





Article

Obtaining 2,3-Dihydrobenzofuran and 3-Epilupeol from *Ageratina pichinchensis* (Kunth) R.King & Ho.Rob. Cell Cultures Grown in Shake Flasks under Photoperiod and Darkness, and Its Scale-Up to an Airlift Bioreactor for Enhanced Production

Mariana Sánchez-Ramos ^{1,*}, Silvia Marquina-Bahena ², Laura Alvarez ² , Antonio Bernabé-Antonio ³ , Emmanuel Cabañas-García ⁴, Angélica Román-Guerrero ¹  and Francisco Cruz-Sosa ^{1,*} 

¹ Department of Biotechnology, Metropolitan Autonomous University-Iztapalapa Campus, Av. Ferrocarril de San Rafael Atlixco 186, Col. Leyes de Reforma 1a. Sección, Alcaldía Iztapalapa, Mexico City 09310, Distrito Federal, Mexico

² Chemical Research Center-IICBA, Autonomous University of the State of Morelos, Av. Universidad 1001, Col. Chamilpa, Cuernavaca 62209, Morelos, Mexico

³ Department of Wood, Pulp and Paper, University Center of Exact Sciences and Engineering, University of Guadalajara, Km 15.5 Guadalajara-Nogales, Col. Las Agujas, Zapopan 45100, Jalisco, Mexico

⁴ Scientific and Technological Studies Center No. 18, National Polytechnic Institute, Blvd. del Bote 202 Cerro del Gato, Ejido La Escondida, Col. Ciudad Administrativa, Zacatecas 98160, Zacatecas, Mexico

* Correspondence: marianas_ramos_06@xanum.uam.mx (M.S.-R.); cuhp@xanum.uam.mx (F.C.-S.)



Citation: Sánchez-Ramos, M.; Marquina-Bahena, S.; Alvarez, L.; Bernabé-Antonio, A.; Cabañas-García, E.; Román-Guerrero, A.; Cruz-Sosa, F. Obtaining 2,3-Dihydrobenzofuran and 3-Epilupeol from *Ageratina pichinchensis* (Kunth) R.King & Ho.Rob. Cell Cultures Grown in Shake Flasks under Photoperiod and Darkness, and Its Scale-Up to an Airlift Bioreactor for Enhanced Production. *Molecules* **2023**, *28*, 578. <https://doi.org/10.3390/molecules28020578>

Academic Editors: Simona Fabroni, Krystian Marszałek and Aldo Todaro

Received: 14 November 2022

Revised: 29 December 2022

Accepted: 1 January 2023

Published: 6 January 2023

Abstract: *Ageratina pichinchensis* (Kunth) R.King & Ho.Rob. is a plant used in traditional Mexican medicine, and some biotechnological studies have shown that its calluses and cell suspension cultures can produce important anti-inflammatory compounds. In this study, we established a cell culture of *A. pichinchensis* in a 2 L airlift bioreactor and evaluated the production of the anti-inflammatory compounds 2,3-dihydrobenzofuran (**1**) and 3-epilupeol (**2**). The maximum biomass production (11.90 ± 2.48 g/L) was reached at 11 days of culture and cell viability was between 80% and 90%. Among kinetic parameters, the specific growth rate (μ) was 0.2216 days⁻¹ and doubling time (td) was 3.13 days. Gas chromatography coupled with mass spectrometry (GC-MS) analysis of extracts showed the maximum production of compound **1** (903.02 ± 41.06 μ g/g extract) and compound **2** (561.63 ± 10.63 μ g/g extract) at 7 and 14 days, respectively. This study stands out for the significant production of 2,3-dihydrobenzofuran and 3-epilupeol and by the significant reduction in production time compared to callus and cell suspension cultures, previously reported. To date, these compounds have not been found in the wild plant, i.e., its production has only been reported in cell cultures of *A. pichinchensis*. Therefore, plant cell cultured in an airlift reactor can be an alternative for the improved production of these anti-inflammatory compounds.

Keywords: medicinal plant; anti-inflammatory compounds; cell cultures; airlift bioreactor; photoperiod; absolute darkness



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The secondary metabolites of plants play an important role in their development and survival, since they participate in the defense against insects, microorganisms, and other abiotic conditions [1–6]. However, the production of these metabolites is variable over time due to seasonal and environmental factors [7–9]. On the other hand, plants have been an important resource for humans due to their nutritional and medicinal attributes, such as antimicrobial, antioxidant, anti-inflammatory, antitumor, antiviral, antiparasitic, and antidiabetic [10–18]. Therefore, the interest in the use and study of medicinal plants and

their bioactive metabolites has increased in the last decades since it also represents a viable field for the exploration of new bioactive molecules despite low yields [19,20].

In this regard, through the application of plant cell culture technology, it has been possible to obtain and improve the biomass and bioactive production of compounds, and there are several reports in which important results have been obtained, highlighting some advantages such as constant and controlled production in reduced spaces [21–31]. Moreover, using biotic and abiotic elicitors, it is possible to increase compound production; in particular, light conditions have been studied as an elicitor to increase biomass production, and bioactive compounds such as alkaloids and phenolics [32–34]. Another advantage of cell cultures is the ability to scale up to bioreactors to reduce culture times and increase the production of the compounds of interest. For instance, the production of taxol, resveratrol, berberine, diosgenin, vinblastine, and vincristine was significantly increased when cells were scaled up from flasks to bioreactors [35–41]; in fact, these are produced industrially [42–45]. These success stories show that it is possible to produce and even increase the production of compounds of interest in reactors, which contributes to the supply of necessary drugs.

Among other plants, *Ageratina pichinchensis* (Kunth) R.King & Ho. Rob. (Asteraceae) is an endemic species from the state of Morelos, Mexico, and it is used in traditional medicine as an antifungal, anti-inflammatory, and healing agent [46,47]. Previous studies have reported the production of triterpene, benzochromene, benzofuran, flavonoid, sterol, and eudesmane-type compounds, and their ethnomedical uses have already been validated [48–55].

In this regard, we have previously reported the establishment of callus cultures and cell suspension cultures of *A. pichinchensis* that maintained the ability to produce bioactive compounds. For instance, the (2*S*,3*R*)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (**1**) compound (Figure 1A) has shown potential to inhibit the activation of NF- κ B, IL-6, and TNF- α secretion as well as suppression of NF- κ B factor expression that have been linked to disorders such as rheumatoid arthritis, chronic hepatitis, and pulmonary fibrosis [56,57]. On the other hand, the 3-epilupeol (**2**) compound (Figure 1B) has been attributed anti-inflammatory, anti-HIV, and anti-tuberculous effects [58–60]. For *A. pichinchensis*, the production of these compounds has only been obtained in callus cultures or cell suspension cultures [61,62].

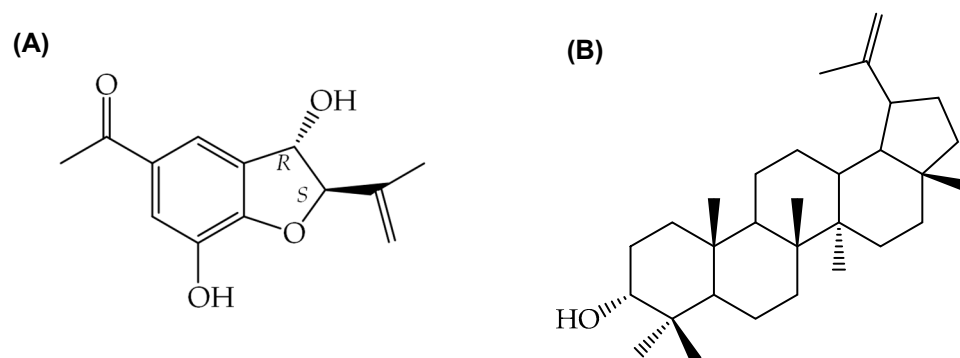


Figure 1. Anti-inflammatory compounds isolated from *A. pichinchensis* cell cultures. (A) (2*S*,3*R*)-5-acetyl-7,3 α -dihydroxy-2 β -(1-isopropenyl)-2,3-dihydrobenzofuran (**1**); (B) 3-epilupeol (**2**).

This study aimed to cultivate cells of *A. pichinchensis* in shake flasks to evaluate the production of anti-inflammatory compounds under photoperiod and dark conditions, and then to use the best-established conditions to scale up the cell culture to an airlift bioreactor to increase production of the anti-inflammatory compounds **1** and **2**.

2. Results and Discussion

2.1. Cell Cultures in Shake Flasks under Photoperiod and Absolute Darkness

2.1.1. Yield of Biomass

Biomass growth showed a similar behavior in its growth phases both under photoperiod and absolute darkness conditions (Figure 2A). For the cells cultured under photoperiod conditions, the adaptation phase was observed from 0 to 4 days, the exponential phase from 6 to 16 days, the stationary phase from 16 to 18 days in which the maximum dry-weight biomass (DW) was observed at day 18 (13.52 ± 0.44 g/L DW), and finally, the death phase was observed. The specific growth rate was 0.1281 days^{-1} with a doubling time of 5.41 days. For the cells cultured under absolute darkness conditions, the adaptation phase was on days 0 to 4, the exponential phase stopped at day 14 and then the stationary phase was maintained until day 20 and later the death phase was presented. Under these conditions, the maximum accumulation of biomass (10.33 ± 0.78 g/L DW) was also observed on day 18. In fact, the μ was 0.1518 days^{-1} and td was 4.57 days.

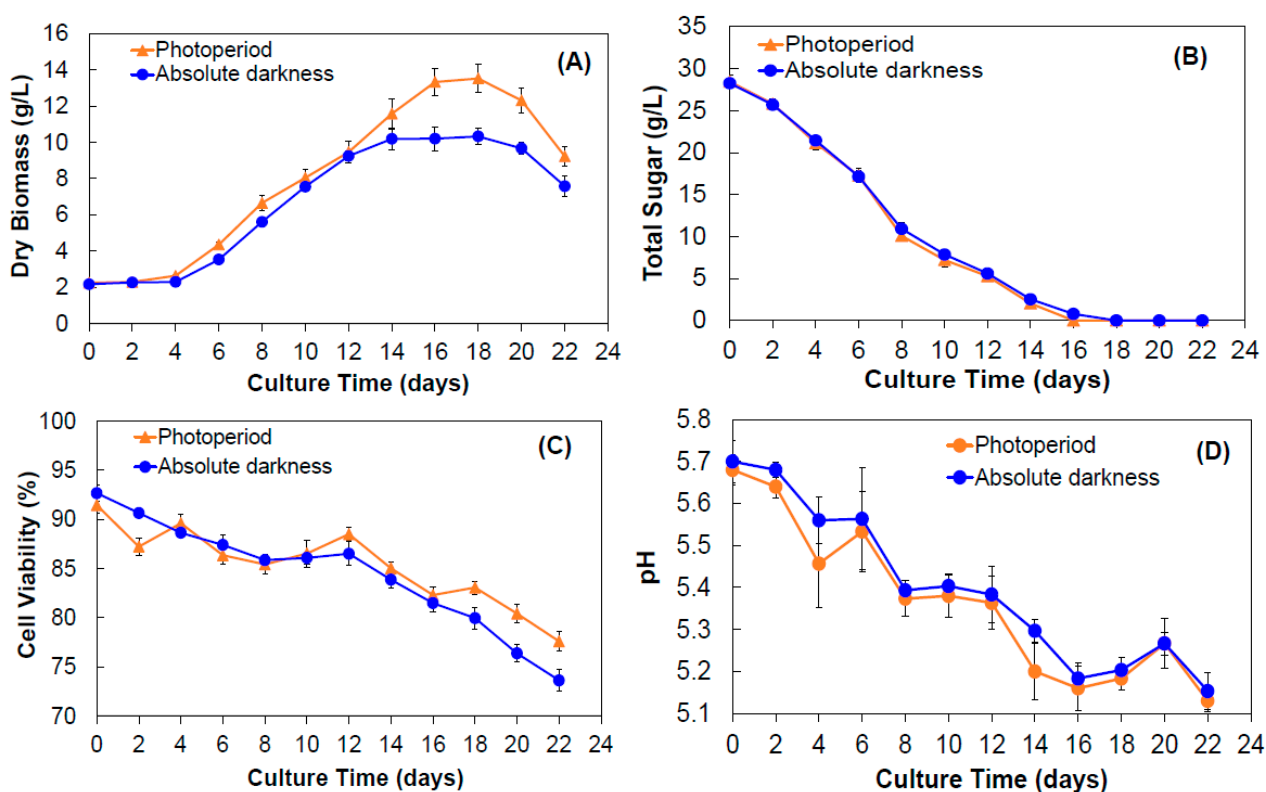


Figure 2. Growth kinetics of *A. pichinchensis* cell culture produced in shaken flasks under photoperiod and absolute darkness conditions for 22 days of culture. (A) Yield of dry biomass; (B) sucrose consumption; (C) cell viability; (D) pH values of the culture medium.

These results are similar to those previously reported for cultures of *A. pichinchensis* [62]. For instance, cell suspension cultures of *Ficus deltoidea* yielded high biomass production under photoperiod compared to dark condition [63]. Similarly, *Basella rubra* callus cultures exhibited higher biomass production when grown under photoperiod [64].

2.1.2. Sugar Consumption, Cell Viability, and pH

Regarding sucrose levels, the behavior was very similar in both incubation conditions, observing a constant decrease from day 2 to 16, after which there was a total consumption until day 22 (Figure 2B). In addition, a relationship between biomass growth and sucrose consumption as a carbon source was observed.

On the other hand, cell viability had slight changes over culture time, when compared between both incubation conditions (Figure 2C). The initial cell viability (day 0) had an average 92% and decreased to 75.5% at day 22. This behavior is common in cultures reported in other species [64–66]. The pH of the culture medium is another relevant factor; some authors mention that pH value is a determining factor for secondary metabolite production [67–69]. In this case, the pH values had slight variation and remained between 5.6 and 5.2 for both incubation conditions (Figure 2D).

2.1.3. Production of 2,3-Dihydrobenzofuran and 3-Epilupeol

The photoperiod stimulated the production of 2,3-dihydrobenzofuran and 3-epilupeol compounds (Figure 3), particularly during the exponential phase of culture growth. Higher production of 2,3-dihydrobenzofuran was achieved with photoperiod at day 8, obtaining $495.04 \pm 22.85 \mu\text{g/g DW}$ (Figure 3A). Despite the same behavior occurring under dark conditions, the maximum production at day 8 was reduced by 36.3%.

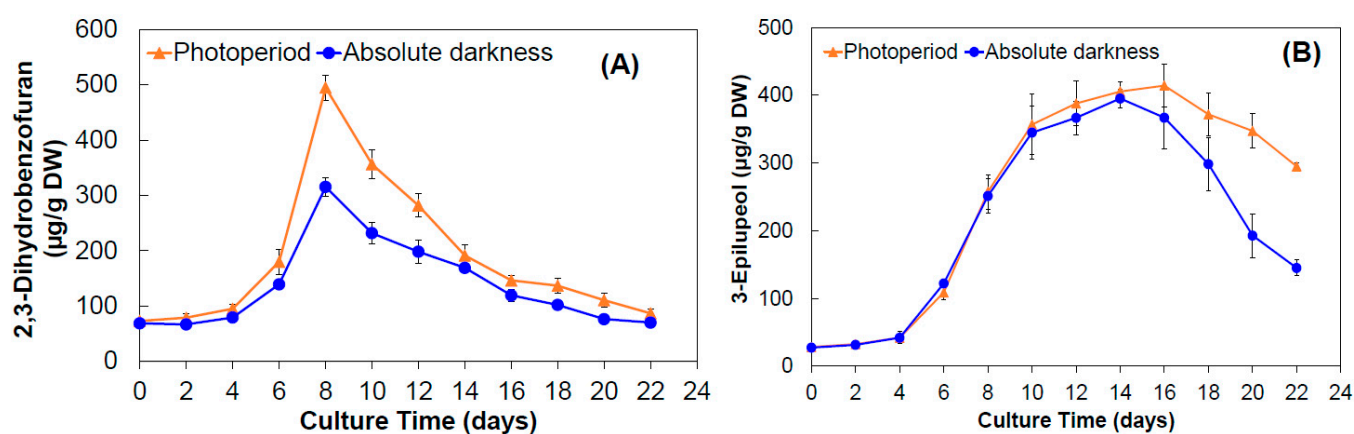


Figure 3. Production kinetics of compounds in cellular biomass of *A. pichinchensis* cultured in shaken flasks under photoperiod or absolute darkness for 22 days. (A) 2,3-dihydrobenzofuran (1); (B) 3-epilupeol (2).

Regarding 3-epilupeol, its production was closely related to crop growth, although no significant differences were observed by effect of photoperiod and absolute darkness (Figure 3B). The maximum production in photoperiod and absolute darkness was obtained during the stationary phase for both photoperiod and darkness treatments, with yields of 414.24 ± 31.56 and $395.14 \pm 13.32 \mu\text{g/g DW}$, respectively.

Cell suspension cultures have demonstrated to be suitable for improving the cell growth, producing a higher yield of biomass and not only higher contents but also great variety of bioactive molecules [70–74], when cell cultures are submitted to photoperiod conditions [62,75–83]. Similar results have been reported in cell suspension cultures for the production of antioxidant compounds in *Melastoma malabathricum* [84], lignans in *Linum usitatissimum* [85], and alkaloids in *Hyoscyamus muticus* [86], where higher contents were achieved when cultured under photoperiod conditions. Likewise, cell suspension cultures of *Rosa hybrida* cv. Charleston significantly enhanced the anthocyanin production when exposed to light conditions, but anthocyanin yield was drastically reduced when dark conditions were used [87]. *Tecoma stans* callus cultures also exhibited an increase in the production of antioxidant compounds when photoperiod conditions were used [88]. In this study, our results for *A. pichinchensis* showed that the biomass production and anti-inflammatory compounds in shaken flasks is favored under photoperiod conditions, whereby we decided to culture cells in an airlift bioreactor under the same conditions. However, some reports state that cell culture under darkness can promote the production of biomass and secondary metabolites, and even improve the morphology of cell suspensions

by reducing cell aggregation and oxidation; however, this response depends on the type of compound looked for and the plant genotype [89–95].

2.2. Cell Culture in an Airlift Bioreactor under Photoperiod Conditions

2.2.1. Growth Kinetics and Biomass Yield

A. pichinchensis cell culture was grown in the airlift bioreactor for 15 days, the adaptation phase was not observed, instead, the exponential growth was shown from day 0 to day 11, stationary phase lasted until day 12, and by day 13, the death phase was observed and lasted until day 15 (Figure 4A). The maximum biomass production occurred between days 11 and 12, with a yield of 11.90 ± 0.19 and 11.88 ± 0.44 g/L DW, respectively, time significantly shorter than that achieved for the cell culture in shake flasks. The growth index was 0.2216 days^{-1} and the doubling time lasted 3.13 days.

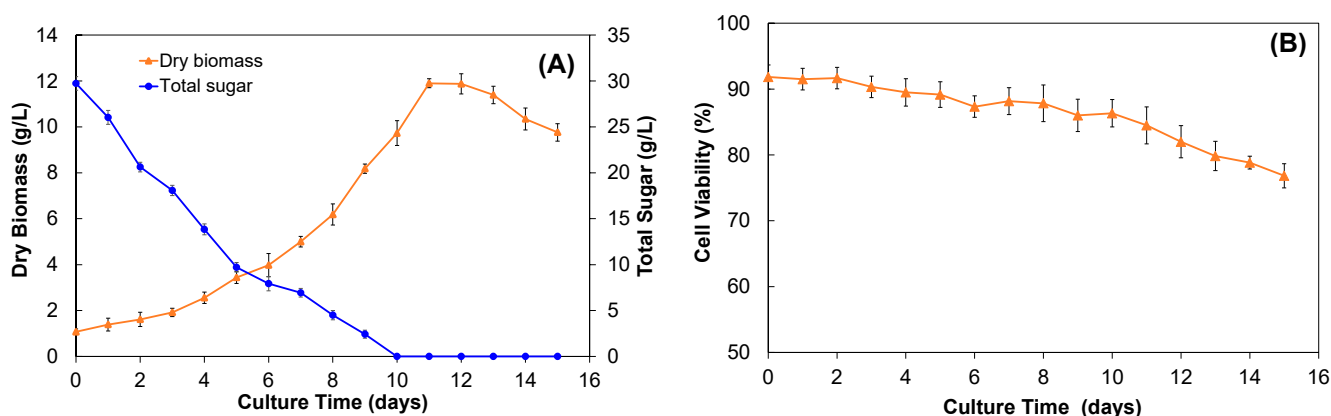


Figure 4. (A) Growth kinetics and (B) viability of cell culture of *A. pichinchensis* grown in an airlift bioreactor for 15 days under photoperiod conditions.

On the other hand, an inverse relationship between the consumption of the carbon source and biomass production was observed, where the carbon source was depleted at the end of the exponential phase, which agrees with the decrease in cell growth or the death phase; moreover, cell viability was maintained between 91.83 to 76.8% throughout the culture growth (Figure 4B).

Cell culture of *Haematococcus pluvialis* exhibited a specific growth rate of 0.31 days^{-1} [95]; meanwhile, cell cultures of *Taxus wallichiana* grown in a 20-L airlift bioreactor for 28 days reported a growth rate of 0.16 days^{-1} with cell viability between 100% and 85% [96,97]. *Solanum chrysotrichum* cells cultured in an airlift bioreactor for 21 days displayed a specific growth rate at $0.03\text{--}0.08 \text{ days}^{-1}$, the pH remained between 5.7 and 5.3, and the cell viability was maintained between 100% and 70% [98]. Similar results were shown in *Panax notoginseng* cell cultures, in which, an enhancement in cell density and specific growth rate was obtained when cultured in an airlift bioreactor and modifying the composition culture medium conditions [99].

Regarding biomass production, no important changes were observed during the first three days of growth (Figure 5A), but after 4 days there was an increase in cell density and a slightly yellow coloration (Figure 5B). As growth increased, morphological changes in the cells were observed. Then, during the exponential phase the biomass was beige (Figure 5C).

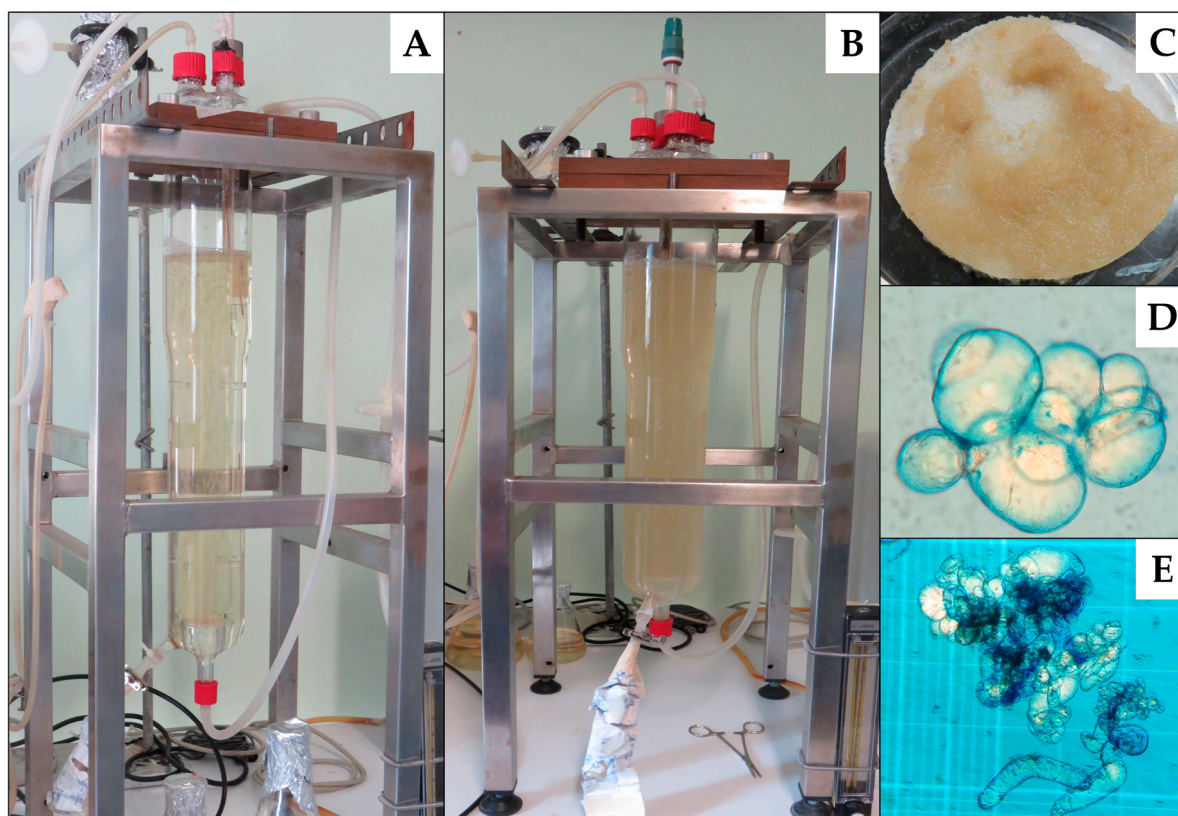


Figure 5. Cell culture of *A. pichinchensis* produced in an airlift bioreactor. (A) Cell culture during the first three days of growth; (B) cell culture after 4 days; (C) biomass appearance of exponential phase; (D) micrograph of the cell culture at 6 days (40X); (E) micrograph of the cell culture at 12 days (10X).

Microscopic analysis with Evan’s blue showed circular aggregates made up of 5 to 9 cells (Figure 5D). Subsequently, the size of the aggregates increased in the stationary phase, observing more elongated cells and the viability also decreased (Figure 5E).

During crop growth, the pH was maintained at 5.5–5.8, avoiding sudden changes that could affect the cell culture. The formation of foam in the airlift bioreactor was controlled with the addition of antifoam agents [96] to reduce the limitations on the mass transfer processes.

2.2.2. Production of 2,3-Dihydrobenzofuran and 3-Epilupeol

The extracts from the biomass cultivated in the airlift bioreactor showed that the production of 2,3-dihydrobenzofuran occurred from day 1, reaching its maximum production on day 7 with $903.02 \pm 41.06 \mu\text{g/g DW}$ and gradually reducing its content until the end of the culture (Figure 6). This production is almost double that produced in flasks with only $495.04 \pm 22.85 \mu\text{g/g DW}$ on day 8.

For the compound identified as 3-epilupeol, it was quantified from day 3, showing association with growth behavior, displaying a maximum production at day 14 with $561.63 \pm 10.16 \mu\text{g/g DW}$. Major production was observed in the exponential and stationary phase, which may be due to a response of the cells to nutrient limitations. The yielding of 3-epilupeol was significantly higher than that found in flask cultures ($414.24 \pm 31.56 \mu\text{g/g DW}$). In a previous study of suspension cultures of *A. pichinchensis*, similar results were reported for these compounds, except for callus cultures where lower production was obtained [61,62]. Figures S1 and S2 shows the chromatograms of the extracts of days 7 and 14, culture times where higher production of both compounds was found.

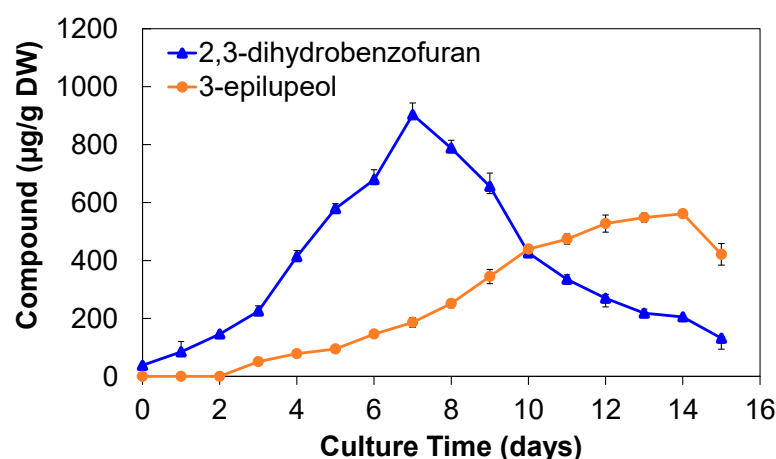


Figure 6. Kinetics of 2,3-dihydrobenzofuran (1) and 3-epilupeol (2) production in a cell culture of *A. pichinchensis* grown in an airlift bioreactor for 15 days.

Table 1 shows a comparison of the production of compounds 1 and 2 obtained in different cell culture production systems of *A. pichinchensis*. The airlift bioreactor significantly improved the production of the compounds from the cell culture. Compound 1 increased its production by 1.82 times on day 7 compared to cultures in flasks whose maximum production occurred on day 8. Likewise, cultures in the dark showed that the production of compounds in the reactor showed an increase of 2.86 times in 7 days, while this was 8 days for the flask cultures. On the other hand, compound 2 produced 1.36 times the compound of day 14 in the reactor in relation to the production monitored on day 16 of cultures in flasks under the photoperiod. Additionally, compound production in the reactor showed a 1.42-fold improvement over quantification in flask cultures under dark on day 16.

Table 1. Comparison of 2,3-dihydrobenzofuran and 3-epilupeol content in different in vitro culture systems of *A. pichinchensis* on specific days of maximum production.

Culture System	Culture Time (Day)	2,3-Dihydrobenzofuran (µg/g DW)	Culture Time (Day)	3-Epilupeol (µg/g DW)
Callus culture in jars/photoperiod *	30	650.00 ± 11.00	30	201.10 ± 15.00
Cell culture in flasks/photoperiod	8	495.04 ± 22.85	16	414.24 ± 31.56
Cell culture in flasks/absolute darkness	8	315.44 ± 16.72	16	395.14 ± 13.32
Cell culture in airlift bioreactor/photoperiod	7	903.02 ± 41.06	14	561.63 ± 10.16

* Reference: [61].

These results highlight that cell suspension cultures of *A. pichinchensis* can be scalable to bioreactors to increase the production of bioactive compounds. In other studies, an increase in the production of compounds in the bioreactor has been reported. For instance, cell suspension cultures of *Scrophularia striata* revealed that phenylethanoid glycosides and phenolic compounds were produced in greater amounts when grown in bioreactors rather than flask cultures [100,101]. Suspension cells of *Prunella vulgaris* showed increased production of antioxidant compounds when cultured under different photoperiod regimes and sucrose concentrations in an airlift bioreactor compared to flask cultures [79].

Likewise, the production of ajmalicin in *Catharanthus roseus* cells cultured in an airlift bioreactor was increased on day 14 [102]. On the other hand, the shikonin production of *Arnebia* sp. was improved in an airlift bioreactor under dark conditions and pH 5.75 [103]. The protein brazzein was produced and its production was even improved by transgenic *Daucus carota* cells that were grown in an airlift bioreactor compared to animal cells that also produce the protein, which opens the panorama of plant cell applications [104]. Cell density and ginseng production of *Panax notoginseng* have also been dramatically increased when cultured in an airlift reactor after 15 days of culture [105]. It has also been reported

that the addition of CO₂ and ethylene to suspension cultures of *Thalictrum rugosum* grown in an airlift bioreactor increased alkaloid production [106].

Ocimum basilicum cells grown in a 2.5 L airlift bioreactor increased rosmarinic acid accumulation compared to those grown in a flask [107]. The production of paclitaxel and baccatin III from suspension cell cultures of *Taxus wallichiana* was successfully carried out in a 20 L airlift bioreactor, significantly improved compared to flasks [97]. Squalene has also been produced prominently in *Santalum album* cultures grown in an airlift bioreactor compared to those in flasks [108]. On the other hand, the production of antifungal saponins from *Solanum chrysotrichum* plant cells increased when they were grown in an airlift bioreactor compared to the yields found in flask cultures [98].

3. Materials and Methods

3.1. Obtaining Plant Material

Cell suspension cultures (CSC) of *Ageratina pichinchensis* were previously established in shake flasks by our working group [62]. To increase biomass, CSCs were subcultured every 15 days in 500 mL Erlenmeyer flasks containing 100 mL of liquid MS [109] basal medium with an inoculum size of 10% (*v/v*). Sterile MS contained 30 g/L sucrose, 1.0 mg/L α -naphthaleneacetic acid (NAA), and 0.1 mg/L furfurylaminopurine (KIN) at pH 5.7. Cultures were maintained under the same shaking and incubation conditions [62].

3.2. Cell Culture in Shake Flask under Photoperiod and Absolute Darkness Conditions

CCS were cultured under photoperiod and absolute darkness (absence of light during growth kinetics) conditions to compare the production of 2,3-dihydrobenzofuran (**1**) and 3-epilupeol (**2**) compounds. For both incubation conditions, growth kinetics were performed using several 250 mL flasks using the same culture medium composition. Each flask containing 50 mL of MS medium was inoculated with 3 g of cells and incubated on an orbital shaker at 120 rpm at 25 \pm 2 °C under photoperiod (16 h light/8 h darkness with 50 μ M m⁻² s⁻¹ fluorescent light intensity) or under absolute darkness. Subsequently, three flasks were harvested every two days, and the culture was allowed to grow for 22 days. Biomass was washed with distilled water, filtered through a cellulose filter (Whatman No. 1) using a vacuum pump, and then dried in an oven at 55 °C until reaching a constant weight. Dry weight data were recorded and used to perform the growth curve. The specific growth rate was calculated using the natural logarithm (ln) of biomass dry weights of the exponential phase versus time, the doubling time was determined with the equation $dt = \ln 2 / \mu$. In addition, cell viability was monitored during culture growth and samples of the filtered cell culture medium were also obtained to determine the content of total sugars as mentioned below.

3.3. Bioreactor

3.3.1. Bioreactor Characteristics

Airlift bioreactors (ALB) are commonly used for suspension culture of plant cells [110]. In this study, a 2 L capacity airlift bioreactor (Applikon Holland) was used (Figure 7), equipped with a suction tube placed in the center of the reactor through which air was administered through a diffuser using a suction pump submersible water (Zhiquan CMH042, Eiko Co. Ltd., Shanghai, China). The pH was measured using an InPro4260/SG/225/PT100 electrode (Mettler-Toledo, New York, OH, USA). The air flow rate was controlled with a Rotameter (Total Temperature Instrumentation, Inc., Vermont, USA). A MasterFlex[®] 77601-10 peristaltic pump was used to feed the culture medium and obtain the samples.

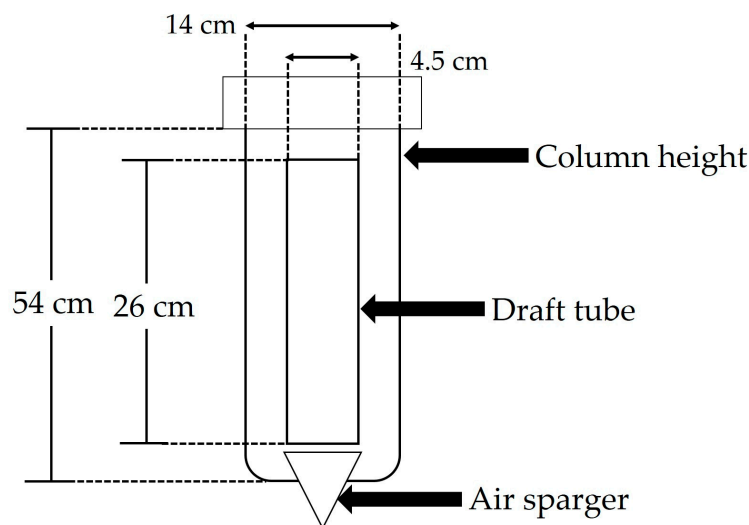


Figure 7. Scheme of an airlift bioreactor used for cell culture of *A. pichinchensis*.

3.3.2. Cell Cultures and Bioreactor Operating Conditions

Culture medium consisted of sterile MS supplemented with 30 g/L sucrose, 1.0 mg/L NAA, 0.1 mg/L KIN, and pH value was adjusted to 5.7. The bioreactor was operated with a total volume of 1.7 L. To characterize cell culture growth, the bioreactor was inoculated with fresh cell biomass (5%, *w/v*) with 10 days of growth in flask (exponential phase). The bioreactor was maintained in an incubation room at 25 ± 2 °C under photoperiod (16 h light/8 h darkness with $50 \mu\text{M m}^{-2} \text{s}^{-1}$ fluorescent light intensity). The pH values were maintained at 5.5–5.8 by adding NaOH solution at 0.1 M. The bioreactor was operated at 0.5 volumes of air/medium volume/min (vvm) incorporating a sterile gas with an air pump through a flow meter and a sterile filter (0.2 μm).

3.4. Cell Viability in Shake Flask and Bioreactor

The cell viability was determined for both cell cultures grown in shake flasks and bioreactor. Viability was determined considering the integrity of the membrane by using Evan's blue at 0.25% [111]. A 1 mL sample of cell suspension was obtained from each flask or bioreactor, mixed with the dye, incubated for 5 min, and then cell counted. Viable cells were considered those that were not stained. All experiments were repeated three times, with three replicates each.

3.5. Sugar Quantification in Shake Flask and Bioreactor

Sugar quantification was determined in the culture media of both cells grown in flasks and bioreactor. The culture medium filtered from the cell suspension cells of the growth kinetics was used to determine the total sugars by the phenol–sulfuric method [112]. For each sampling of growth kinetics an aliquot of 250 μL of culture medium was diluted in distilled water, a 500 μL aliquot was obtained and 500 μL of phenol 5% (*w/v*) was added in digester tubes and placed in a rack submerged in a cold water bath. Then, 5 mL of concentrated sulfuric acid was added to the mixture and allowed to stand for 15 min and analyzed in a spectrophotometer at 490 nm against a blank. A calibration curve was performed using sucrose as a standard at concentration of 0.1 to 1.0 $\mu\text{g/mL}$.

3.6. Extraction of 2,3-Dihydrobenzofuran and 3-Epilupeol of Cell Cultures

Both the biomasses of the growth kinetics harvested at days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 of the growth kinetics in shake flasks, and at days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16 of the growth kinetics in airlift reactor were filtered, washed and dried in an oven at 55 °C until constant weight. Each sample was extracted three times by sonication with 25 mL ethyl acetate, then concentrated on a rotary evaporator.

3.7. Quantification of 2,3-Dihydrobenzofuran and 3-Epilupeol by GC-MS

Extracts were analyzed in an HP Agilent Technologies 6890 gas chromatograph equipped with an MSD 5973 quadrupole mass detector (HP Agilent, Markham, ON, Canada), equipped with a capillary column HP-5MS (length: 30 m; inside diameter: 0.25 mm; film thickness: 0.25 μ M). The helium carrier gas was set to the column (1 mL per minute at constant flow). The inlet temperature was set at 250 °C while oven temperature was initially at 40 °C (held for 1 min) and increased to 280 at 10 °C/min. The mass spectrometer was operated in positive electron impact mode with ionization energy of 70 eV. Detection was performed in selective ion-monitoring (SIM) mode and peaks were identified and quantitated using target ions. Quantification was performed in triplicate using a calibration curve of 2,3-dihydrobenzofuran (**1**) at 2.2, 1.1, 0.55, 0.275, 0.1375, and 0.06875 mg/mL and 3-epilupeol (**2**) at 0.350, 0.175, 0.0875, 0.04375 and 0.02187 mg/mL as reference standards, previously purified and characterized by our work group [62]. Calibration curve showed acceptable linearity with correlation coefficients $r^2 = 0.9926$ and 0.9997, respectively. The quantification of compounds in the extracts was expressed in terms of μ g per g of biomass dry (μ g/g DW).

3.8. Statistical Analysis

All dates are presented as the mean values \pm standard deviation of at least three replicates. Growth parameters and the production of compounds **1** and **2** were compared by means of one-way of analysis of variance (ANOVA), factors with a $p \leq 0.05$ were considered significant. Significant differences in the experiments were calculated by Tukey test (GraphPad Prism[®] version 9.3.1 software, Dotmatics, San Diego, CA, USA).

4. Conclusions

A. pichinchensis has been studied from different approaches due to its ethnomedical uses, which makes it interesting for the development of crops that allow sustainable production of its bioactive compounds. In this work, a laboratory-scale bioreactor of a cell culture of *A. pichinchensis* is reported for the first time. We found that photoperiod conditions were more suitable than absolute darkness to produce biomass and compounds **1** and **2** in both production systems for *A. pichinchensis*. In addition, the production of compounds in an airlift bioreactor showed a significant increase in the production of both compounds (1.82 and 1.35 folds, respectively) under photoperiod conditions, and the production time was reduced by half. This work opens an encouraging path to improve the production of these compounds using other biotechnological alternatives, such as the use of elicitors using this system, which can contribute to the development of sustainable alternatives for the use of medicinal plants and is currently a priority need due to the rapid reduction in wild populations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28020578/s1>, Figure S1: GC-MS chromatograms for standard compound and EtOAc extract. (a) 2,3-dihydrobenzofuran profile used a standard; (b) EtOAc extract profile from *A. pichinchensis* cell culture suspension cultivated in an airlift bioreactor at 7 days of culture showing the peak of 2,3-dihydrobenzofuran. Figure S2: GC-MS chromatograms for standard compound and EtOAc extract. (a) 3-epilupeol profile used a standard; (b) EtOAc extract profile from *A. pichinchensis* cell culture suspension cultivated in an airlift bioreactor at 14 days of culture showing the peak of 3-epilupeol.

Author Contributions: Conceptualization, M.S.-R. and F.C.-S.; methodology, investigation and formal analysis, S.M.-B., L.A. and A.R.-G.; data curation, A.B.-A. and E.C.-G.; visualization, F.C.-S. and L.A.; writing—original draft preparation, M.S.-R. and A.B.-A.; writing—review and editing, M.S.-R., F.C.-S., S.M.-B., L.A., A.B.-A., E.C.-G. and A.R.-G.; supervision, A.R.-G. and E.C.-G.; project administration, E.C.-G. and S.M.-B.; funding acquisition, L.A. and S.M.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Laboratorio Nacional de Estructura de Macromoléculas (CONACYT 279905) for the use of gas chromatography coupled with mass spectrometry and Maria Gregoria Medina Pintor (Centro de Investigaciones Químicas, UAEM) for skillful technical assistance in the analysis and GC/MS experiments.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

References

1. Borges, C.V.; Minatel, I.O.; Gomez-Gomez, H.A.; Pereira, L.G.P. Medicinal plants: Influence of environmental factors on the content of secondary metabolites. In *Medicinal Plants and Environmental Challenges*; Ghorbanpour, M., Varma, A., Eds.; Springer: Cham, Switzerland, 2017; pp. 259–277.
2. Pant, P.; Pandey, S.; Dall'Acqua, S. The influence of environmental conditions on secondary metabolites in medicinal plants: A literature review. *Chem. Biodivers.* **2021**, *18*, e2100345. [[CrossRef](#)] [[PubMed](#)]
3. Takshak, S.; Agrawal, S.B. Defense potential of secondary metabolites in medicinal plants under UV-B stress. *J. Photochem. Photobiol* **2019**, *193*, 51–88. [[CrossRef](#)]
4. Mishra, T. Climate change and production of secondary metabolites in medicinal plants: A review. *Int. J. Herb. Med.* **2016**, *4*, 27–30.
5. Pagare, S.; Bhatia, M.; Tripathi, N.; Pagare, S.; Bansal, Y.K. Secondary metabolites of plants and their role: Overview. *Curr. Trends Biotechnol* **2015**, *3*, 293–304.
6. Faehnrich, B.; Franz, C.; Nemaz, P.; Kaul, H.-P. Medicinal plants and their secondary metabolites—State of the art and trends in breeding, analytics and use in feed supplementation—With special focus on *German chamomile*. *J. Appl. Bot. Food Qual.* **2021**, *94*, 61–74.
7. Soni, U.; Brar, S.; Gauttam, V.K. Effect of seasonal variation on secondary metabolites of medicinal plants. *Int. J. Pharm. Sci. Res.* **2015**, *6*, 3654–3662.
8. Jain, C.; Khatana, S.; Vijayvergia, R. Bioactivity of secondary metabolites of various plants: A review. *Int. J. Pharm. Sci. Res.* **2019**, *10*, 494–504.
9. Mahajan, M.; Kuiry, R.; Pal, P.K. Understanding the consequence of environmental stress for accumulation of secondary metabolites in medicinal and aromatic plants. *J. Appl. Res. Med. Aromat. Plant.* **2020**, *18*, 100255. [[CrossRef](#)]
10. Wink, M. Medicinal plants: A source of anti-parasitic secondary metabolites. *Molecules* **2012**, *17*, 12771–12791. [[CrossRef](#)]
11. Kumar, M.; Radha, S.P.; Kumari, N.; Pundir, A.; Punia, S.; Saurabh, V.; Choudhary, P.; Changan, S.; Dhupal, S.; Pradhan, P.C.; et al. Beneficial role of antioxidant secondary metabolites from medicinal plants in maintaining oral health. *Antioxidants* **2021**, *10*, 1061. [[CrossRef](#)]
12. Bahmani, M.; Golshahi, H.; Saki, K.; Rafieian-Kopaei, M.; Delfan, B.; Mohammadi, T. Medicinal plants and secondary metabolites for diabetes mellitus control. *Asian Pac. J. Trop. Dis.* **2014**, *4*, S687–S692. [[CrossRef](#)]
13. Sholikahah, E.N. Indonesian medicinal plants as sources of secondary metabolites for pharmaceutical industry. *J. Med. Sc.* **2016**, *48*, 226–239.
14. Kumari, P.; Kumari, C.; Singh, P.S. Phytochemical screening of selected medicinal plants for secondary metabolites. *Int. J. Life Sci. Res.* **2017**, *4*, 1151–1157. [[CrossRef](#)]
15. Bernstein, N.; Akram, M.; Daniyal, M.; Koltai, H.; Fridlender, M.; Gorelick, J. Antiinflammatory potential of medicinal plants: A source for therapeutic secondary metabolites. *Adv. Agron.* **2018**, *150*, 131–183.
16. Twilley, D.; Rademan, S.; Lall, N. A review on traditionally used South African medicinal plants, their secondary metabolites and their potential development into anticancer agents. *J. Ethnopharmacol.* **2020**, *261*, 113101. [[CrossRef](#)]
17. Velu, G.; Palanichamy, V.; Rajan, A.P. Phytochemical and pharmaceutical importance of plant secondary metabolites in modern medicine. In *Bioorganic Phase in Natural Foods: An Overview*; Roopan, S., Madhumitha, G., Eds.; Springer: Cham, Switzerland, 2018; Chapter 8.
18. Maridass, M. Survey of phytochemical diversity of secondary metabolism in selected wild medicinal plants. *Ethnobot. Leaflet.* **2010**, *14*, 616–625.
19. Rungsung, W.; Ratha, K.K.; Dutta, S.; Ditix, A.K.; Hazra, J. Secondary metabolites of plants in drugs discovery. *World J. Pharm. Res.* **2015**, *7*, 604–613.
20. Sree, N.V.; Udayasri, P.; Kumar, Y.A.; Babu, B.R.; Kumar, Y.P.; Varma, M.V. Advancements in the production of secondary metabolites. *J. Nat. Prod.* **2010**, *3*, 112–123.
21. Cardoso, J.C.; Oliveira, M.E.B.S.; Cardoso, F.C.I. Advances and challenges on the in vitro production of secondary metabolites from medicinal plants. *Hortic. Bras.* **2019**, *37*, 124–132. [[CrossRef](#)]

22. Tripathi, L.; Tripathi, J.N. Role of biotechnology in medicinal plants. *Trop. J. Pharm. Res.* **2003**, *2*, 243–253. [CrossRef]
23. Gandhi, S.G.; Mahajan, V.; Bedi, Y.S. Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants. *Planta* **2014**, *2*, 303–317. [CrossRef]
24. Yoshimatsu, Y. Tissue culture of medicinal plants: Micropropagation, transformation and production of useful secondary metabolites. *Stud. Nat. Prod. Chem.* **2008**, *34*, 647–752.
25. Chandana, B.C.; Nagaveni, H.C.; Kumari; Lakshmana, D.; Shashikala, S.K.; Heena, M.S. Role of plant tissue culture in micropropagation, secondary production and conservation of some endangered medicinal crops. *J. Pharmacog. Phytochem.* **2018**, *3*, 246–251.
26. Pérez-González, M.Z.; Jiménez-Arellanes, M.A. Biotechnological processes to obtain bioactive secondary metabolites from some Mexican medicinal plants. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 6257–6274. [CrossRef] [PubMed]
27. Smetanska, I. Production of secondary metabolites using plant cell cultures. *Adv. Biochem. Eng. Biotechnol.* **2008**, *111*, 187–228. [PubMed]
28. Vanisree, M.; Lee, C.-Y.; Lo, S.-F.; Nalawade, S.M.; Lin, C.Y.; Tsay, H.-S. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot. Bull. Acad. Sin.* **2008**, *45*, 1–22.
29. Kolowe, M.; Gaurav, V.; Roberts, S.C. Pharmaceutical active natural products synthesis and supply via plant cell culture technology. *Mol. Pharm.* **2008**, *5*, 243–256. [CrossRef] [PubMed]
30. Weathers, P.; Towler, M.J.; Xu, J. Bench to batch: Advances in plants cell culture for producing useful products. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1339–1351. [CrossRef]
31. Georgiev, M.I.; Weber, J. Bioreactors for plants cells: Hardware configuration and internal environment optimization as tools for wider commercialization. *Biotechnol. Lett.* **2014**, *36*, 1359–1367. [CrossRef] [PubMed]
32. Wu, C.-H.; Murthy, H.N.; Hahn, E.-J.; Paek, K.-Y. Enhanced production of caftaric acid, chlorogenic acid and cichoric acid in suspension cultures of *Echinaceae purpureae* by the manipulation of incubation temperature and photoperiod. *Biochem. Eng. J.* **2007**, *36*, 301–303. [CrossRef]
33. Bong, F.B.; Subramaniam, S.; Chew, B.L. Effects of light illumination and subculture frequency on biomass production in cell suspension cultures of *Clinacanthus nutans*. *Malays. Appl. Biol.* **2021**, *50*, 197–204. [CrossRef]
34. Ibrahim, M.M.; Danial, N.; Matter, M.A.; Rady, M.R. Effect of light and methyl jasmonate on the accumulation of anticancer compound in cell suspension cultures of *Catharanthus roseus*. *Egypt. Pharm. J.* **2022**, *20*, 294–302.
35. Khosroushahi, A.Y.; Valizadeh, M.; Ghasempour, A.; Khosrowshahli, M.; Naghdibadi, H.; Dadpour, M.R.; Omid, Y. Improved taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biol. Int.* **2006**, *30*, 262–269. [CrossRef]
36. Donezz, D.; Kim, K.-H.; Antonie, S.; Conreux, A.; De Luca, V.; Jeandet, P.; Clément, C.; Courot, E. Bioproduction of resveratrol and viniferins by an elicited grapevine cell culture in a 2 L stirred reactor. *Process Biochem.* **2011**, *46*, 1056–1062. [CrossRef]
37. Nair, A.J.; Sudhakaran, P.R.; Rao, M.; Ramakrishna, S.V. Berberine synthesis by callus and cell suspension cultures of *Cosciniun fenestratum*. *Plant Cell Tissue Organ Cult.* **1992**, *29*, 7–10. [CrossRef]
38. Khan, T.; Krupadanam, D.; Anwar, S.Y. The role of phytohormone on the production of berberine in the calli cultures of an endangered medicinal plant, turmeric (*Cosciniun fenestratum* I.). *Afr. J. Biotechnol.* **2008**, *7*, 3244–3246.
39. Nazir, R.; Kumar, V.; Gupta, S.; Dwivedi, P.; Pandey, D.K.; Dey, A. Biotechnological strategies for the sustainable production of diosgenin from *Dioscorea* spp. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 569–585. [CrossRef]
40. Mekky, H.; Al-Sabahi, J.; Abdel-Kreem, M.F.M. Potentiating biosynthesis of the anticancer alkaloids vincristine and vinblastine in callus cultures of *Catharanthus roseus*. *S. Afr. J. Bot.* **2018**, *114*, 29–31. [CrossRef]
41. Ataei-Azimi, A.; Hashemloian, B.D.; Ebrahimzadeh, H.; Majd, A. High in vitro production of ant-canceric indole alkaloids from periwinkle (*Catharantus roseus*) tissue culture. *Afr. J. Biotechnol.* **2008**, *7*, 2834.
42. Valdiani, A.; Hansen, O.K.; Nielsen, U.B.; Johannsen, V.K.; Shariat, M.S.; Georgiev, M.I.; Omidvar, V.; Ebrahimi, M.; Dinanai, E.T.; Abiri, R. Bioreactor-based advances in plant tissue and cell culture: Challenges and prospects. *Crit. Rev. Biotechnol.* **2018**, *39*, 20–34. [CrossRef]
43. Chandran, H.; Meena, M.; Barupal, T.; Sharma, K. Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. *Biotechnol. Rep.* **2020**, *26*, e00450. [CrossRef]
44. Mukta, S.; Ahmed, S.R.; Afrin, D. Plant tissue culture- the alternative and efficient way to extract plant secondary metabolites. *J. Sylhet Agril. Univ.* **2017**, *4*, 1–13.
45. Nartop, P. Engineering of biomass accumulation and secondary metabolite production in plant cell and tissue cultures. In *Plant Metabolites and Regulation under Environmental Stress*; Ahmad, P., Ahanger, M.A., Singh, V., Tripathi, D., Alam, P., Alyemini, M.N., Eds.; Elsevier: Amsterdam, The Netherlands, 2018; Chapter 9; pp. 169–194.
46. BDMTM. Atlas de las Plantas de la Medicina Tradicional Mexicana. 1994. Available online: <http://www.medicinatradicionalmexicana.unam.mx/index.html> (accessed on 20 October 2022).
47. WFO. *Ageratina pichinchensis* (Kunth) R.M.King & H.Rob. 2022. Available online: <http://www.worldfloraonline.org/taxon/wfo-0000122234> (accessed on 20 October 2022).
48. Navarro, V.M.; González, A.; Fuentes, M.; Avilez, M.; Ríos, M.Y.; Zepeda, G. Antifungal activities of nine traditional Mexican medicinal plants. *J. Ethnopharmacol.* **2003**, *87*, 85–88. [CrossRef] [PubMed]

49. Torres-Barajas, L.; Rojas-Vera, J.; Morales-Méndez, A.; Rojas-Fermín, L.; Lucena, M.; Buitrago, A. Chemical composition and evaluation of antibacterial activity of essential oils of *Ageratina jahnii* and *Ageratina pichinchensis* collected in Mérida, Venezuela. *Bol. Latinoam. Caribe Plantas Med. Aromat.* **2013**, *12*, 92–98.
50. Sánchez-Mendoza, M.E.; Reyes-Trejo, B.; Sánchez-Gómez, P.; Rodríguez-Silverio, J.; Castillo-Henkel, C.; Cervantes-Cuevas, H.; Arrieta, J. Bioassay-guided isolation of an anti-ulcer chromene from *Eupatorium aschenbornianum*: Role of nitric oxide, prostaglandins and sulfhydryls. *Fitoterapia* **2010**, *81*, 66–71. [[CrossRef](#)] [[PubMed](#)]
51. Sánchez-Mendoza, M.E.; Rodríguez-Silverio, J.; Rivero-Cruz, J.F.; Rocha-González, H.I.; Pineda-Farías, J.B.; Arrieta, J. Antinociceptive effect and gastroprotective mechanisms of 3,5-diprenyl-4-hydroxyacetophenone from *Ageratina pichinchensis*. *Fitoterapia* **2013**, *87*, 11–19. [[CrossRef](#)]
52. Romero-Cerecero, O.; Zamilpa, A.; González-Cortazar, M.; Alonso-Cortés, D.; Jiménez-Ferrer, E.; Nicasio-Torres, P.; Aguilar-Santamaría, L.; Tortoriello, J. Pharmacological and chemical study to identify wound-healing active compounds in *Ageratina pichinchensis*. *Planta Med.* **2013**, *79*, 622–627. [[CrossRef](#)]
53. Romero-Cerecero, O.; Zamilpa-Álvarez, A.; Ramos-Mora, A.; Alonso-Cortés, D.; Jiménez-Ferrer, J.E.; Huerta-Reyes, M.E.; Tortoriello, J. Effect on the wound healing process and in vitro cell proliferation by the medicinal mexican plant *Ageratina pichinchensis*. *Planta Med.* **2011**, *77*, 979–983. [[CrossRef](#)]
54. Romero-Cerecero, O.; Zamilpa, A.; Díaz-García, E.R.; Tortoriello, J. Pharmacological effect of *Ageratina pichinchensis* on wound healing in diabetic rats and genotoxicity evaluation. *J. Ethnopharmacol.* **2014**, *156*, 222–227. [[CrossRef](#)]
55. Aguilar-Guadarrama, B.; Navarro, V.; León-Rivera, I.; Ríos, M.Y. Active compounds against tinea pedis dermatophytes from *Ageratina pichinchensis* var. *bustamenta*. *Nat. Prod. Res.* **2009**, *16*, 1559–1565. [[CrossRef](#)]
56. Dong, R.; Yuan, J.; Wu, S.; Huang, J.; Xu, X.; Wu, Z.; Gao, H. Anti-inflammation furanoditerpenoids from *Caesalpinia minax* Hance. *Phytochemistry* **2015**, *117*, 325–331. [[CrossRef](#)] [[PubMed](#)]
57. Kanwar, J.R.; Kanwar, R.K.; Burrow, H.; Baratchi, S. Recent advances on the roles of NO in cancer and chronic inflammatory disorders. *Curr. Med. Chem.* **2009**, *16*, 2373–2394. [[CrossRef](#)] [[PubMed](#)]
58. Nicholas, C.; Batra, S.; Vargo, M.A.; Voss, O.H.; Gavrilin, M.A.; Wewers, M.D.; Guttridge, D.C.; Grotewold, E.; Doseff, A.I. Apigenin blocks lipopolysaccharide-induced lethality in vivo and proinflammatory cytokines expression by inactivating NF- κ B through the suppression of p65 phosphorylation. *J. Immunol.* **2007**, *179*, 7121–7127. [[CrossRef](#)] [[PubMed](#)]
59. Akihisa, T.; Franzblau, S.G.; Ukiva, M.; Okuda, H.; Zhang, F.; Suzuki, T.; Kimura, Y. Antitubercular activity of triterpenoids from Asteraceae flowers. *Biol. Pharm. Bull.* **2005**, *28*, 158–160. [[CrossRef](#)]
60. Romero-Estrada, A.; Maldonado-Magaña, A.; González-Christen, J.; Marquina-Bahena, S.; Garduño-Ramírez, M.L.; Rodríguez-López, V.; Alvarez, L. Anti-inflammatory and antioxidative effects of six pentacyclic triterpenes isolated from the Mexican copal resin of *Bursera Copallifera*. *BMC Complem. Altern. Med.* **2016**, *16*, 422–432. [[CrossRef](#)]
61. Sánchez-Ramos, M.; Marquina-Bahena, S.; Romero-Estrada, A.; Bernabé-Antonio, A.; Cruz-Sosa, F.; González-Christen, J.; Acevedo-Fernández, J.J.; Perea-Arango, I.; Álvarez, L. Establishment and phytochemical analysis of a callus culture from *Ageratina pichinchensis* (Asteraceae) and its anti-inflammatory activity. *Molecules* **2018**, *23*, 1258. [[CrossRef](#)]
62. Sánchez-Ramos, M.; Alvarez, L.; Romero-Estrada, A.; Bernabé-Antonio, A.; Marquina-Bahena, S.; Cruz-Sosa, F. Establishment of a cell suspension culture of *Ageratina pichinchensis* (Kunth) for the improved production of anti-inflammatory compounds. *Plants* **2020**, *9*, 1398. [[CrossRef](#)]
63. Ling, O.S.; Kiong, A.L.P.; Hussein, S. Establishment and optimization of growth parameters for cell suspension cultures of *Ficus deltoidea*. *Am. Eurasian J. Sustain. Agric.* **2008**, *2*, 38–49.
64. Açıkgöz, M.A. Establishment of cell suspension cultures of *Ocimum basilicum* L. and enhanced production of pharmaceutical active ingredients. *Ind. Crops Prod.* **2020**, *148*, 112278. [[CrossRef](#)]
65. Corchete, P.; Almagro, L.; Gabaldón, J.A.; Pedreño, M.A.; Palazón, J. Phenylpropanoids in *Silybum marianum* cultures treated with cyclodextrins coated with magnetic nanoparticles. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 2393–2401. [[CrossRef](#)]
66. Tahizadeh, M.; Nasibi, F.; Kalantari, K.M.; Benakahani, F. Callogenesis optimization and cell suspension culture establishment of *Dracocephalum polychaetum* Bornm. and *Dracocephalum kotschyi* Bois.: An in vitro approach for secondary metabolite production. *S. Afr. J. Bot.* **2020**, *132*, 79–86. [[CrossRef](#)]
67. Malik, S.; Bhushan, S.; Sharma, M.; Ahuja, P.S. Physico-chemical factors influencing the shikonin derivatives production in cell suspension cultures of *Arnebia eucroma* (Royle) Johnston, a medicinally important plant species. *Cell Biol. Int.* **2011**, *35*, 153–158. [[CrossRef](#)]
68. Zhao, J.-L.; Zhou, L.-G.; Wu, J.-Y. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 137–144. [[CrossRef](#)] [[PubMed](#)]
69. Mimura, T.; Shindo, C.; Kato, M.; Yokota, E.; Sakano, K.; Ashihara, H.; Shimmen, T. Regulation of cytoplasmic pH under extreme acid conditions in suspension culture cells of *Catharanthus roseus*: A possible role of inorganic phosphate. *Plant Cell Physiol.* **2000**, *41*, 424–431. [[CrossRef](#)] [[PubMed](#)]
70. Nieto-Trujillo, A.; Cruz-Sosa, F.; Luria-Pérez, R.; Gutiérrez-Rebolledo, G.A.; Román-Guerrero, A.; Burrola-Aguilar, C.; Zepeda-Gómez, C.; Estrada-Zúñiga, M.E. *Arnica montana* cell culture establishment, and assessment of its cytotoxic, antibacterial, α -amylase inhibitor, and antioxidant in vitro bioactivities. *Plants* **2021**, *10*, 2300. [[CrossRef](#)]

71. Pérez-González, M.Z.; Nieto-Trujillo, A.; Gutiérrez-Rebolledo, G.A.; García-Martínez, I.; Estrada-Zúñiga, M.E.; Bernabé-Antonio, A.; Jiménez-Arellanes, M.A.; Cruz-Sosa, F. Lupeol acetate production and antioxidant activity of a cell suspension culture from *Cnidioscolus chayamansa* leaves. *S. Afr. J. Bot.* **2019**, *125*, 30–38. [[CrossRef](#)]
72. Sajid, Z.A.; Aftab, F. An efficient method for the cell suspension culture in potato (*Solanum tuberosum* L.). *Pak. J. Bot.* **2016**, *48*, 1993–1997.
73. Shyam, C.; Tripathi, M.K.; Tiwari, S.; Ahuja, A.; Tripathi, N.; Gupta, N. In vitro regeneration from callus and cell suspension cultures in Indian mustard [*Brassica juncea* (Linn.) Czern & Coss]. *Int. J. Agric. Technol.* **2021**, *17*, 1095–1112.
74. Songserm, P.; Klanrit, P.; Klanrit, P.; Phetcharaburanin, J.; Thanonkeo, P.; Apiraksakorn, J.; Phomphrai, K.; Klanrit, P. Antioxidant and anticancer potential of bioactive compounds from *Rhinacanthus nasutus* cell suspension culture. *Plants* **2022**, *11*, 1994. [[CrossRef](#)]
75. Fouad, A.; Hegazy, A.E.; Azab, E.; Khojah, E.; Kapiel, T. Boosting of antioxidants and alkaloids in *Catharanthus roseus* suspension cultures using silver nanoparticles with expression of CrMPK3 and STR genes. *Plants* **2021**, *10*, 2202. [[CrossRef](#)]
76. Moniruzzaman, M.; Zhong, Y.; Huang, Z.; Yan, H.; Yuanda, L.V.; Jiang, B.; Zhong, G. Citrus cell suspension culture establishment, maintenance, efficient transformation and regeneration to complete transgenic plant. *Plants* **2021**, *10*, 664. [[CrossRef](#)] [[PubMed](#)]
77. Bernabé-Antonio, A.; Sánchez-Sánchez, A.; Romero-Estrada, A.; Meza-Contreras, J.C.; Silva-Guzmán, J.A.; Fuentes-Talavera, F.J.; Hurtado-Díaz, I.; Alvarez, L.; Cruz-Sosa, F. Establishment of a cell suspension culture of *Eysenhardtia platycarpa*: Phytochemical screening of extracts and evaluation of antifungal activity. *Plants* **2021**, *10*, 414. [[CrossRef](#)] [[PubMed](#)]
78. Lertphadungkit, P.; Suksiriworapong, J.; Satitpatipan, V.; Sirikantaramas, S.; Wongrapanich, A.; Bunsupa, S. Enhanced production of bryonic acid in *Trichosanthes cucumerina* L. (Thai cultivar) cell cultures by elicitors and their biological activities. *Plants* **2020**, *9*, 709. [[CrossRef](#)] [[PubMed](#)]
79. Fazal, H.; Abbasi, B.H.; Ahmad, N.; Ali, M.; Ali, S. Sucrose induced osmotic stress and photoperiod regimes enhanced the biomass and production of antioxidant secondary metabolites in shake-flask suspension cultures of *Prunella vulgaris* L. *Plant Cell Tissue Organ Cult.* **2015**, *124*, 573–581. [[CrossRef](#)]
80. Ali, H.; Khan, M.A.; Khan, R.S. Impacts of hormonal elicitors and photoperiod regimes on elicitation of bioactive secondary volatiles in cell cultures of *Ajuga bracteosa*. *J. Photochem. Photobiol.* **2018**, *183*, 242–250. [[CrossRef](#)]
81. Kumar, S.S.; Arya, M.; Mahadevappa, P.; Giridhar, P. Influence of photoperiod on growth, bioactive compounds and antioxidant activity in callus cultures of *Basella rubra* L. *J. Photochem. Photobiol.* **2020**, *209*, 111937. [[CrossRef](#)]
82. Khan, T.; Abbasi, B.H.; Khan, M.A. The interplay between light, plant growth regulators and elicitors on growth and secondary metabolism in cell cultures of *Fagonia indica*. *J. Photochem. Photobiol.* **2018**, *185*, 153–160. [[CrossRef](#)]
83. Zahir, A.; Ahmad, W.; Nadeem, M.; Giglioli-Guivarc'h, N.; Hano, C.; Abbasi, B.H. In vitro cultures of *Linum usitatissimum* L.: Synergistic effects of mineral nutrients and photoperiod regimes on growth and biosynthesis of lignans and neolignans. *J. Photochem. Photobiol.* **2018**, *187*, 141–150. [[CrossRef](#)]
84. Chan, L.K.; Koay, S.S.; Boey, P.L.; Bhatt, A. Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. *Biol. Res.* **2010**, *43*, 127–135. [[CrossRef](#)]
85. Anjum, S.; Abbasi, B.H.; Doussot, J.; Favre-Réguillon, A.; Hano, C. Effects of photoperiod regimes and ultraviolet-C radiations on biosynthesis of industrially important lignans and neolignans in cell cultures of *Linum usitatissimum* L. (Flax). *J. Photochem. Photobiol.* **2017**, *167*, 216–227. [[CrossRef](#)]
86. Aly, U.I.; El-Shabrawi, H.M.; Hanafy, M. Impact of culture conditions on alkaloid production from undifferentiated cell suspension cultures of *Egyptian Henbane*. *Aust. J. Basic. Appl. Sci.* **2010**, *4*, 4717–4725.
87. Hennayake, C.K.; Takagi, S.; Nishimura, K.; Kanechi, M.; Uno, Y.; Inagaki, N. Differential expression of anthocyanin biosynthesis genes in suspension culture cells of *Rosa hybrida* cv. Charleston. *Plant Biotechnol. J.* **2006**, *23*, 379–385. [[CrossRef](#)]
88. López-Laredo, A.R.; Ramírez-Flores, F.D.; Sepúlveda-Jiménez, G.; Trejo-Tapia, G. Comparison of metabolite levels in callus of *Tecoma stans* (L.) Juss. Ex Kunth. cultured in photoperiod and darkness. *Vitr. Cell Dev. Biol. Plant* **2009**, *45*, 550–558. [[CrossRef](#)]
89. Mukherjee, S.; Ghosh, B.; Jha, S. Establishment of forskolin yielding transformed cell suspension cultures of *Coleus forskohlii* as controlled by different factors. *J. Biotechnol.* **2000**, *76*, 73–81. [[CrossRef](#)] [[PubMed](#)]
90. Fakhari, S.; Sharifi, M.; Yousefzadi, M.; Beshamgan, E. Effect of some phytohormones on podophyllotoxin production in cell and plantlets cultures of *Linum album*. *J. Med. Plant By Prod.* **2013**, *1*, 83–89.
91. Andi, S.A.; Gholami, M.; Fors, C.M.; Maskani, F. The effect of light, phenylalanine and methyl jasmonate, alone or in combination, on growth and secondary metabolism in cell suspension cultures on *Vitis vinifera*. *J. Photochem. Photobiol.* **2019**, *199*, 111625. [[CrossRef](#)]
92. Figueiredo, A.C.; Pais, M.S.S. *Achillea millefolium* (yarrow) cell suspension cultures: Establishment and growth and growth conditions. *Biotechnol. Lett.* **1991**, *13*, 63–68. [[CrossRef](#)]
93. Beigmohamadi, M.; Movafeghi, A.; Sharafi, A.; Jafari, S.; Danafar, H. Cell suspension culture of *Plumbago europaea* L. Towards production of plumbagin. *Iran. J. Biotech.* **2019**, *17*, e2169. [[CrossRef](#)]
94. Arias, J.P.; Zapata, K.; Rojano, B.; Arias, M. Effect of light wavelength on cell growth, content of phenolic compounds and antioxidant activity in cell suspension cultures of *Thevetia peruviana*. *J. Photochem. Photobiol.* **2016**, *163*, 87–91. [[CrossRef](#)]
95. Kaewpintong, K.; Shotipruk, A.; Powtongsook, S.; Pavasant, P. Photoautotrophic high-density cultivation of vegetable cells of *Haematococcus pluvialis* in airlift bioreactor. *Bioresour. Technol.* **2007**, *98*, 288–295. [[CrossRef](#)]

96. Navia-Osorio, A.; Garden, H.; Palazón, J.; Alfermann, A.W.; Piñol, M.T. Production of paclitaxel and baccatin III a 20-L airlift bioreactor by a cell suspension of *Taxus wallichiana*. *Planta Med.* **2002**, *68*, 336–340. [[CrossRef](#)]
97. Navia-Osorio, A.; Garde, H.; Cudisó, R.M.; Palazón, J.; Alfermann, A.W.; Piñol, M.T. Taxol[®] and baccatin III production in suspension cultures of *Taxus baccata* and *Taxus wallichiana* in an airlift bioreactor. *J. Plant Physiol.* **2002**, *159*, 97–102. [[CrossRef](#)]
98. Salazar-Magallón, J.A.; de la Peña, A.H. Production of antifungal saponins in an airlift bioreactor with a cell line transformed from *Solanum chrysotrichum* and its activity against strawberry phytopathogens. *Prep. Biochem. Biotechnol.* **2020**, *50*, 204–214. [[CrossRef](#)]
99. Woragidbumrung, K.; Sae-Tang, P.; Yao, H.; Han, J.; Chauvatcharin, S.; Zhong, J.-J. Impact of conditioned medium on cell cultures of *Panax notoginseng* in an airlift bioreactor. *Process Biochem.* **2001**, *37*, 209–213. [[CrossRef](#)]
100. Ahmadi-Sakha, S.; Sharifi, M.; Niknam, V. Bioproduction of phenylethanoid glycosides by plant cell culture of *Scrophularia striata* Boiss.: From shake-flasks to bioreactor. *Plant Cell Tissue Organ Cult.* **2015**, *124*, 275–281. [[CrossRef](#)]
101. Ahmadi-Sakha, S.; Sharifi, M.; Niknam, V.; Ahmadian-Chashmi, N. Phenol compounds profiling in shake flask and bioreactor system cell cultures of *Scrophularia striata* Boiss. *Vitr. Cell. Dev. Biol.* **2018**, *54*, 444–453. [[CrossRef](#)]
102. Fulzele, D.P.; Heble, M.R. Large-scale cultivation of *Catharanthus roseus* cells: Production of ajmalicine in a 20L airlift bioreactor. *J. Biotechnol.* **1994**, *35*, 1–7. [[CrossRef](#)] [[PubMed](#)]
103. Gupta, K.; Garg, S.; Singh, J.; Kumar, M. Enhanced production of naphthoquinone metabolite (shikonin) from cell suspension culture of *Arnebia* sp. and its up-scaling through bioreactor. *3 Biotech* **2014**, *4*, 263–273. [[CrossRef](#)]
104. Han, J.-E.; Lee, H.; Ho, T.-T.; Park, S.-Y. Brazzein protein production in transgenic carrot cells using air-lift bioreactor culture. *Plant Biotechnol. Rep.* **2022**, *16*, 161–171. [[CrossRef](#)]
105. Han, J.; Zhong, J.-J. High density cell culture of *Panax notoginseng* for production of ginseng saponin and polysaccharide in an airlift bioreactor. *Biotechnol. Lett.* **2002**, *24*, 1927–1930. [[CrossRef](#)]
106. Kim, D.I.; Pedersen, H. Cultivation of *Thalictrum rugosum* cell suspension in an improved airlift bioreactor: Stimulatory effect of carbon dioxide and ethylene on alkaloid production. *Biotechnol. Bioeng.* **1991**, *38*, 331–339. [[CrossRef](#)]
107. Kintzios, S.; Kollias, H.; Stratouris, E.; Makri, O. Scale-up micropropagation of sweet basil (*Ocimum basilicum* L.) in an airlift bioreactor and accumulation of rosmarinic acid. *Biotechnol. Lett.* **2004**, *26*, 521–523. [[CrossRef](#)] [[PubMed](#)]
108. Rani, A.; Meghana, R.; Kush, A. Squalene production in the cell suspension cultures of Indian sandalwood (*Santalum album* L.) in shake flasks and air lift bioreactor. *Plant Cell Tissue Organ Cult.* **2018**, *135*, 155–167. [[CrossRef](#)]
109. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with Tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [[CrossRef](#)]
110. Palomares, L.A.; Ramírez, O.T. Bioreactor scale-up. In *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology*; The Wiley Biotechnology Series; Flickinger, M.C., Ed.; John Wiley and Sons: Hoboken, NJ, USA, 2009; pp. 183–201.
111. Rodríguez-Monroy, M.; Galindo, E. Broth rheology, growth and metabolite production of *Beta vulgaris* suspension culture: A comparative study between cultures grown in flasks and in a stirred tank. *Enzym. Microb. Technol.* **1999**, *24*, 687–693. [[CrossRef](#)]
112. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.