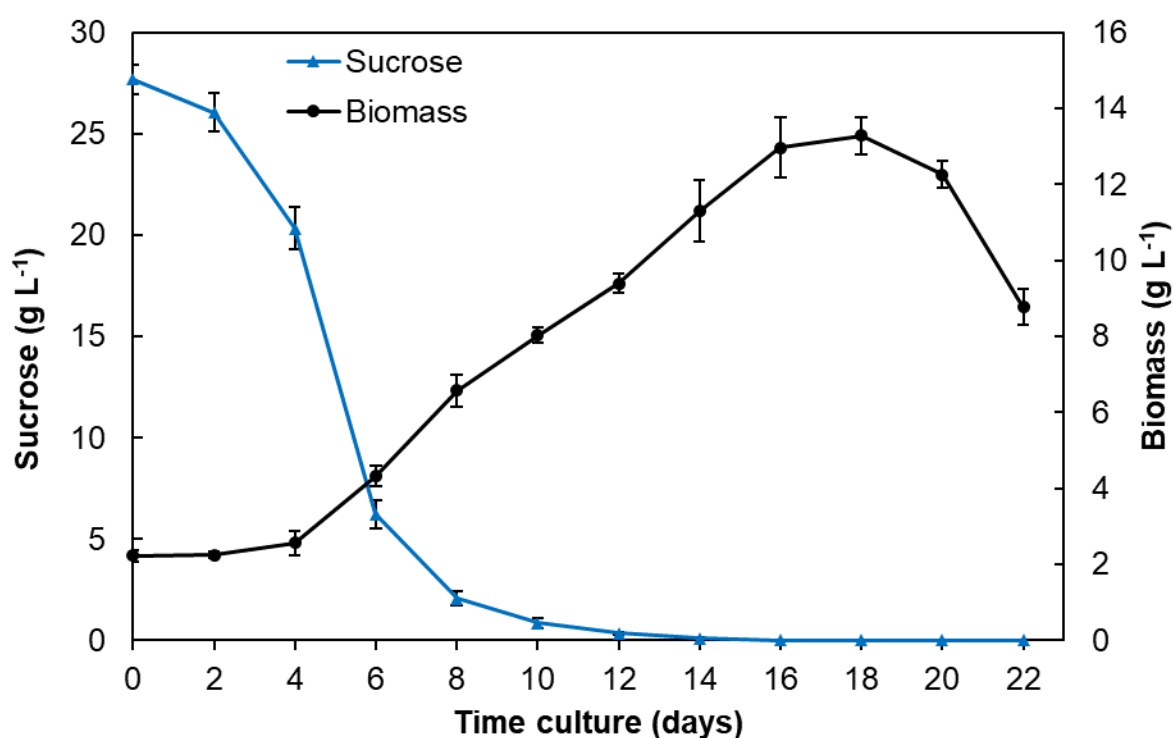


Figure 2. Growth kinetics and consumption of sucrose of *A. pichinchensis* cell suspension culture during grown for 22 days in MS medium with 1.0 mg L⁻¹ NAA and 0.1 mg L⁻¹ KIN.

Growth kinetics is similar to that for *Spilanthes acmella*, in which, the cell suspension culture reached a specific growth rate of 0.279 days⁻¹, during the exponential phase the doubling time was 2.50 days and the maximum biomass was 8.5 g L⁻¹ at day 15 [32]. On the other hand, of *Satureja khuzistanica* cell suspension cultures reached a maximum dry biomass of 19.7 g L⁻¹ at 21 days with a specific growth rate of 1.5 days⁻¹ and doubling time was 7.6 days⁻¹ [33]. Similarly, cell suspension cultures of *Helianthus annuus*, produced 12.7 g L⁻¹ dry biomass at 9 days of culture showing a specific growth rate of 0.21 days⁻¹ and doubling time of 3.31 days⁻¹ [34]. These results suggest that *A. pichinchensis* cell cultures have similar tendency to other cell suspension cultures and although biomass yield differs for each species, it can also be produced in a short time.

2.2. Cell viability and pH In the Culture Medium

Cell viability was suitable in the cell suspension culture, which decreased slightly during the 22 days of culture. At the beginning of the culture, 89.57% of viability was presented and at 22 days it decreased only to 77.19% (Fig. 3). These results confirm that the establishment of *A. pichinchensis* cell suspension culture was successful since the viability was greater than 50%. In other species such as *Taxus globosa*, at the end of the growth culture the viability decreases from 45 to 50% [35], which was lower than *A. pichinchensis* culture. The microscopic image showed that the *A. pichinchensis* cell suspension culture produced single cells and some small aggregates (Fig. 1d). In a study conducted in *Taxus cuspidate* cell cultures [35], they found that size of cell aggregates is important since at average sizes greater than 800 μm there is a decrease in the production of paclitaxel; nevertheless, in our study the aggregates were smaller (Fig. 1d).

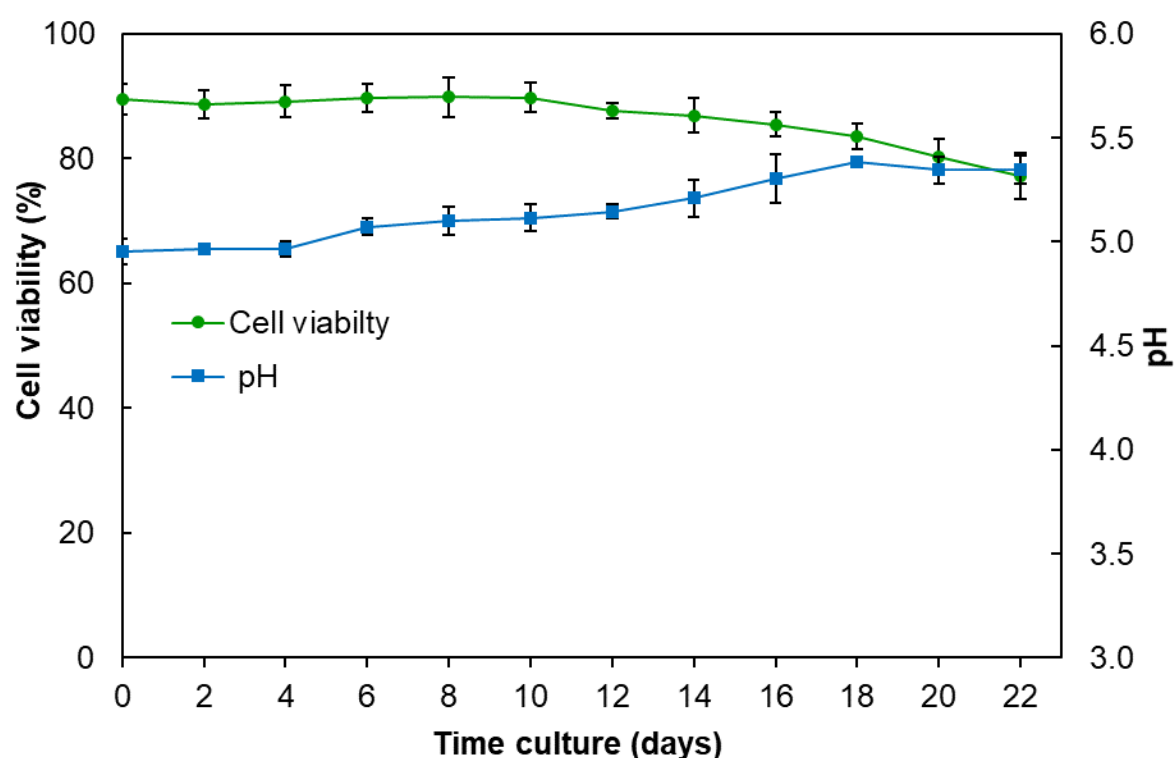


Figure 3. Viability of cell suspension culture and pH of the culture medium for 22 days of culture growth of *A. pichinchensis*.

On the other hand, there was an increase in the pH values the exponential phase (from day 4 to 18). Subsequently, between days 18 and 22 the pH values were stable, but the cell viability and the increase in pH values (Fig. 3). In cell suspension cultures of *Dioscorea deltoidea* reported there was an acidification of the culture medium during the lag phase, an alkalization during the exponential phase and a steady state of pH in the stationary phase. At the same time, they mentioned that the exponential growth of the cell culture medium [36]. The observed changes in pH values may be related to the use of ammonium and nitrate or the absorption of sugar by the transport mechanism with H^+ [37]. Moreover, plant cells can also modify the external pH they can increase or decrease the values according to the pH range in which they growth, until a balance is produced [38].

2.3. Phytochemical Analysis of the Cell Suspension Cultures.

The phytochemical analysis of the ethyl acetate and methanol extracts obtained from biomass on day 20, led to the identification of 17 known compounds (Fig. 4), including the benzochromenes: desmethoxyencecalin (1), (-)-artemesinol (6), (-)-artemesinol glucoside (7), and encecalin (10); three triterpenes: 3-epilupeol (3), 24-methylene-9,19-cyclolanastan-3 β -ol (8), and 24-methylenecycloartan-3-one (9); two sterols: β -sitosterol (4) and stigmasterol (5); the benzofuran (2*S*,3*R*)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2); the 3,5-diprenyl-acetophenone (11); the fatty acids: palmitic acid (12), hexadecanoic acid, methyl ester (13), isopropyl palmitate (14), palmitamide (15), oleamide (16), and the amino acid derivative methyl pyroglutamate (17). Compounds 1-5 and 10-14 were identified in the ethyl acetate extract. Compounds 1-5 were isolated by column chromatography as described in the experimental part, these were identified by analysis of their ^1H , ^{13}C NMR data and by comparison with the literature data [27]. While compounds 10-14 were identified by GC-MS analysis (NIST 1.7a). Compounds 4-5 and 10-11 were previously reported as constituents of the aerial parts of *A. pichinchensis* wild plant [15, 17], compounds 1-3 and 12-13 were previously reported in a callus culture of *A. pichinchensis* [27].

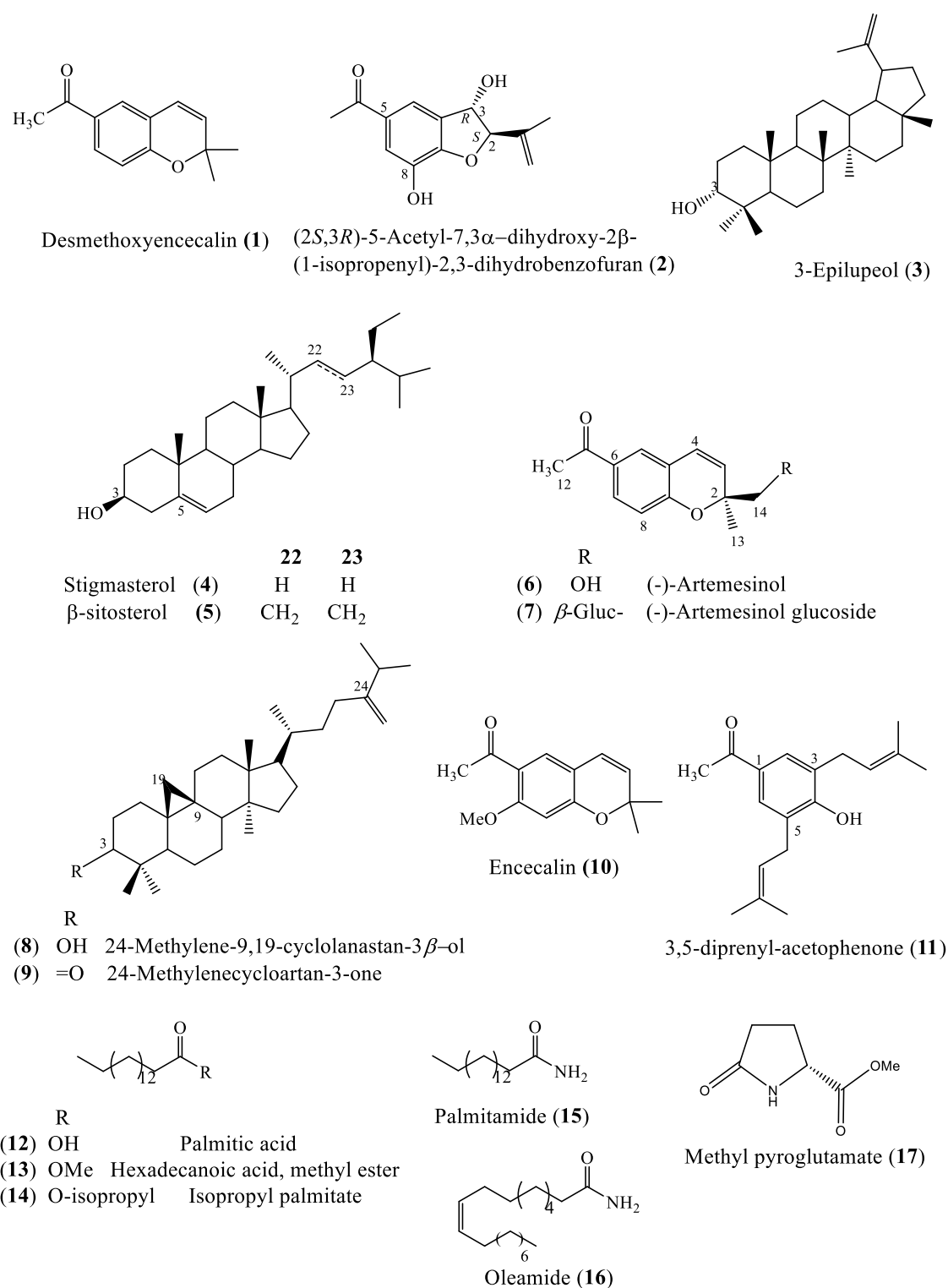


Figure 4. Chemical structures of compounds identified in cell suspension culture of *A. pichinchensis*

The MeOH extract was fractionated by column chromatography (described in experimental part), from the chromatographic work 5 groups of fractions were obtained (MSR-M-1 to MSR-M-5). The MSR-M-1 group was analyzed by GC-MS, and compounds 15-17 were identified. The MSR-M-2 and MSR-M-3 groups were purified by column chromatography (described in the experimental part). From these groups, compounds 1 and 2 were isolated and identified, in the mixtures of 4 and 5. The MSR-M-4 group subjected to purification process allowed obtaining 3 groups of fractions:

MSR-M-4A (1-32; 21.2 mg), MSR-M-4B (33-42; 32.7 mg) and MSR-M -4C (43-68; 48.4 mg). The MSR-M-4A group was analyzed by GC-MS, and compounds 8-9 were identified. The MSR-M-4B group was subjected to silica gel column chromatography and compound 6 was obtained as a semi-solid mass. The ^1H NMR spectrum of (6) displayed three signals at δ 1.30 (3H, s, CH_3 -13), δ 2.44 (3H, s, CH_3 -12), and δ 3.53 (2H, s, CH_2 -14), which suggests the presence of CH_3 -, CH_3CO and $-\text{CH}_2\text{OH}$ groups in the skeleton. In the aromatic proton region, signals of a ABX system at δ 7.65 (1H, dd, J = 8.4, 2.2 Hz, H-7), δ 7.51 (1H, d, J = 2.2 Hz, H-5), δ 6.73 (1H, d, J = 8.5 Hz, H-8) indicated the presence of a three-substituted aromatic ring. The olefinic AB system at δ 5.58 (1H, d, J = 10 Hz, H-4) and δ 6.40 (1H, d, J = 10 Hz, H-3), established the presence of a double bond with *cis*-configuration (Spectrum S1, Supplementary material). Moreover, the ^{13}C NMR spectrum (Spectrum S2, Supplementary material) showed 13 signals, including an acetoxy carbonyl carbon (δ 196.84; CO), two oxygenated carbons (δ 80.86, 68.99; CO and CH_2OH), five methine protons at δ 127.94, 130.97, 127.45, 116.67, 124.32 (C-7, C-3, C-5, C-4 and C-8), four quaternary carbons δ 157.38, 130.21, 120.64 and 80.86 (C-9, C-6, C-10 and C-2) and two methyl groups in δ 26.32 and 23.49 (C-12 and C-13). According to the data obtained in ^1H , and ^{13}C NMR, compound 6 was characterized as (-)-Artemesinol (6) [40]. Compound 7 was obtained as a semi-solid mass from MSR-M-4C fractions group. The ^1H NMR spectrum of (7) displayed signals in δ 1.30 (3H, s, CH_3 -13), δ 2.44 (3H, s, CH_3 -12), δ 3.91 (1H, d, J =10.6 Hz, H-14a), δ 3.58 (1H, d, J =10.7 Hz, H-14b), a symmetric AB system δ 5.67 (1H, d, J = 10 Hz, H-3) and 6.42 (1H, d, J = 10 Hz, H-4), as well as, hydrogens in the aromatic region at δ 7.69 (1H, dd, J = 8.4, 2.2 Hz, H-7), 7.56 (1H, d, J = 2.2 Hz, H-5) and δ 6.77 (1H, d, J = 8.5 Hz, H-8). The ^1H , COSY, DEPT and ^{13}C NMR (S3, S4, S5 and S6 Spectra, Supplementary material) displayed a compound similar to (-)-Artemesinol (6), but with the additional presence of a pyranose sugar moiety (δ 4.46, 1H, d, J = 7.8 Hz, H-1'), δ 3.87-3.18 (6H, m, H-2' to H-6'). The chemical shift of the proton at -1' (δ 4.46) and the coupling constant $J_{1',2'}$ of 7.8 Hz indicated that the glycosidic bond β -configure. The direct connections between the protons and carbons were identified by the HSQC spectrum (Spectrum S7, Supplementary material). In the HMBC spectrum (Spectrum S8, Supplementary material), the glycosidic linkage was located at C-14, based on the correlations between H-1' (δ 4.46, 1H, d, J = 7.8 Hz) and C-14 (δ 75.19), as well as the hydrogens H-14a (δ 3.91, 1H, d, J =10.6 Hz) and H-14b (δ 3.58, 1H, d, J =10.7 Hz) with C-2 (δ 79.95), C-13 (δ 23.79) and C-3 (δ 127.77). The optical rotation $[\alpha]_{20\text{D}} = -8.3^\circ$ (c 0.8, CH_3OH) allows to suggest a relative "R" configuration for C-2 of compound 7. These evidences indicated that the structure of compound 7 is 6-acetyl-(S)-2-methyl-2-(R)-glucopyranosil chromene or Artemesinol glucopyranoside [41]. It is important to note that the 1D (^{13}C , DEPT) and 2D NMR data (COSY, HSQC and HMBC) of compound 7 have not been described in the literature. Minority compounds (8-11), identified in the *A. pichinchensis* cell culture, have been employed in cosmetic preparations [17, 43, 44].

2.4. Production of Bioactive Compounds During Growth Kinetics.

The GC-MS analysis of the EtOAc extract obtained from biomass of *A pichinchensis* suspension cell culture indicated that the main bioactive compounds (2S,3R)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2) and 3-epilupeol (3) are produced during all growth phases (Fig. 5). The quantification of compound 2 and 3 was

performed by analyzing the peaks at RT= 20.67 min (compound 2) and 38.70 min (compound 3), and the molecular ion peaks at m/z = 234 for 2 and 426 for 3 observed in GC-MS (Supplementary material, S9 and S10).

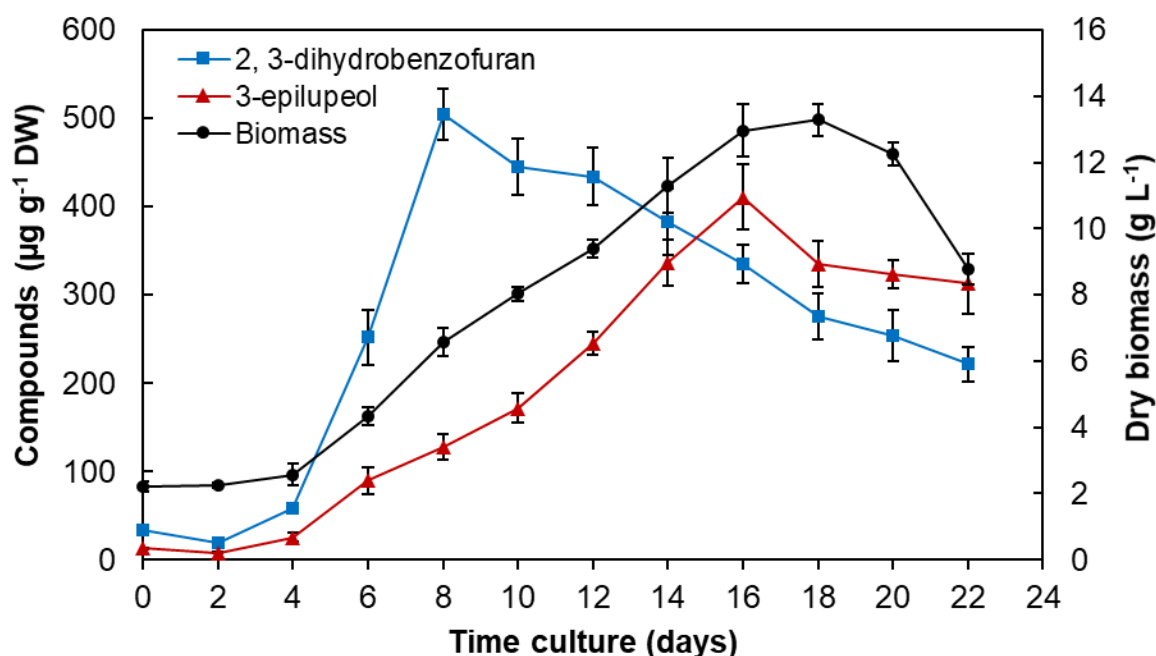


Figure 5. Relationship of the production of 2,3-dihydrobenzofuran and 3-epilupeol and culture growth of *A. pichinchensis* for 22 days.

The production of compound 2 started from the lag phase and the maximum production ($510.75 \pm 29.10 \mu\text{g g}^{-1}$ of dry biomass) occurred on day 8 of the exponential phase; then it gradually decreased until day 22. Similar results have been observed in *Capsicum chinense* Jacq suspension cell cultures, in which, the capsaicin compound reaches a maximum production of $567.4 \mu\text{g g}^{-1}$ of dry biomass at 25 days [44]. On the other hand, in *Celosia cristata* cell suspension cultures, the betalaine production was observed at the beginning of the exponential phase and then decreased, remaining stable during the exponential and stationary phases [45].

Regarding to the 3-epilupeol compound (2), an association with the culture growth was observed, obtaining its maximum yield ($410.59 \pm 36.91 \mu\text{g g}^{-1}$ of dry biomass) at day 16 (Fig. 5). Likewise, the production of the fatty acid amide spilanthol by cell suspension cultures of *Spilanthes acmella* Murr., presented a trend associated with its growth, reaching a maximum yield during the exponential phase and subsequently decreasing rapidly due to the lack of nutrients and consequently cellular death [46]. In another species such as *Eurycoma longifolia*, was reported that cell suspension cultures produce the quassinoid eurycomanone and this also occurs from the beginning of the growth kinetics reaching its maximum (1.7 mg g^{-1} of dry biomass) [47].

These results are important given that they improve those reached by callus cultures whose maximum production was identified on day 30, producing $650 \mu\text{g g}^{-1}$ of dry biomass for compound 2 and $201.10 \mu\text{g g}^{-1}$ of dry biomass of compound 3 [27], reducing the time of production of the

compounds is a desirable characteristic in suspension cell cultures, in addition to being more homogeneous cultures compared to callus cultures, it is additionally possible to increase the production of bioactive compounds by adding elicitors, additionally it is it can then be scaled up to reactors to mass produce the compounds [48-50].

On the other hand, enecalinal (10) (m/z 232, RT = 18.5 min) and 3,5-diprenyl-1,4-hydroxyacetophenone (11) (m/z 272, RT = 21.87 min), were detected in very low concentration during the exponential phase (day 8) (Fig. 6). Antifungal, gastroprotective and antinociceptive effects have been reported for compounds 10 and 11 [43, 17]. The highlight of both compounds is that their production on *in vitro* cultures is reported for the first time. Based on the importance of its biological effects, the cell suspension cultures of *A. pichinchensis* is a useful alternative for production of compound 10 and 11, which could be increased by inductors.

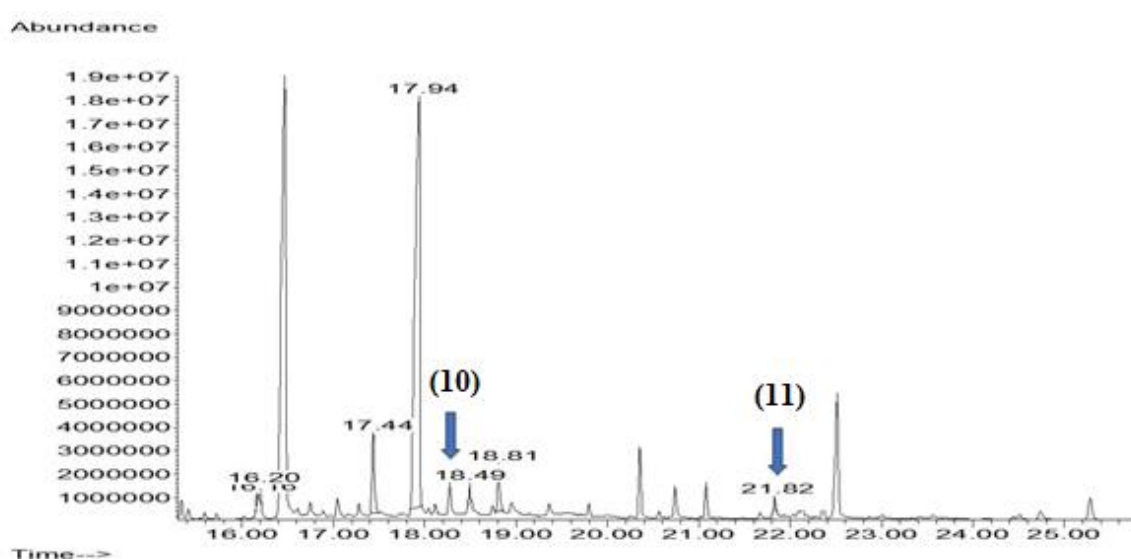


Figure 6. GC-MS chromatogram of the ethyl acetate extract from day 8 of suspension cells cultures, shows the compounds enecalinal (10) and 3,5-diprenyl-4-hydroxyacetophenone (11).

2.5. *In vitro* Anti-inflammatory Activity

The anti-inflammatory activity of the ethyl acetate extracts of the biomass of *A. pichinchensis* suspension cell culture was assessed at different times of the growth kinetics (8, 12 and 16 days). Firstly, the extracts were evaluated for their effect on viability of RAW 264.7 cells at different concentrations (5, 10, 20, 30 and 40 $\mu\text{g mL}^{-1}$). All extracts did not exhibit a significant reduction in the viability of macrophages compared with the control group while the positive control (etoposide) showed a significant reduction in the cellular viability at 40 $\mu\text{g mL}^{-1}$ (Fig. 7).

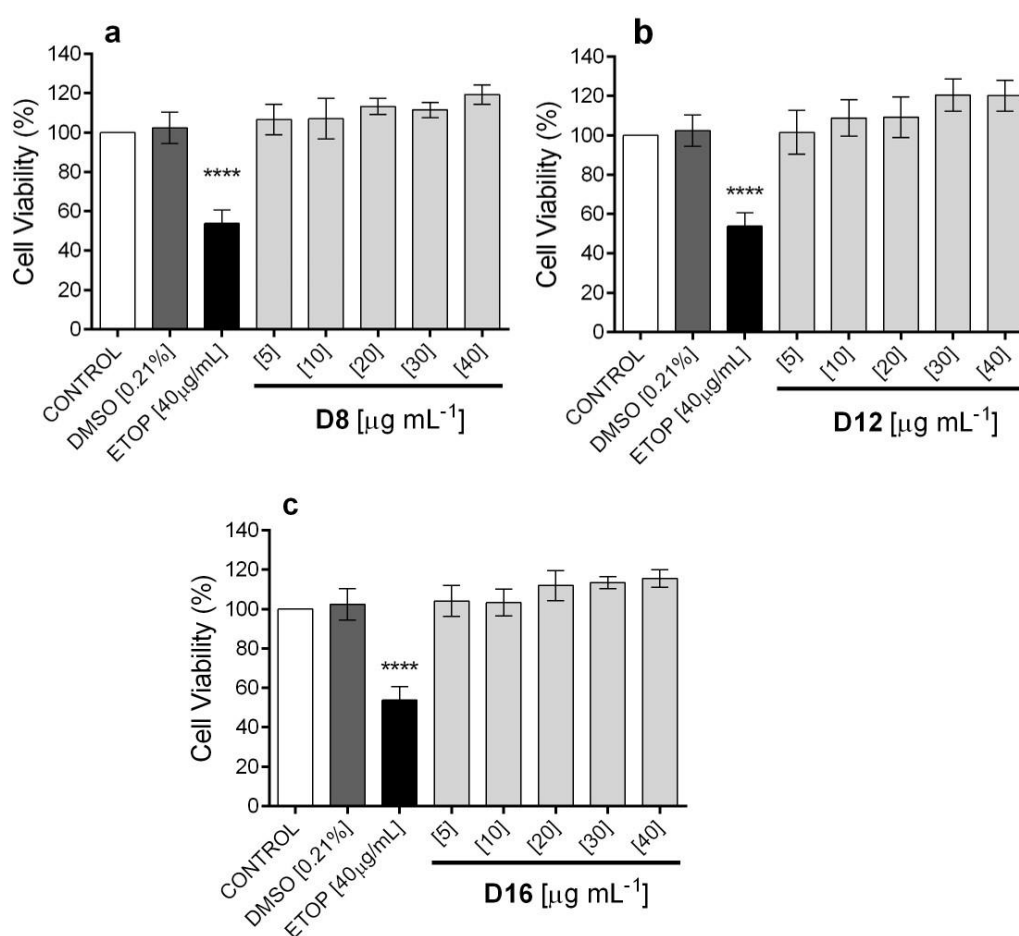


Figure 7. Effect of extracts D8, D12 and D16 on the viability of RAW 264.7 cells. The values are expressed as the mean \pm SD of three independent experiments ($n=3$). Significance was determined using ANOVA followed by Dunnett's multiple comparison test (**** $p < 0.0001$ DMSO, ETOP (etoposide) and extracts compared with control group).

To assess the effect of the extracts from D8, D12 and D16 on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells, cells were treated with the extracts at the same concentrations used in the viability assay. The experimental results showed that NO level was increased in LPS-stimulated RAW cells, and this effect was decreased significantly by treatment with extracts at the concentrations tested (Fig. 8). The results showed that D12 and D16 extracts were the most active to inhibit NO production at 40 $\mu\text{g mL}^{-1}$ with $35.14 \pm 7.55\%$ and $34.42 \pm 7.15\%$ inhibition, respectively. On the other hand, the extract from day 8 (D8) showed $24.69 \pm 6.17\%$ inhibition in NO production, at this point of kinetics compound 2 is produced in greater quantity compared to compound 3 ($510.75 \pm 29.10 \mu\text{g g}^{-1}$ of dry biomass and $127.85 \pm 14.86 \mu\text{g g}^{-1}$ of dry biomass, respectively), while in the extract from day 12 (D12) the concentration of the compounds was $434.01 \pm 32.25 \mu\text{g g}^{-1}$ of dry biomass (compound 2) and $244.89 \pm 13.34 \mu\text{g g}^{-1}$ of dry biomass (compound 3), finally it was observed that the extract from day 16 (D16) contains in greater abundance compound 3 ($410.60 \pm 36.91 \mu\text{g g}^{-1}$ of dry biomass) which has shown more outstanding effect with respect to compound 2 that was identified with $335.45 \pm 21.72 \mu\text{g g}^{-1}$ of dry biomass, this result corroborates the outstanding effect of compound 3, as it increases its contained in the extracts, the anti-inflammatory effect

proportionally also increases. However, in a pure way, both compounds have an important anti-inflammatory effect. The indomethacin (positive control) showed an inhibition of $47.45 \pm 7.41\%$ at $30 \mu\text{g mL}^{-1}$ (Fig. 8). The results obtained in this work are of great interest because the (2*S*,3*R*)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2) and 3-epilupeol (3) compounds have important pharmacological properties. Compound 2 inhibits the secretion of NO, IL-6 and TNF- α in RAW 264.7 macrophages, as well as the activation of NF- κ B in RAW-blue macrophages [27], while compound 3 has shown antiviral [51], anti-inflammatory [52], antitubercular [53] and cytotoxic activity [54]. About the anti-inflammatory activity, the 3-epilupeol (3) compound has shown marked inhibition of the edema induced by TPA in mice and exhibits the nitric oxide (NO) production inhibitory activity in LPS-activated macrophages [52, 55]. Nitric oxide (NO) is a short-lived bioactive molecule which plays an important role in host defense response against various pathogens such as bacteria, viruses, fungi and parasites, and is thought to be various pathophysiological processes such as neuronal communication, vasodilatation, and neurotoxicity [56].

The importance of the anti-inflammatory effects of 3-epilupeol (3), lies in that the excessive production of NO causes tissue damage, extensive systemic vasodilatation and hypotension [57]. In addition, NO is involved in inflammatory disorders including bowel diseases, rheumatoid arthritis, chronic hepatitis, pulmonary fibrosis and colon cancer [58-60].

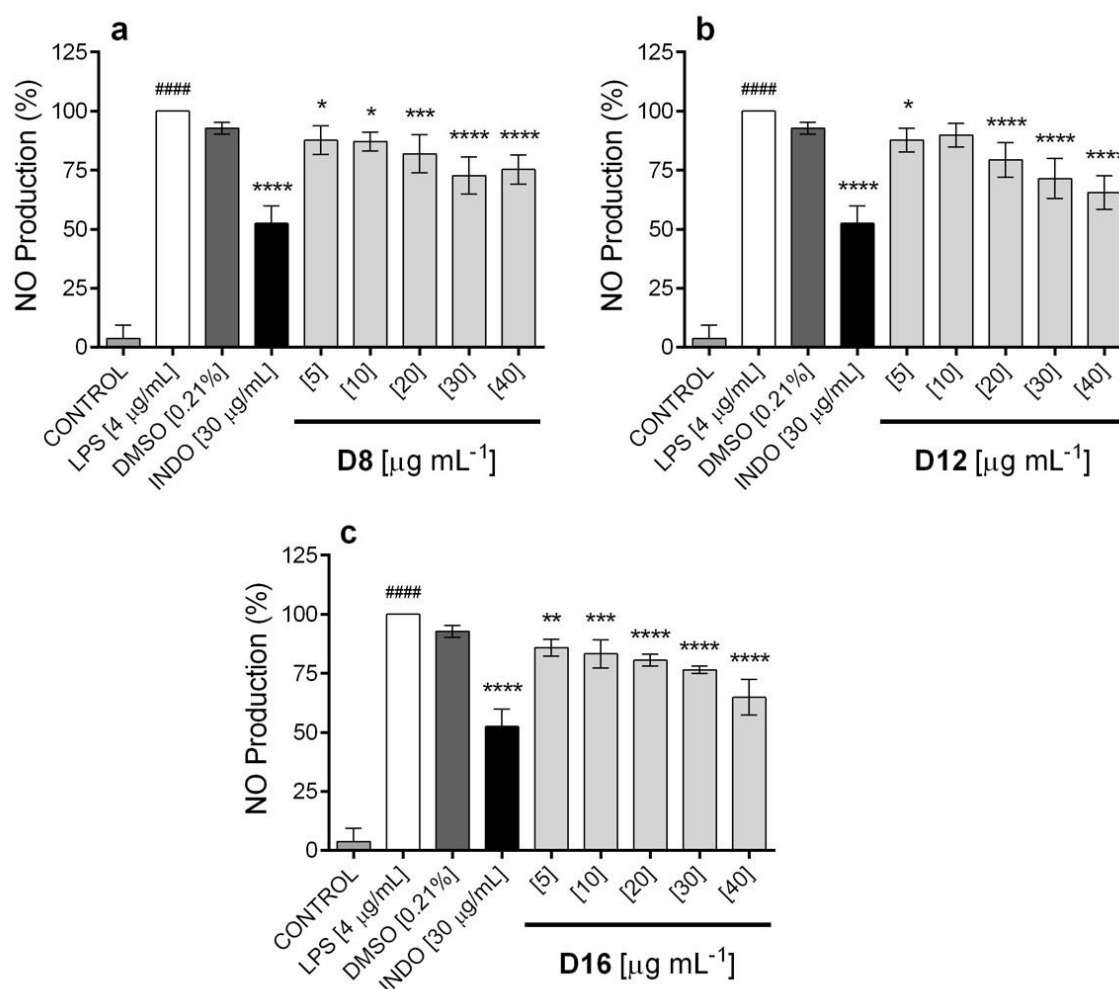


Figure 8. Effect of extracts D8, D12 and D16 on NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The values are expressed as the mean \pm SD of three independent experiments test ($n=3$). Significance was determined using ANOVA followed by Dunnett's multiple comparisons test (#### $p < 0.0001$ LPS compared with control group; * $p < 0.05$ and *** $p < 0.0001$ DMSO, INDO (indomethacin) and extracts compared with LPS group).

3. Materials and Methods

3.1 General Procedures

Compounds 1-7 were isolated by column chromatography (silica gel 70-230 mesh; Merck) and the purity of compounds were checked by GC-EM; detection was performed at UV light (366 and 254 nm), and after spraying with $\text{Ce}(\text{SO}_4)_2 \cdot 2 (\text{NH}_4)_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma-Aldrich) followed by heating. The ^1H - and ^{13}C -, DEPT and 2D NMR (COSY, HSQC and HMBC) experiments were recorded on Varian Unity Inova 200 MHz and a Bruker AVANCE IIIHD 500 at 500 MHz, using CDCl_3 and CD_3OD with tetramethylsilane (TMS) as internal standard. Optical rotations were measured on a 241 digital polarimeter at 25 °C (Perkin Elmer, Waltham, MA, USA) equipped with a sodium lamp (589 nm) and microcell. Indomethacin (indo), dimethyl sulfoxide (DMSO), etoposide, lipopolysaccharide (LPS) from *Escherichia coli* serotype 055: B5, sodium nitrite (NaNO_2), phosphoric acid (H_3PO_4), N-(1-naphtyl) ethylenediamine dihydrochloride, and sulfanilamide were purchased from Sigma-Aldrich (Mexico City, Mexico). DMEM/F12 (Dulbecco's Modified Eagle's Medium/Nutrient

Mixture F-12), and fetal bovine serum (FBS) were from GIBCO (Waltham, MA USA). [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS] was from Promega Co (Fitchburg, WI, USA). Murine macrophage cell line RAW 264.7 (Tib-71™) was from ATCC® (Georgetown, Washington, DC, USA). Compounds 8-15 were identified by GC-MS. Compounds 2 and 3 were quantitated by GC-MS using a calibration curve of authentic samples following the method described in *A. pichinchensis* callus culture [27].

3.2. Plant Material

Plants and seeds of *A. pichinchensis* were collected in Tepoztlán Morelos, México in march 2018 (19° 00' 43.88" N, 99.05' 38.66" W), and were identified by Biol. Gabriel Flores Franco and deposited at the HUMO Herbarium of the Universidad Autónoma del Estado de Morelos (UAEM), with the voucher number 33913.

3.3. Establishment of Cell Suspension Cultures

Friable callus cultures of *A. pichinchensis* was previously established by our working group [27]. Callus were subcultured in the same MS semisolid culture medium containing 3% sucrose, 1.0 mg L⁻¹ α -naphthaleneacetic acid (NAA) and 0.1 mg L⁻¹ 6-furfurylaminopurine (KIN). The 20-day-old callus were used to establish the cell suspension cultures. Fresh callus (5 g) were transferred to 250 mL Erlenmeyer flasks containing 50 mL of MS liquid culture medium using the same conditions and plant growth regulator as in the callus cultures. Cell suspension cultures were placed on an orbital shaker at 110 rpm and incubated at 25 \pm 2 °C under a photoperiod of 16 h with white fluorescent light (50 μ mol m⁻² s⁻¹). When an increase of biomass was shown in the flasks, cells were harvested and screened with 200 μ m nylon mesh filters (Whatman No. 1) to obtain a homogeneous cell culture. To increase the biomass, the cells were subcultured every 15 days for 6 months using an inoculum size of 10% (v/v) in 500 mL Erlenmeyer flasks with 100 mL of liquid culture medium.

3.3.1. Growth Kinetics

The growth kinetics of cell suspension culture was carried out in 250 mL flasks without modifying the composition of the culture medium. Each flask containing 50 mL of medium MS was inoculated with 2 g of fresh cells and incubated in the same conditions mentioned above. Three flasks were harvested every two days and the culture growth was allowed until 22 days. Harvested cells were washed with distilled water, filtered with a cellulose filter (Whatman No. 1), and dried in an oven at 55 °C for 24 h. Then, dry weight biomass (DW) data were recorded to perform the culture growth curve. The specific cell growth rate (μ) was calculated by plotting the natural logarithm of the cell growth data versus time. The doubling time (td) was computed from the μ exponential data.

3.3.2. Cell Viability

The cell viability of cell suspension culture was measured by Evan's blue day exclusion method [61]. A sample of 1 mL of cell suspension was taken from each flask and incubated into 0.25% Evan's blue stain for 5 min and at least 700 cells were counted. Viable cells were considered those that were not stained. All the experiments were repeated 3 times with three replicates each.

3.3.3. Sugar Quantification and pH Measurement

During each sampling of growth kinetics, 5 mL aliquots from the residual culture medium of each biomass sample were taken, their pH was measured with a potentiometer (Science Med S_-25CW) and total sugar content by phenol-sulfuric method [62]. A calibration curve was performed using sucrose as a standard at concentrations of 0.1 to 1.0 $\mu\text{g mL}^{-1}$. A sample aliquot (2 mL) of sample was mixed with 2 mL of phenol reagent at 5% in digester tubes and placed in a rack submerged in a cold-water bath. Then, 5 mL of H_2SO_4 concentrated was added to the mixture and allowed to stand for 15 min and analyzed in a spectrophotometer at 490 nm against a blank.

3.4. Extraction and Isolation of Compounds from Cell Suspension Cultures

Biomass harvested on day 20 was dried in an oven at 40 °C (12.30 g) and extracted with 100 mL of ethyl acetate by sonication (30 min), the extraction process was performed in triplicate. The excess of solvent was eliminated in a rotatory evaporator under reduced pressure and a brown residue (1.3 g) was obtained. A second extraction was carried out with methanol (3 X 100 mL), the solvent was removed by distillation giving rise to a resinous residue (2.4 g).

The ethyl acetate extract (1.2 g) was fractionated in an open chromatographic column previously packed with silica gel (36 g, 70-230 mesh, Merck) and eluted with a n-hexane, ethyl acetate gradient system (100:00, 95:05, 90:10, 85:15, 80:20, 75:25 and 00:00) v/v). Fractions of 20 mL were collected to obtain 64 fractions that were monitored by TLC (ALUGRAM® SIL G/UV254 silica gel plates). Fractions that showed TLC similarity were grouped obtaining 6 groups: MSR1 (1–9; 241.4 mg), MSR2 (10–18; 138.4 mg), MSR3 (19–32; 122.3 mg), MSR4 (33–42; 147.2 mg), MSR5 (43–55; 216.5 mg) and MSR6 (56–64; 270.6 mg). The MSR1 fraction showed a single spot in TLC, and its GC-MS analysis indicated that this fraction was constituted by the mixture of palmitic acid (12), hexadecanoic acid methyl ester (13) and isopropyl palmitate (14). The MSR2 fraction (120 mg) obtained in an n-hexane/ethyl acetate system (95:05), was purified using column chromatography, silica gel (4 g) and an n-hexane/ethyl acetate gradient system (98:02 → 86:14), 28 fractions of 3 mL each were obtained, in fractions 16-22 obtained in a n-hexane/ethyl acetate system (92:08) a single spot was observed in TLC, these were combined obtaining 18.3 mg (0.1%) of a colorless oil. Analysis of their ^1H , ^{13}C -NMR data allowed to identify desmethoxyencecalin (1). The MSR3 fraction (120 mg) was purified by column chromatography, silica gel and a system of gradients with n-hexane/ethyl acetate (96:04 → 80:20). From this purification process, 53 fractions were obtained. The mixture of stigmasterol (4) and β -sitosterol (5) mixture (41.8 mg; 0.3%) was obtained as a white amorphous solid in the fractions 42-48 eluted with n-hexane/ethyl acetate (87:13). An aliquot of 115 mg of column chromatography. Silica gel was used as the stationary phase and a n-hexane/ethyl acetate gradient system (90:10 → 80:20) was used as the mobile phase. 48 fractions were obtained, the purification process was monitored by TLC, which allowed obtaining 5 groups of fractions (MSR4A to MSR4E). In the group MSR4B (10.3 mg) obtained in the 86:14 system (n-hexane/ethyl acetate) the mixture of sterols (4) and (5) was identified, from the group MSR4C (37.6 mg) obtained in 84:16 (n-hexane/ethyl acetate) a crystalline solid was obtained, it was analyzed by ^1H , ^{13}C -NMR and its data allowed identifying as 3-epilupeol (3). The MSR4D group (8 mg) obtained in an 82:18 system (n-hexane/ethyl acetate) was analyzed by GC-MS and the compounds 24-methylene-9,19-cyclolanastan-3 β -ol (8) and 24-methylenecycloartan-3-one (9) were identified. Column chromatographic purification of the MSR5 fraction (216.5 mg), using a n-hexane-EtOAc

gradient system (95:05 → 70:30), provided 64 fractions, these were combined according to their chemical profile observed in TLC in 7 groups of fractions MSR5A (1-20; 34.6 mg), MSR5B (21-25; 23.4 mg), MSR5C (26-33; 19.2 mg), MSRD (34-42; 16.9 mg), MSR5E (43-50; 18.1 mg), MSR5F (51-58; 28.7 mg) and MSR5G (59-64; 44.2 mg). Fractions 51-58 eluted with 76:24 n-hexane-EtOAc contained a viscous liquid and a single spot was observed on TLC. This was analyzed by ^1H , ^{13}C -NMR, analysis of their data led to the identification of compound as (2*S*,3*R*)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2).

The MeOH extract (2.4 g) was fractionated in an open chromatographic column previously packed with silica gel (72 g, 70–230 mesh; Merck) and eluted with a CH_2Cl_2 :MeOH gradient system (100:00, 95:05, 90:10, 85:15, 80:20, 75:25 and 00:100, v/v). Fractions of 50 mL were collected to obtain 118 fractions and monitored by TLC. Fractions that showed similarity in TLC analysis were grouped obtaining 5 groups: MSR-M-1 (1-60; 548.3 mg), MSR-M-2 (61-83; 218.6 mg), MSR-M-3 (84-86; 314.8 mg), MSR-M-4 (87-95; 112.6 mg) and MSR-M-5 (96-118; 878.4 mg). The MSR-M-1 group was analyzed by GC-MS and compounds palmitamide (15), oleamide (16) and methyl pyroglutamate (17) were identified. By successive chromatography of fraction MSR-M-2, using silica gel as stationary phase and a system of gradients (95:05 → 88:12) n-hexane/ethyl acetate, compound 1 (8 mg) was identified. Fractions 84-86 (MSR-M-3; 314.8 mg) were obtained in a gradient system of increasing polarity with CH_2Cl_2 : MeOH (90:10 → 85:15), this fraction was purified by column chromatography, silica gel and an n-hexane/ethyl acetate elution system (90:10 → 80:20), of the fractions obtained in the n-hexane/ethyl acetate system (86:14), the mixture of sterols was identified 4 and 5 (46.8 mg) and in the fractions obtained from the (82:18) system, compound 2 (6 mg) was obtained. The MSR-M-4 fraction (112.6 mg) was purified by column chromatography, silica gel and eluted with CH_2Cl_2 :MeOH (98:02 → 70:30) gradient system, 68 fractions were obtained. Fractions that showed TLC similarity were grouped obtaining 3 groups: MSR-M-4A (1-32; 21.2 mg), MSR-M-4B (33-42; 32.7 mg), MSR-M-4C (43-68; 48.4 mg). The MSR-M-4A group was analyzed by GC-MS and compounds 24-methylene-9,19-cyclolanastan-3 β -ol (8) and 24-methylenecycloartan-3-one (9) were identified. The MSR-M-4B fraction (32.2 mg) obtained in a CH_2Cl_2 /MeOH system (88:12 → 82:18) was purified by column chromatography, silica gel and an isocratic system with CH_2Cl_2 /MeOH (84:16) obtaining a semisolid (14 mg). Analysis by ^1H , ^{13}C -NMR allowed the compound to be identified as (-)-Artemesinol (6). The MSR-M-4C fraction (48.4 mg) obtained in a CH_2Cl_2 /MeOH system (80:20 → 74:26) was purified by column chromatography, silica gel and an isocratic system with CH_2Cl_2 /MeOH (75:25) obtaining a semisolid (34 mg). Analysis of NMR in 1D (^1H , ^{13}C) and 2D (COSY, HSQC and HMBC) allowed the compound to be identified as (-)-Artemesinol glucoside (7).

Compounds 1-5 were identified using ^1H - and ^{13}C -NMR and comparing with reported data and by direct comparison with authentic samples available in our laboratory [27].

3.4.1. (-)-Artemesinol (6)

Compound (6) was isolated as a semi-solid mass. ^1H NMR (200 MHz, CDCl_3 : CD_3OD) δ 7.65 (1H, dd, J = 8.4, 2.2 Hz, H-7), 7.51 (1H, d, J = 2.2 Hz, H-5), 6.73 (1H, d, J = 8.5 Hz, H-8), 6.40 (1H, d, J = 10 Hz, H-4), 5.58 (1H, d, J = 10 Hz, H-3), 3.53 (2H, s, CH_2 -14), 2.44 (3H, s, CH_3 -12) and 1.3 (3H, s,

CH₃-C-O). ¹³C NMR (50 MHz, CDCl₃:CD₃OD) δ 196.84 (C-11), 157.38 (C-9), 130.21 (C-6), 127.94 (C-7), 130.97 (C-3), 127.45 (C-5), 116.67 (C-4), 120.64 (C-10), 124.32 (C-8), 80.86 (C-2), 68.99 (C-14), 26.32 (C-12), 23.49 (C-13). Spectra of ¹H, ¹³C NMR are in supplementary material (S1, S2) Stereochemistry at C-2 has not been determined, configuration relative (*R*) is suggested, compared to the optical rotation [α]_D = -4.5 ° (c 0.89, CHCl₃) reported for this compound [40].

3.4.2. (\pm)-Artemesinol Glucoside (7)

Compound (7) was isolated as a semi-solid mass. ¹H NMR (500 MHz, CDCl₃:CD₃OD) δ 7.69 (1H, dd, *J* = 8.4, 2.2 Hz, H-7), 7.56 (1H, d, *J* = 2.2 Hz, H-5), 6.77 (1H, d, *J* = 8.5 Hz, H-8), 6.42 (1H, d, *J* = 10 Hz, H-4), 5.67 (1H, d, *J* = 10 Hz, H-3), 4.46 (1H, d, *J* = 7.8 Hz, H-1'), 3.91 (1H, d, *J* = 10.6 Hz, H-14a), 3.87-3.18 (6H, m, H-2' to H-6'), 3.58 (1H, d, *J* = 10.7 Hz, H-14b), 2.48 (3H, s, CH₃-12) and 1.39 (3H, s, CH₃-13). The ¹H NMR data were compared with those reported [41]. ¹³C NMR (126 MHz, CDCl₃:CD₃OD) δ 197.44 (C-11), 157.31 (C-9), 130.71 (C-6), 130.66 (C-7), 127.77 (C-3), 127.30 (C-5), 123.71 (C-4), 120.69 (C-10), 116.12 (C-8), 103.57 (C-1'), 79.55 (C-2), 76.18 (C-5'), 75.90 (C-3'), 75.19 (C-14), 73.51 (C-2'), 69.85 (C-4'), 62.19 (C-6'), 26.39 (C-12), 23.79 (C-13). Spectra of ¹H, ¹³C, COSY, DEPT, HSQC and HMBC are in supplementary material (S3 to S8).

3.5. Quantification of Compounds 2 and 3 by GC-MS

Biomass collected of the growth kinetics (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 days) was dried in an oven at 55 °C for 24 h. subsequently, each sample were extracted by sonication with EtOAc (25 mL X 3) and MeOH (25 mL X 3), and concentrated in a rotatory evaporator. Maximum production of compounds 2 and 3 was identified in ethyl acetate extracts on days 8 (D8) and 16 (D16), respectively. For the quantitative analysis, a standard curve of compounds 2 and 3 was prepared in triplicate and analyzed by GC-MS. Concentrations of 2.2, 1.1, 0.55, 0.275, 0.1375, 0.06875 mg mL⁻¹ were used for compound 2 and 0.350, 0.175, 0.0875, 0.04375, 0.02187 mg mL⁻¹ for compound 3. Each standard solution was analyzed in triplicate to calculate the peak area ratio (*y*) and relative concentration (*x*), these data were used to construct the linear calibration curve, which showed acceptable linearity with correlation coefficients *r*² = 0.9926 and *r*² = 0.9997 respectively (Fig. S11 and S2). The quantification of compound 2 and 3 in the extracts was expressed as µg g⁻¹ dry biomass (µg g⁻¹ DW).

3.6. In vitro Anti-inflammatory Activity

3.6.1. Cell Culture

RAW 264.7 cells were maintained in DMEM/F12 medium supplemented with 10% heat inactivated FBS, without antibiotics. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ and sub-cultured by scraping and seeding them in 25 cm² flasks of 96 -well plates.

3.6.2. Assay for Cell Viability

Cells (1 X 10⁴ cell/ well in 100 µL of medium) were seeded in a 96-well plate and incubated for 24 h. Then, the cells were incubated for 22 h in presence of extracts at various concentrations (5- 40 µg mL⁻¹) or vehicle (DMSO, 0.21%, v/v) or etoposide (40 µg mL⁻¹) which served as a positive control and cells without treatment were considered (negative control). Cell viability was determined by

MTS solution was added to each well, and the cells were incubated for another 2 h. The optical density was measured at 490 nm on a microplate reader.

3.6.3. Treatments with LPS

Cells (2 X 10⁴ cells/ well in 200 μ L of medium) were plated and incubated for 24 h into 96-well plates. After that, the cells were incubated for one hour in presence of extracts at not cytotoxic concentrations (5 - 40 μ g mL⁻¹) or vehicle (DMSO, 0.21%, v/v) or indomethacin (30 μ g mL⁻¹) with served as a positive control and cells without treatment were considered (negative control). Then, the cells were incubated at 37 °C for 20 h with LPS at 4 μ g mL⁻¹ (for wells with extracts, vehicle, indomethacin and 100% stimulus control) as a pro-inflammatory stimulus and without LPS (negative control). Finally, cell-free supernatants were collected and used to NO quantification in fresh.

3.6.4. Determination of NO Concentration

Nitrite, the stable end-product of NO, was used as an indicator of NO production in the cell-free supernatants and was measured according to Griess reaction. Briefly, 50 μ L of each supernatant were mixed with 100 μ L of Griess reagent [50 μ L of 1.0% sulfanilamide and 50 μ L of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid solution] in a new 96-well plate and incubated for 10 min at room temperature. The optical density at 540 nm (OD₅₄₀) was measured with a microplate reader and nitrite concentration in the samples were calculated by comparison with the OD₅₄₀ of a standard curve of NaNO₂ in fresh culture medium.

3.7. Statistical Analysis

The results shown were obtained at least by three independent experiments and are presented as means \pm SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. For suspension cell cultures, the values of each experiment are means and the bars represent the standard error of triplicate determinations. All statistical analyses were performed using the GraphPad Prism®, version 8.0 software, p values <0.5 were considered to indicate statistical significance. The values of each experiment are means and the bars represent the standard error of triplicate determinations. Microsoft® Excel® for office 364 MSO (16.0.11425.20242) 32 bits software was used for analysis.

4. Conclusions

A suspension cell culture of *A. pichinchensis* was successfully established in MS culture medium with 1.0 mg L⁻¹ NAA and 0.1 mg L⁻¹ KIN. The production of (2S,3R)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (2) and 3-epilupeol (3) anti-inflammatory were identified and quantified. Compound 2 was obtained on day 8 (510.74 \pm 29.10 μ g g⁻¹ of dry biomass) and compound 3 on day 16 (410.59 \pm 36.91 μ g g⁻¹ dry biomass). The production of compound (2) and (3) was found to be associated with cell growth. Phytochemical analysis of dry cells showed the production of 17 secondary metabolites, of which, compounds 2-5 and 8-11 were previously described with anti-inflammatory, antimicrobial, antifungal and gastroprotective properties. Cell suspension cultures of *A. pichinchensis* offers a tool for studying bioreactors and obtaining secondary metabolites of higher added value

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: ^1H NMR (200 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (6), Figure S2: ^{13}C NMR (50 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (6), Figure S3: ^1H NMR (500 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S4: COSY (500 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S5: DEPT (125 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S6: ^1H NMR (125 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S7: HSQC (500 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S8: HMBC (500 MHz, $\text{CDCl}_3:\text{CD}_3\text{OD}$) of compound (7), Figure S9: GC-MS chromatograms for standard compound and EtOAc extract. a) 2,3-dihydrobenzofuran profile used as a standard; b) EtOAc extract profile from *A. pichinchensis* cell culture suspension at 8 days of culture showing the peak of 2,3-dihydrobenzofuran compound, Figure S10: GC-MS chromatograms for standard compound and EtOAc extract. a) 3-epilupeol profile used as a standard; b) EtOAc extract profile from *A. pichinchensis* cell culture suspension at 16 days of culture showing the peak of 3-epilupeol compound.

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