

**PRODUCING MEDICINAL
PHYTOCHEMICALS
FROM *Phyllanthus
acuminatus* IN PLANT-
CELL SUSPENSION
CULTURES**

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Abstract: Pharmaceutical use is not feasible for important medicinal compounds derived from certain plant materials, including *Phyllanthus acuminatus* roots, due to their low natural abundance. New technologies in non-traditional biomass generation are needed to produce these remarkable natural compounds. Therefore, this article describes a methodology for establishing *Phyllanthus acuminatus* plant-cell suspensions from callus cultures: An evaluation on inoculum concentration and agitation speed displayed significant changes in plant cell growth kinetics. It was determined that treatment with 2 g of inoculum in 25 mL of medium and 100 rpm agitation creates the best conditions for generating thick cell suspensions. Likewise, treatment with 2 g of inoculum and 120 rpm agitation produces the best conditions for establishing fine cell suspensions. Phytochemical comparison through high-resolution mass spectrometry of *P. acuminatus* roots and plant cell suspension extracts confirmed presence in the plant cell culture of multiple phyllanthostatins of pharmaceutical interest. Here, we demonstrate that *Phyllanthus acuminatus* can be cultured in plant cell suspensions to produce secondary metabolites of medical interest – technology that could be scaled up for implementation in industrial bioprocesses.

Keywords: Plant cell suspension, secondary metabolites, *Phyllanthus acuminatus*, phyllanthostatins.

INTRODUCTION

Plants as sessile organisms, like a defense mechanism, synthesize a wide range of secondary metabolites with important physiological and ecological effects. These metabolites have acted as a reliable source of numerous medicinal compounds with diverse biological activities, including antimicrobial,

immunosuppressive, anticancer, and anti-inflammatory properties, many of which could have been developed as therapeutic agents, if not for their low concentrations in plant tissues, which makes the use of plant extracts unfeasible for their industrialization (Guerriero et al., 2018; Pham et al., 2019).

The *Phyllantaceae* family is widely distributed in tropical and subtropical areas, such as tropical Africa, the tropical Americas, Asia, and Oceania (Mao et al, 2016). Around 14 different species of *Phyllanthus* have been found in Costa Rica (Navarro et al, 2017), including *Phyllanthus acuminatus*. This is a shrub-like plant, reaching 2-8 m high, and can be found between 0-1800 meters above sea level in valleys, in both hot and temperate climates, and in both dry and humid environments (Moreira-González, 2014).

The phytochemistry for several species of the *Phyllanthus* genus has been extensively studied (Qi et al., 2014), and many classes of organic compounds have been isolated and characterized (Gutiérrez, 2011; Jamal et al., 2008); over 500 compounds have been isolated from plant extracts (Duarte et al., 2018; Mao et al., 2016).

Additionally, Muthusamy and his collaborators (2016) conducted studies on the impact of plant growth regulators and precursors on generating bioactive lignans and antioxidant content on in vitro cultures of *Phyllanthus amarus* and *Phyllanthus urinaria*.

Recently, natural compounds of bioactivity relevance, including flavonoids, ellagitannins, flavan-3-ols, and phenolic acids, have been identified on *Phyllanthus acuminatus* leaf plant material (Navarro et al., 2017).

Plant cell and tissue culture techniques offer environmentally friendly alternatives for the production of secondary metabolites, particularly when their natural supply is limited or their chemical synthesis is unviable (Arias et al., 2009; Gonçalves & Romano, 2018).

This is the case of trees, including *Phyllanthus acuminatus*, whose roots produce many metabolites of interest at low concentrations (García-Hernández et al., 2017).

In similar studies, Mendoza and collaborators (2020) analyzed bioactive metabolites of *Thevetia peruviana*, comparing and finding similarities between the secondary metabolite profiles of cell suspension cultures and explant extracts. Also, Yang and collaborators (2019) investigated the phytochemical characteristics of extracts from callus suspensions of *Helicteres angustifolia* and their bioactive properties. They demonstrated that, as compared to the equivalent wild roots, the callus cultures yielded larger amounts of phytochemicals as total phenolics, flavonoids, and saponins.

The plant cell suspension technique has been studied on *Phyllanthus debilis* (Malayaman et al., 2017) and *Phyllanthus pulcher* (Danaee et al., 2015), without specific identification of bioactive metabolites in cell culture extracts. Therefore, suitable conditions have not been reported for the *Phyllanthus acuminatus* plant cell suspension culture.

Consequently, this work presents optimization for *Phyllanthus acuminatus* plant cell suspension culture, by evaluating culture conditions including the amount of initial inoculum and agitation speed and confirming via high-resolution mass spectrometry the generation of specific bioactive phyllanthostatin in comparison with *Phyllanthus acuminatus* root extracts.

METHODOLOGY

Obtaining friable calli: Calli were induced from 1 cm stem segments, obtained from previously established *Phyllanthus acuminatus* seedlings in vitro. Said segments were introduced in a Murashige & Skoog (MS) (1962) medium containing 3% m/v of sucrose and 4.2 g/L of Gelzan™ gelling agent, and

supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 2 mg/L.

Establishing thick cell suspensions: After a literature review of suspension studies conducted with other *Phyllanthus* species and similar plants, the conditions chosen for this experiment were 1g/2g inoculum and 100rpm/120rpm. Two different inoculum concentrations and agitation speeds were evaluated. Working with three repetitions of each treatment, callus segments were added to 125 mL flasks with liquid MS (1962) medium supplemented with 0.5 mg / L of 2,4-D. The work volume for all treatments was 25 mL and all treatments were placed in dark conditions at 27 °C.

Establishing fine cell suspensions: After two weeks of growth as a thick cell suspension, the suspensions settle for 30 minutes. The supernatant was discarded and fresh culture medium added, until a volume of 25 mL was again achieved. Once the fresh medium had been added, the suspensions were passed through ss # 30 sieve filters (TecnoSagot S.A catalog), to separate the larger cell aggregates and then to obtain fine suspensions.

Plant cell counting: 250 µL aliquots of both fine and thick cell suspensions were taken three times a week. The aliquots settled for 30 minutes and then the culture medium was removed with a micropipette, which was replaced by phosphate-buffered saline (PBS). These were incubated for 3 minutes with 250 µL of 0.4% trypan blue stain, as indicated by Kamal et al. (2015), as a cell viability indicator. The cells were counted using a Neubauer chamber and an optical microscope at 4X magnification.

Preparing cells and plant tissue for metabolites extraction: After 3 weeks of growth as fine cell suspension, the suspensions were filtered with Whatman® #3 filters. Once the medium was removed, the cells were placed in 50 mL conical tubes and stored for

12 hours at -4 °C. Subsequently, the samples were lyophilized for 96 h with a Christ model Alpha 1-2 LD Plus® lyophilizer. For *P. acuminatus* roots, cleaned and dried material was pulverized with a Lamilpa artisanal mechanical mill.

Extracting metabolites: Mechanical maceration of the lyophilized material was conducted with a Retsch® mill (Type MM400), adjusted to 17 strokes per second for 2 series of 4.5 minutes each for cell suspension, and 24 strokes per second for 2 series of 5 minutes each to extract *P. acuminatus* roots. 0.26 g of lyophilized material, 13.84 mL of absolute ethanol, and a 13.58 g pellet were placed in each equipment support, with a capacity of 25 mL. Both extracts were filtered and recovered for analysis through chromatography. The extracts were concentrated using a speed vacuum system and stored at -80 °C, until mass spectrometry analysis.

IDENTIFYING PHYLLANTHOSIDES USING HIGH-RESOLUTION MASS SPECTROMETRY

Samples were reconstituted and then measured using a Xevo G2-XS quadrupole time of flight mass spectrometer (Waters Corporation, Wilmslow, UK), coupled with an ACQUITY UPLC H-Class. A 1 µL sample injection was separated with a BEH-C18 column (2.1x100 mm, 1 µm) using a mobile phase gradient of A (water, 0.05% formic acid) and B (acetonitrile, 0.05% formic acid), and setting a flow of 0.5 mL/min. The gradient consisted of maintaining 5% B for 5 min, then increasing to 80% at 10 min and, subsequently, to 100% at 11 minutes and holding until 13 minutes, to finish by equilibrizing the column to initial conditions. The column temperature was set to 50 °C. The mass spectrometer was configured to use a capillary voltage of 2 kV, a 40 V sampling cone, and a source offset of 80 V. Source temperatures were set at 125 °C

and 600 °C for the desolvation temperature, and gas flows were set to 150 L/h for the cone gas and 1000 L/h for the desolvation gas. Measurements were made using the MS^e independent data acquisition mode in positive and negative polarities, and high-resolution mode with a mass range from 50-1000 m/z, scan time to 0.25 sec, and a ramp of collision energy from 20 V to 40 V for the high-energy function.

Data were analyzed using Progenesis QI software with Progenesis MetaScope for compound identification, using an in-house structure-data file (SDF) comprising a database of 290 compounds containing previous reported phyllanthosides structures (Geethangili & Ding, 2018; Mao et al., 2016). Theoretical fragmentation matching was achieved by setting precursors and fragment tolerance to 10 ppm.

Statistical analysis: To determine the statistically significant differences between growth curves, both for thick and fine cell suspensions, the Cross Tabulation tool and Chi² function of the Minitab 19 software were used, determining with 95% confidence whether growth kinetics present different behaviors with each other. The growth rate and doubling time were calculated based on the formulas:

$$\mu = \frac{\ln\left(\frac{x}{x_0}\right)}{t} \quad y \quad td = \frac{\ln 2}{\mu}$$

Where: μ corresponds to the growth rate in days⁻¹, X corresponds to the final biomass produced in the exponential phase, X₀ corresponds to the biomass at which the exponential phase begins, t corresponds to the duration of the phase in days, and td to the doubling time of the cells in days. Source: Shuichi et al., 2003.

To compare cell doubling times, biomass production, and percentage of cell viability, the statistical assumptions were verified

for the obtained data's homoscedasticity, randomization, and residue normality. The ANOVA statistical test, from the same software, was used and determined with 99% confidence that the means of the analyzed parameters were statistically different from each other between each treatment.

RESULTS

To optimize *Phyllanthus acuminatus* plant cell suspension culture, an evaluation was made on multiple growth conditions. First, as thick cell suspensions, seeking higher biomass production and better cell viability; then, optimizing a fine cell suspension culture and targeting for increased biomass generation and cell viability, as a culture stage in which interesting metabolites are produced. The evaluation on cell morphology, growth kinetics, cell viability, and the identification of specific metabolites is further described.

CELL MORPHOLOGY

Most *Phyllanthus acuminatus* cells were observed to form cell aggregates (figure 1). The aggregates were present in both thick and fine suspensions, with differences in the number of cells present per aggregate, which was reduced by filtering the suspensions. Thick cell suspension presented a size and cell amount considerably larger in comparison to fine cell suspension, in which the aggregates seldom presented more than 20 cells. Additionally, after filtering, the cells in fine suspension showed a gradual increase in size – a phenomenon that did not occur during the thick suspension culture.

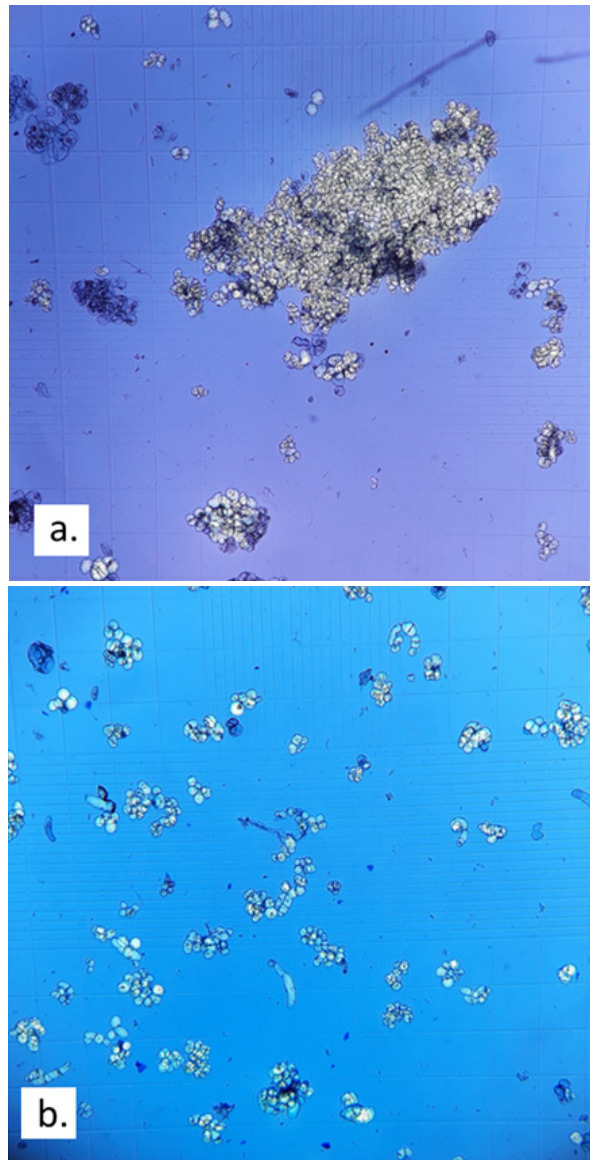


Figure 1. *P. acuminatus* cell aggregates of thick cell suspension (a) and fine suspension (b), 4x magnification.

THICK CELL SUSPENSION GROWTH AND VIABILITY

Growth curves were obtained for *Phyllanthus acuminatus* thick cell suspension during two weeks of culture, according to different culture conditions. (Figure 2).

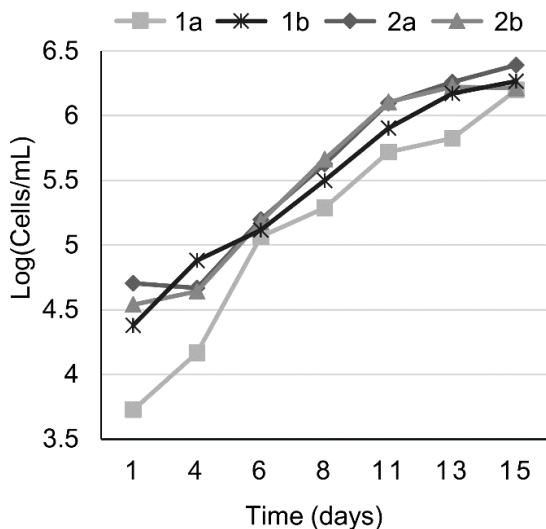


Figure 2. Growth kinetics of the different treatments of thick cell suspensions of *P. acuminatus*. Treatments are described in table 1.

Treatments 2a and 2b had a latency phase from days 1 to 4, an exponential phase from days 4 to 11, and a stationary phase from days 11 to 15. For treatments 1a and 1b, exponential phases of variable slopes were presented from days 1 to 13 (1a) and 1 to 15 (1b).

The Chi² statistical test determined that the 4 kinetics presented are statistically different from each other ($p = 0.00$, 95% confidence), so

that the treatments do cause differing growth kinetics in *P. acuminatus* cells.

With the data obtained, the growth speed and cell doubling times were calculated (Table 1).

Replicate	1a	1b	2a	2b
1	1.2249	2.1372	1.9273	2.1902
2	1.6354	1.6779	1.7894	2.0204
3	1.6988	2.4823	1.9738	2.0498
Mean	1.5210	2.0991	1.8969	2.0868

Table 1. Cell duplication times in days, of the different treatments of thick cell suspensions of *P. acuminatus*.

An ANOVA test with 99% confidence determined that the values do not present significant differences ($p = 0.067$). This means that cell duplication was not affected by the proposed treatments. For this test, the statistical assumptions of homoscedasticity, randomization, and normality of residuals ($p > 0.05$) were previously checked.

After carrying out the cell counts for viable and non-viable cells, the following values were also obtained during the culture (Figure 3).

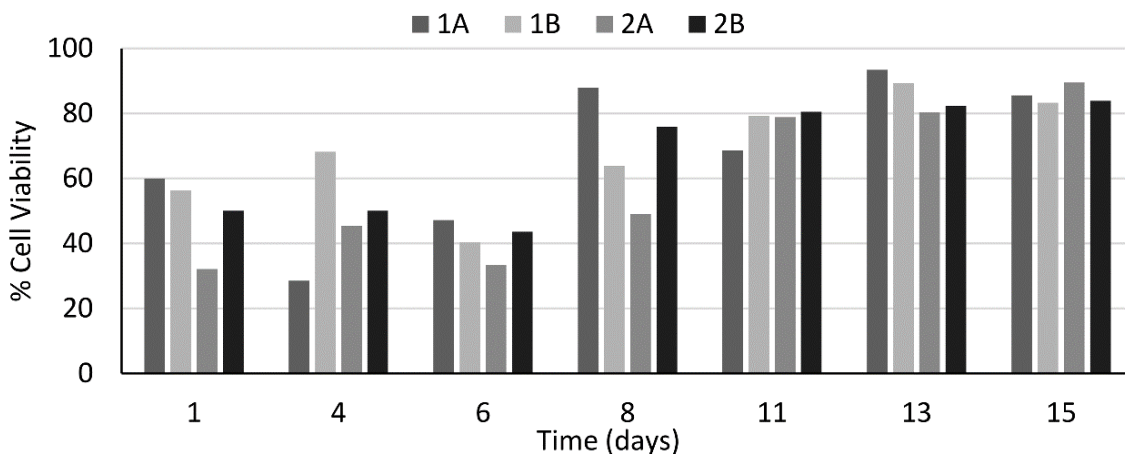


Figure 3. Cell viability according to the treatment of thick cell suspensions of *P. acuminatus*. Treatments are described in table 1.

The mean cell viability percentages obtained were 73.31% for treatment 1b, 67.72% for 1a, and 66.62%, and 66.24% for treatments 2b and 2a, respectively. Regarding percentages of cell viability, the statistical test did not detect significant differences between the four treatments, so it can be stated that the amount of initial inoculum and suspension agitation do not influence this parameter. Even so, all treatments reached maximum cell viability above 80% at the late stage of the culture, together with the maximum of generated biomass.

Fine cell suspension growth and viability

After three weeks of monitoring the fine cell suspensions' growth, the following graph was obtained (Figure 4), which shows the logarithmic value averages for the number of cells per milliliter, for each treatment.

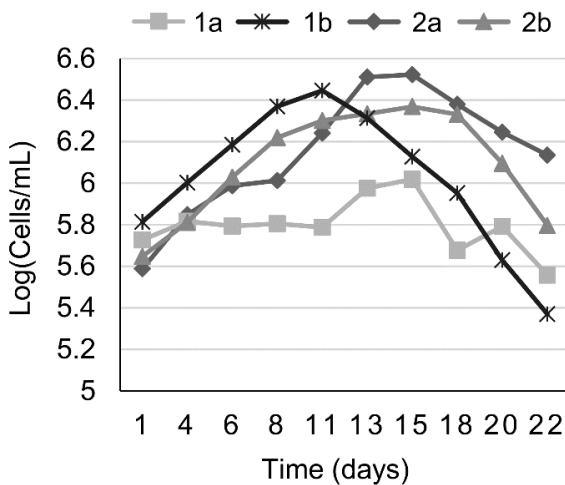


Figure 4. Growth kinetics of the different treatments of fine cell suspensions of *Phyllanthus acuminatus*. Treatments are described in table 1.

Only treatment 1a presented an observable latency phase during the evaluation period, then continued with a biomass increase phase of two days. For treatments 2a, 1b, and 2b, the exponential phase lasted 13, 11, and 8 days, respectively. Treatment 2b showed a stable stationary phase from days 8 to 18

before entering the death phase, this being the treatment with the longest stationary phase. On the other hand, treatments 2a and 1a presented a brief stationary phase, before entering the death stage. Treatment 1b displayed accelerated growth, after which it gradually decreased until the death stage.

It was determined, with 95% confidence, that the 4 growth kinetics presented are statistically different from each other ($p = 0.00$), as with the thick plant cell suspensions. However, as they are fine suspensions, they are intended to have a long and stable stationary phase in which production takes place for the metabolites of interest.

Of all treatments, 2b was the only one that presented a stable stationary phase for a considerable period, while the 2a treatment presented a diauxic growth curve and treatments 1a and 1b presented shorter stationary phases.

The culture in treatment 1b presents a curve in which a stationary phase is not observed; however, this is due to one of the replicates that grew faster than the others. It caused the average of the treatments to be observed as if it were a prolonged exponential phase that dies at the end of its growth. The curves of treatment b individually presented a stationary phase of around five days. Although treatment 2b did not reach a biomass quantity as high as treatments 2a and 1b, it was the most stable in its stationary phase, which makes it the best treatment for fine cell suspensions. Due to the high amount of biomass and the stability of its stationary phase (days 8 to 18), treatment 2b was selected as the most recommended to produce secondary metabolites.

As with the thick cell suspensions, the percentage of cell viability for each of the treatment repetitions was calculated by obtaining an average of the calculated values for each sampled culture time (Figure 5).

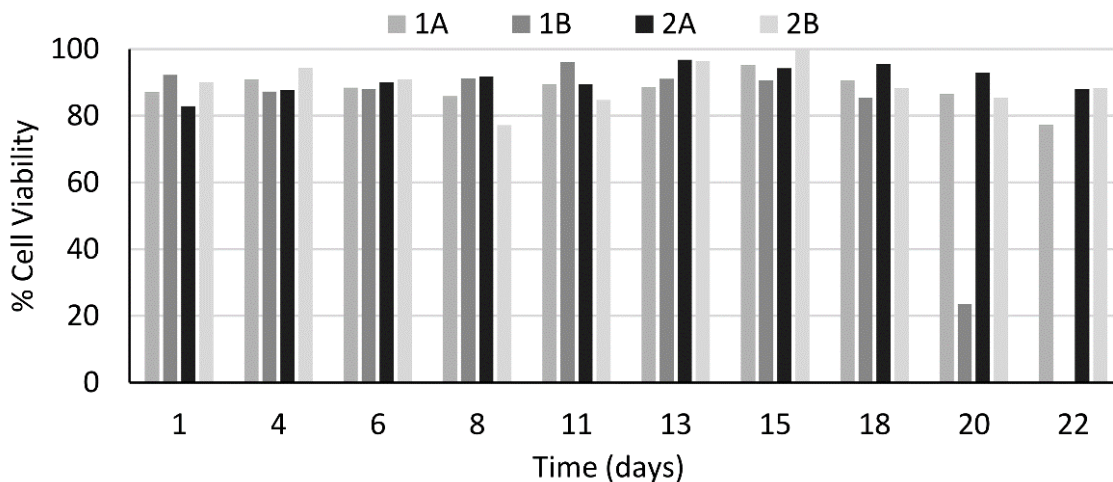


Figure 5. *P. acuminatus* percentages of viability according to the treatment of fine suspensions. Treatments are described in table 1.

The statistical test detected significant differences between treatments ($p < 0.05$). 2a and 2b are the best treatments, with around 90% of average cell viability during culture, with no statistical difference between them.

METABOLITE IDENTIFICATION

Several compounds of medicinal interest were found through mass spectrometry in cell suspension extracts; among them, phyllanthostatins 1, 2, 3, and 6 were the compounds of greatest interest for their anticancer biological activities. Observed extracted ion chromatograms for identified phyllanthostatins are shown in Figure 6. The compounds were assigned identification based on mass accuracy better than 10 ppm, as well as identification of at least 5 fragments from in silico fragmentation of structures from a selected structure database. Identification parameters for all compounds are available in table S1.

A comparison of cell suspension and root extracts was made to confirm phyllanthostatin identifications. Matching fragment structures of Phyllanthostatin 6 in root extracts and cell suspensions confirms its presence in cell

suspension (Figure 7). The fragmentation and identification of other phyllanthostatins can be observed in figures S1 to S3 into supplementary materials.

DISCUSSION

Optimization of plant cell cultures may require observation of cell morphologies, as cellular aggregates are formed and have consequences on cell cultures. At the first stage, a thick suspension culture is used for biomass generation before cell disaggregation via cell filtration into a fine cell suspension for secondary metabolites production. Plant cell growth kinetics and viability of thick and fine suspensions are followed, to find optimal conditions for higher biomass production and a longer stationary phase, in order to increase generation of secondary metabolites. Plant cell suspension extracts were screened via high-resolution mass spectrometry to verify the presence of interesting bioactive compounds in the cell culture, by comparing their signals with measurements of *Phyllanthus acuminatus* root extracts.

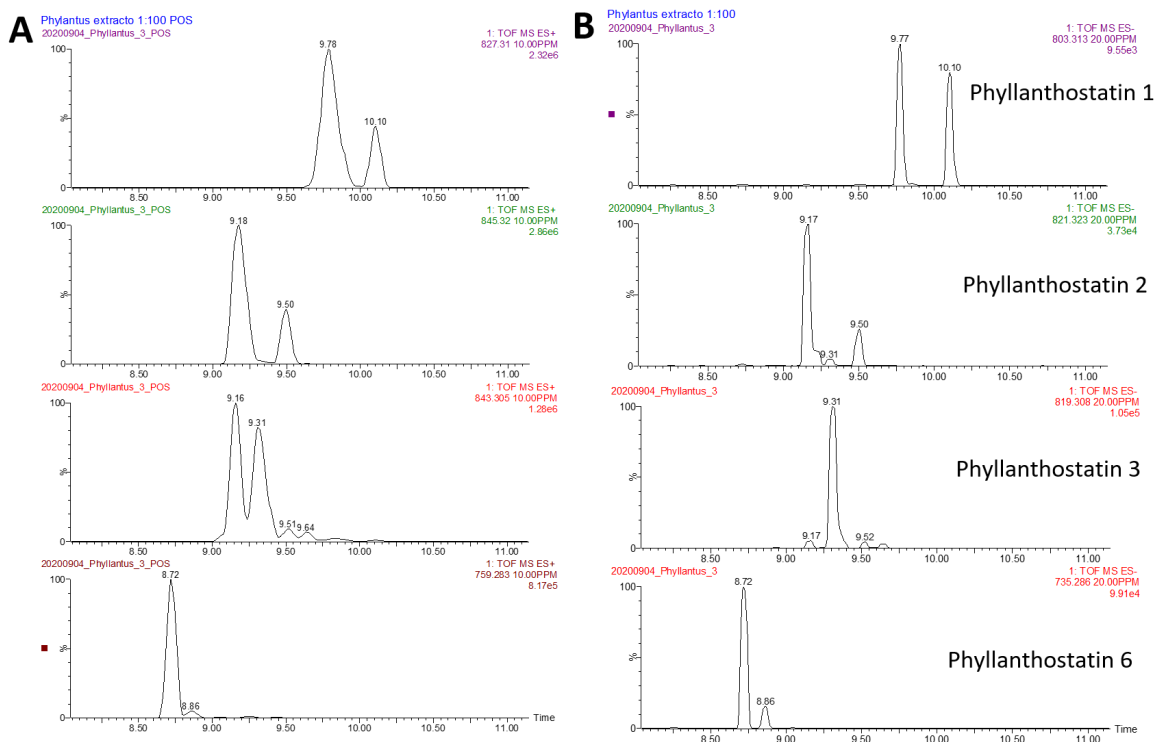


Figure 6. Extracted ion chromatograms from M+Na (A) and M-H (B) adducts of the identified phyllanthosides. Chromatograms peaks correspond to respective phyllanthosides and their structural isomers.

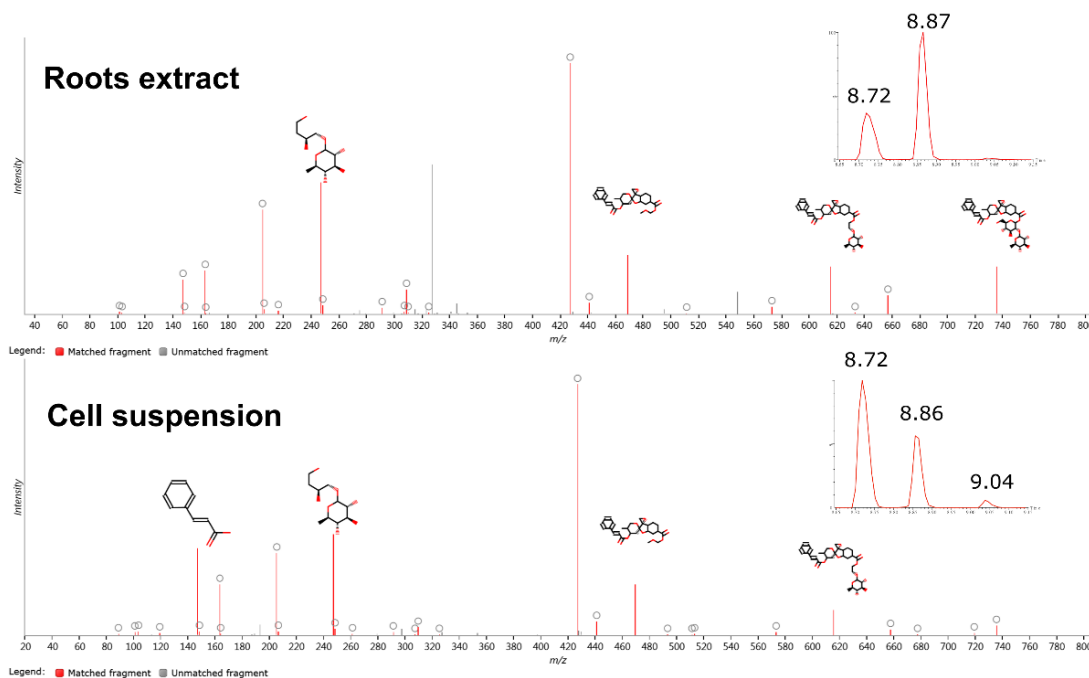


Figure 7. Extracted ion chromatograms and assigned fragmentation spectra for the compound identified as Phyllanthostatin 6 (M-H adduct, retention time 8.72 min), matching compounds found in cells suspension and *Phyllanthus acuminatus* roots extracts. Red-colored fragments represent matched fragments and their structures with mass error less than 10 ppm.

CELL MORPHOLOGY

Spherical cells, as well as some with elongated or isodiametric morphology, were found in both thick and fine cell suspensions, which agrees with Arias Echeverri's (2013) reports on cell suspensions of *Thevetia peruviana*.

Cell aggregates of different sizes were observed in all treatments. The formation of cell aggregates is characteristic of plant cells in suspension, since the cells either do not separate adequately when dividing or they later associate (Mustafa et al., 2011) which are convenient for the large-scale production of fine chemicals in bioreactors and for the study of cellular and molecular processes, as they offer the advantages of a simplified model system for the study of plants when compared with plants themselves or differentiated plant tissue cultures. The commonly used methods of initiation of a callus from a plant and subsequent steps from callus to cell suspension culture are presented in the protocol. This is followed by three different techniques for subculturing (by weighing cells, pipetting and pouring cell suspension. Carreño Campos (2019) points out that the natural tendency of cells to aggregate is regulated by cell wall cohesiveness; this allows for intercellular communication which, in turn, can benefit from the transport of certain intermediaries necessary for the biosynthesis of metabolites.

Carreño (2019) also determined that cells in the *Daucus carota* suspension form small aggregates of approximately 10 to 20 cells during the initial stage, while in the exponential phase, he observed round and elongated cells forming aggregates of more than 100 cells. Another highlight is that, during the death stage, the cells presented loss of cell membrane integrity, which is why they were deformed.

Evaluation on the filtration process of thick cell suspension, for the formation of the fine

cell suspension, may represent an optimization target for bioprocess generation, as it has a clear impact on cell concentration and cell viability, as observed in the growth curve with the maximal growth rate for treatment 1b.

THICK CELL SUSPENSION GROWTH

Data obtained on growth speed (between 0.279 and 0.413 d⁻¹) and doubling time (2.4823 and 1.67 days) are promising, as compared to other studies such as Martínez's (2018), regarding suspensions of *Borojoa patinoi* and in which lower values were reported, the growth rate was 0.11 d⁻¹ and the doubling time 6.5 days. The data obtained in this study are similar to those reported by Arias Echeverri (2013), who obtained a maximum growth speed of 0.215 d⁻¹ with a doubling time of 3.22 days in suspensions of *T. peruviana*.

Such results may be due to the amount of inoculum used. This concurs with results from Ortiz (2011), who found significant differences between the growth rates of *Calendula officinalis* cells in suspension, when using inocula of 1.17 g/L and 2.55 g/L. Ortiz points out that, for a certain amount of nutrients in the first case, the cellular biomass may reach a greater number of divisions, thus leading to a higher growth rate.

Agitation may also have an important influence on cell growth and culture development. Plant cell cultures in a liquid medium under agitation are constantly subjected to hydrodynamic forces. Such forces are generated by the agitation processes required for adequate cell oxygenation and the distribution of the nutrients in the medium. Cultured cells can respond in different ways to these hydrodynamic forces; depending on the magnitude, the production of biomass or secondary metabolites can either be increased or irreparable damage can be done to cells, affecting cell culture viability (Arias Echeverri,

2013). Agitation promotes the growth of the culture by dispersing nutrients to the cells while preventing the accumulation of heat and toxic metabolites. High rates of agitation can be detrimental to cultures susceptible to mechanical stress (Yoshida, 2017).

In thick suspensions, the curves of treatments 1a and 1b did not present an observable latency phase, since a high increase in cells was obtained between measurements on days 1 and 4. It is probable that the latency phase of these treatments lasted less than 2 days, so it was not possible to detect through actual measurements. Carreño (2019) obtained a similar result with suspensions of *D. carota*, in which growth kinetics did not present a latency phase; he pointed out that this may have been due to the origin of the inoculum, as calli were grown in a medium with the same composition as the medium used to establish the suspension, except for the gelling agent.

On the other hand, treatments 2a and 2b presented similar results to those obtained by Malayaman and collaborators (2017), who reported that *Phyllanthus debilis* cell suspension showed a rapid growth curve that revealed a latency phase of 3 days, followed by an exponential phase of 15 days.

The difference between the treatments that presented a lag phase and those that did not coincide with the treatments in which they were inoculated with 2 g and 1 g of callus, respectively. This difference may be due to the ease of adaptation for a smaller number of cells (treatments 1a and 1b), which would have a greater amount of available nutrients and space for duplication and accelerated growth.

By obtaining average cell viability values between 66.24% and 73.31%, these results agree with Martínez Mira's (2018) reports on suspensions of *B. patinoi*, in which cell viability was 61.9% upon the first 6 days of

culture, but later showed a slight viability decrease, which coincided with decrease in the pH of the medium.

The objective of thick suspensions is to multiply the cells as much as possible and to have a good inoculum to later carry out the filtration and convert these into fine suspensions; for this, a long stationary phase is expected for good production of secondary metabolites. Due to this, it was determined that the best treatments to establish thick suspensions are 2a and 1a, and using lower agitation speeds, which favored a high biomass production, shorter cell doubling times, and a high percentage of cell viability.

FINE CELL SUSPENSION

Treatment 1b presented accelerated growth due to good disintegration of cell aggregates from the thick suspension, which increased the number of cells that passed the filter; this consistently represented the culture with the fastest growth, as it had the highest initial cell concentration.

Similarities in both cell viability and growth kinetics were found in treatments 2a and 2b – both, treatments that used 2 g of the initial inoculum. Similar results were obtained by Akalezi and collaborators (1999), who evaluated the effect of the inoculum concentration on cell growth and the production of ginseng saponins in cell suspensions of *Panax ginseng*. They found that the best results for growth were obtained for the highest initial cell concentrations evaluated, which were 4.5 and 6 g/L.

After filtering suspensions from the thick cell culture, average cell viability increased from 70% to 90%. This could be associated not only with viability of the initial inoculum but also with the diminution of cell aggregate size. Cell aggregates should be affected by their microenvironments, which reverberate in cell viability, bioprocess performance, and

metabolites accumulation (Gaid et al., 2016; Kolewe et al., 2011)

Cell viability percentages around 90% were found in treatments 2a and 2b, similar to the results obtained by Kehie and collaborators (2016), who obtained cell viability percentages between 100% and 96% for *Capsicum chinense* cell suspensions, which were evaluated through the same trypan blue staining technique.

Meanwhile, cell viability was over 80% for all culture conditions for fine cell suspension treatments: 2a reached the higher biomass concentration but treatment 2b (2 g inoculum, high agitation) presented the longer and stable stationary phase, thus offering an advantage in secondary metabolite biosynthesis.

METABOLITES IDENTIFICATION

Culture conditions play an important role in the quality and quantity of the material obtained through plant cell suspension. This is why optimization of the culture condition is an important step to improving accumulation of the desired products (Yue et al., 2016).

Several phyllanthosides and phyllanthostatins have been isolated from leaf extracts (Navarro et al., 2017) and root extracts (Pettit et al., 1981). These compounds possess powerful cytotoxic activity, as proven in murine P388 leukemic lymphocytic cells and B16 melanoma cells (Duarte et al., 2018). Pettit and collaborators evaluated phyllanthostatins 2 and 3 against PS leukemia, resulting in 43% (at 12 mg/kg and ED, 0.12 pg/mL) and 41% (at 80 mg/kg) life extensions, respectively.

In extracts of generated plant cell suspension cultures from *Phyllanthus acuminatus*, phyllanthostatins 1, 2, 3, and 6 were identified as mass spectrometry data revealed the presence of adducts with mass accuracy better than 10 ppm, and also more than 5 MS/MS fragments were matched for similarities in structure. Final confirmation was made, as

the same compounds with the same retention time and fragmentation pattern were found in *Phyllanthus acuminatus* root extracts.

Like Mendoza et al. (2020) and Yang et al. (2019), correspondence was observed during metabolite identification of cell suspensions and in vivo material extracts. Matching phyllanthostatins fragmentation spectra and structures found on cell suspensions and root extracts indicate that the presented plant cell suspension culture possesses the ability to produce phytochemicals of medicinal interest. Further phytochemical characterization will be carried out to characterize the bioactive potential of *Phyllanthus acuminatus* cell suspension extracts.

CONCLUSION

This study revealed that *P. acuminatus* cell suspension extracts present an interesting phytochemical profile comparable to ex-vitro cultured plants being able to produce specifically identified phyllanthostatins. This positions *P. acuminatus* cell suspension cultures not only as a viable solution, but also as a promising and profitable alternative for producing medicinal secondary metabolites. It is predicted that cell filtration, inoculum concentration, and stirring speed cause statistically significant changes in growth kinetics for *Phyllanthus acuminatus* cell suspensions. Plant cell growth as a thick suspension was characterized by short doubling times and higher biomass growth rates, while, in fine suspensions, a long and stable stationary phase was presented and observed as essential to the production of secondary metabolites. Further research on optimizing cell filtration, elicitors in culture medium, and deeper phytochemical analysis will be carried out to identify further metabolites and to increase production of interesting secondary metabolites in plant cell suspensions of *Phyllanthus acuminatus*.

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SUPPLEMENTARY MATERIAL

Compound	RT	Formula	M/z	Detected Adducts	Mass error (ppm)	Frag. score	Isotope similarity	Number of assigned Fragments
**Phyllanthostatin 6	8.72	C ₃₆ H ₄₈ O ₁₆	736.2942, 735.2878	[M+Na]; [M-H]	1.19	77	83	20;20
Phyllanthostatin 2	9.31	C ₄₀ H ₅₂ O ₁₈	803.3090/819.3089	[M+Na]; [M-H]	-3.80; 0.88	57; 56	87; 87	12;14
Phyllanthostatin 3	9.14	C ₄₀ H ₅₄ O ₁₈	845.3128/821.3248	[M+Na]; [M-H]	-9.04	13.5;72	83; 77	6 ;25
Phyllanthostatin 1 Phyllanthoside Phyllanthostatin 4	10.1	C ₄₀ H ₅₂ O ₁₇	827.3088/803.3139	[M+Na]; [M-H]	0.88; 1.12	31; 81	72; 81	5 ;13

Table S1. Compound identification parameters for the identified phyllantosides.

Factor	N	Mean	Aggrupation*
2a	30	90.86	A
2b	30	90.45	A
1a	30	82.37	A B
1b	30	72.66	B

*Means that do not share a letter are significantly different.

Table S2. Grouping of mean cell viability percentages, through the Tukey method, at 95% confidence.

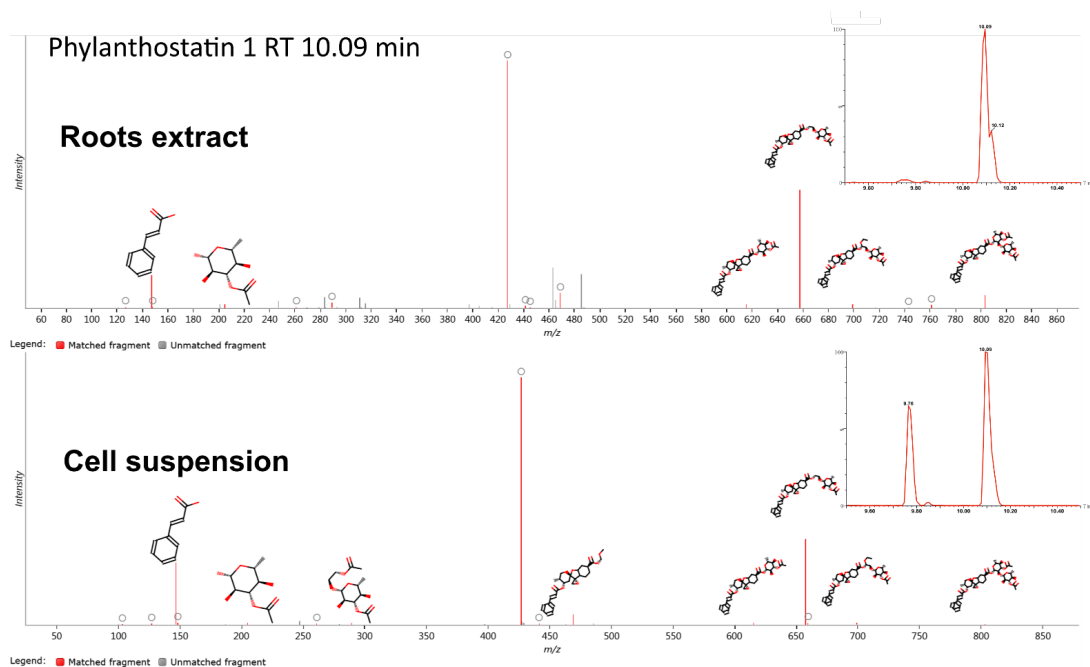


Figure S1. Extracted ion chromatograms and assigned fragmentation spectra for the compound identified as Phyllanthostatin 1 (M-H adduct). Red colored fragments represent matched fragments and their structures with mass error less than 10 ppm. Comparison of fragmentation spectra from roots and cell suspension extracts.

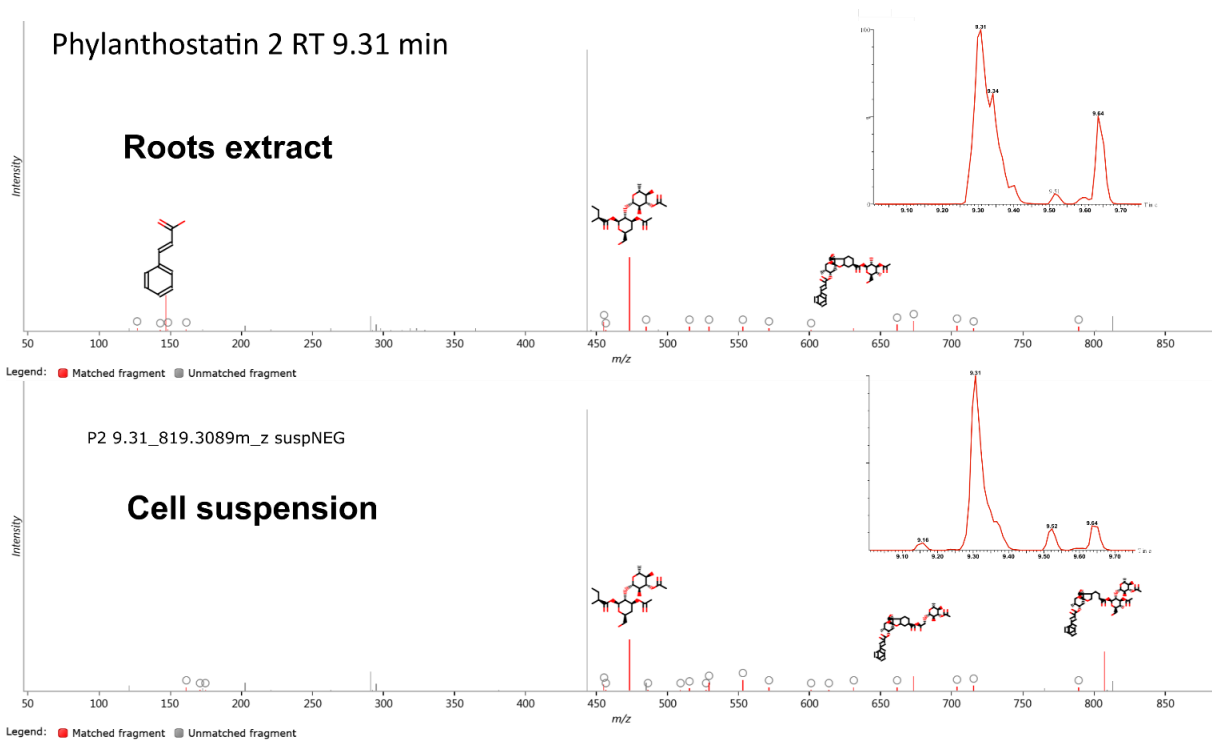


Figure S2. Extracted ion chromatograms and assigned fragmentation spectra for the compound identified as Phyllanthostatin 2 (M-H adduct). Red colored fragments represent matched fragments and their structures with mass error less than 10 ppm. Comparison of fragmentation spectra from roots and cell suspension extracts.

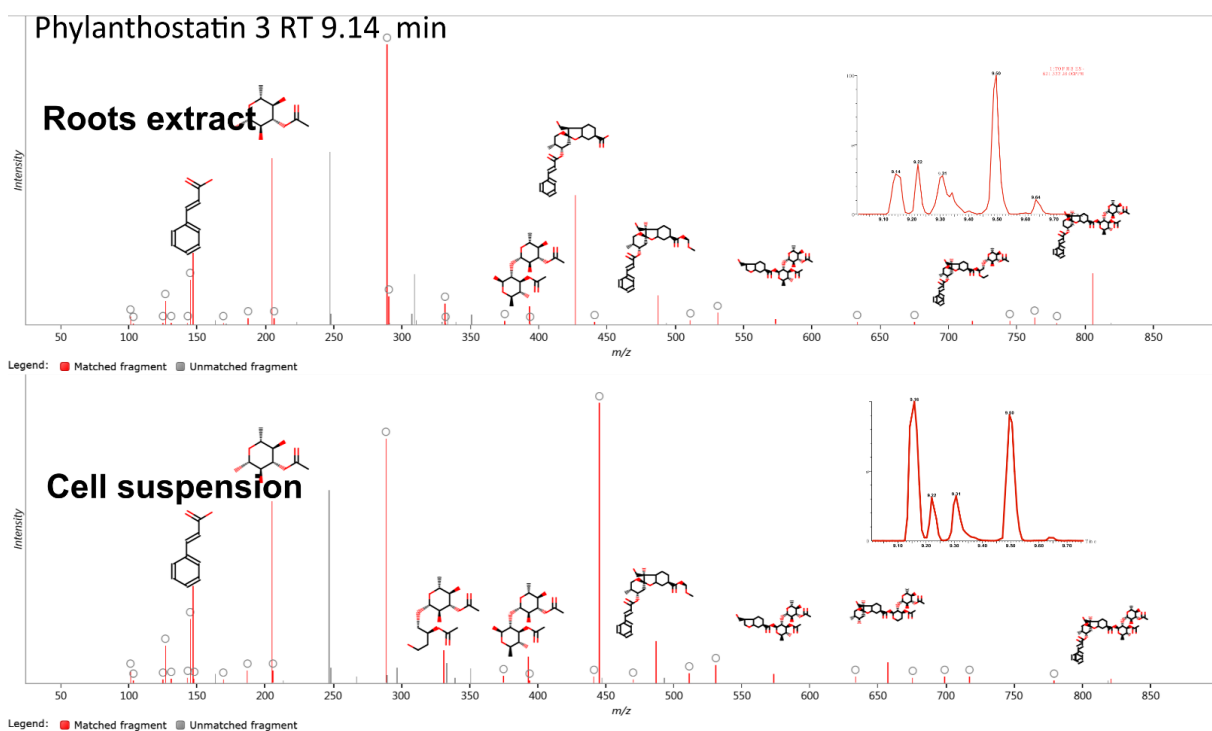


Figure S3. Extracted ion chromatograms and assigned fragmentation spectra for the compound identified as Phyllanthostatin 3 (M-H adduct). Red colored fragments represent matched fragments and their structures with mass error less than 10 ppm. Comparison of fragmentation spectra from roots and cell suspension extracts.