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Phytochemical Screening of Callus and Cell Suspensions Cultures of *Thevetia peruviana*

Dary Mendoza[1,2*]
https://orcid.org/0000-0003-0609-6480

Juan Pablo Arias[2]
https://orcid.org/0000-0001-6760-8617

Olmedo Cuaspud[2]
https://orcid.org/0000-0001-6079-9924

Mario Arias[2]
https://orcid.org/0000-0001-8190-7760

[1]Universidad del Atlántico. Facultad de Ciencias Básicas. Programa de Química. Grupo de Productos Naturales y Bioquímica de Macromoléculas. Puerto Colombia, Atlantico, Colombia. [2]Universidad Nacional de Colombia. Facultad de Ciencias. Escuela de Química. Grupo de Biotecnología Industrial. Medellin, Antioquia, Colombia.

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*Correspondence: darymendoza@mail.uniatlantico.edu.co; Tel.: +57-5- 3853002

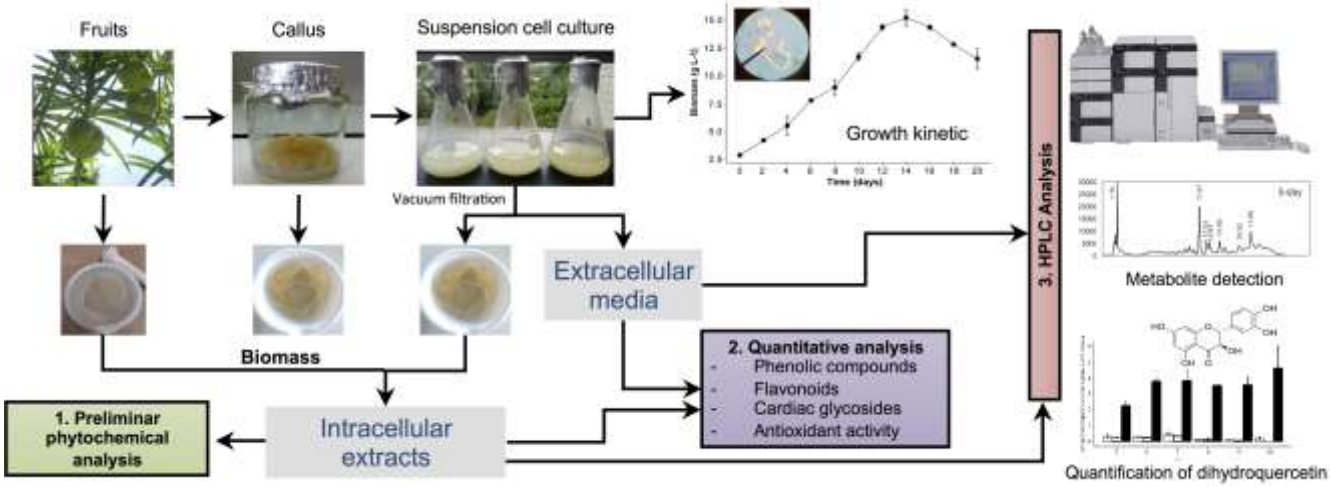
HIGHLIGHTS

- Callus and cell suspensions of *T. peruviana* have similar phytochemical profile to *in vivo* plant.
- Cell culture of *T. peruviana* is a reliable platform for high-value metabolites production.
- Cardiac glycosides and phenolic are the most valuable metabolites detected in plant cell cultures
- Dihydroquercetin production in a free and conjugated form in cell cultures is highlighted

Abstract: *Thevetia peruviana* is an ornamental shrub grown-up in many tropical region of the world. This plant produces secondary metabolites with biological properties of interest for the pharmaceutical industry. The objective was to determine the secondary metabolites profile of callus and cell suspension cultures of T. peruviana and compare them with those from explant (fruit pulp). Extracts in 50% aqueous ethanol and ethyl acetate were prepared. The phytochemical analysis was performed using standard chemical tests and thin layer chromatography. In addition, total phenolic and flavonoids compounds (TPC and TFC), total cardiac glycosides (TCG) and total antioxidant activity (TAA) was determined during the cell suspension growth. Phenolic chemical profile was also analyzed by high performance liquid chromatography (HPLC). Common metabolites (alkaloids, amino acids, antioxidants, cardiac glycosides, leucoanthocyanidins, flavonoids, phenols, sugars and triterpenes) were detected in all samples. The maximum production of extracellular TCG, TPC, TFC and TAA in cells suspensions were at 6-12 days; in contrast, intracellular content was relatively constant during the exponential grown phase (0 to 12-days). HPLC analysis detected one compound with retention time at 11.6 min; this compound was tentatively identified as dihydroquercetin, a flavonoid with anti-cancer properties. These results provide evidence on the utility of the in vitro cell cultures of *T. peruviana* for valuable pharmaceutical compounds production.

Keywords: *Thevetia peruviana*; *in vitro* cell cultures; secondary metabolites; dihydroquercetin; HPLC.

GRAPHICAL ABSTRACT



INTRODUCTION

Thevetia peruviana (Pers.) K. Schum belongs to the family *Apocynaceae* (order: *Gentianales*). It is a native to Central and South America but now it is widely spread through the tropical and subtropical regions of the world [1]. In Colombia, it can be found in the Caribbean and Andean regions, where is mainly used as ornamental plant.

T. peruviana is known for its content of cardiac glycosides compounds, such as peruvoside and thevetoside, particularly concentrated in its fruits and seeds [2,3]. The fact that these metabolites have a positive inotropic effect similar to the digoxin [4], makes the pharmaceutical industry pay special attention to them [5]. *T. peruviana* also produces phenolic compounds potentially used in the development of antimicrobial [6,7] and antineoplastic [8,9] agents. Furthermore, flavonoids with antiviral activity against the human immunodeficiency virus HIV-1 [10] have been identified in its leaves. These properties reveal the need of an ongoing source of good quality biological material for the extraction, purification and screening of relevant metabolites, as well as for research of innovative bioactive compounds.

In vitro culture of plant cells has been implemented as a strategy to increase the production of biologically valuable compounds [11]. Several metabolite groups, including alkaloids, flavonoids, polyphenols, terpenes, triterpenes and cardiac glycosides have been successfully produced in this type of cultures [12]. It has been noted, however, that certain conditions of *in vitro* culture (for example, nutritional media composition, solid and liquid medium, photoperiod and agitation) may modify the cellular metabolism by activating or deactivating the biosynthesis of some compounds or by causing chemical modifications in those previously isolated in the plant [13,14]. For this reason, it is always necessary to perform a phytochemical screening of cultures.

Production of *in vitro* cell cultures of *T. peruviana* has been registered previously [15,16] and its ability to produce cardiac glycosides and total phenolic compounds has already been proven [17-19]. However, there is little knowledge on the ability of these cultures to produce other kind of secondary metabolites. This study shows that *in vitro* cell cultures of *T. peruviana* have a similar phytochemical profile to plants cultured naturally, with the benefit of a greater production of phenolic, flavonoid and antioxidant compounds. Finally, this paper highlights the detection by HPLC of a compound tentatively identified as dihydroquercetin, flavonoid with antioxidant and anti-cancer activity in cell cultures, never registered before in *T. peruviana.*

MATERIAL AND METHODS

Reagents

All solvents used in this study were analytical grade by Merck (Darmstadt, Germany). Analytical standards HPLC grade were purchased in Sigma (Sigma Chemical, St. Louis, MO, USA).

Callus culture

The callus cultures were obtained from *T. peruviana* fruit pulp, collected at the Universidad Nacional de Colombia campus, in Medellin (6º 15' 46.8''N; 75º 34' 41.6''W). Fruits were disinfected and treated according to a previous protocol [15]. Explants were planted aseptically in SH (Shenk and Hildebrandt) medium, supplemented with 2 mg L^{-1} of 2.4-D, 0.5 mg L^{-1} of kinetin, 7 g L^{-1} of agar, 30 g L^{-1} of sucrose and 3 mg L^{-1} of myoinositol (pH 5.8), sterilized at 121 ºC and 20 psi during 20 minutes. Cultures were kept in normal photoperiod (12h light/12h dark) at room temperature (25 ± 2 ºC), carrying out subcultures every 3 weeks until obtaining friable callus.

Plant cell suspension cultures

An inoculum of approximately 10 g of friable callus was transferred to 100 mL of sterile SH medium, previously supplemented with 2 mg L^{-1} of 2.4-D and 0.5 mg L^{-1} of kinetin, 30 g L^{-1} of sucrose and 3 mg L^{-1} of myoinositol (pH 5.8), in 250 mL flasks. Cultures were kept in an orbital shaker at 110 rpm, in normal photoperiod as 12 h light/12 h dark at 25 ± 2 ºC. Subcultures were carried out every 2 weeks.

Growth kinetics

A growth curve of plant cell suspensions was performed in shaken flasks of 250 mL, using a 4-day old inoculum and 3 g L^{-1} initial concentration in 100 mL of supplemented SH medium, under the above described culture conditions. The cell suspension cultures were harvested every 2 days for 20 days, by filtration using a vacuum system and quantitative filter paper. Biomass was rinsed three times with distilled water and was dried in a convection oven at 60 ºC for 48 hours to record the constant dry cell weight. Cell growth was reported in grams of dry biomass per culture liter (g DW L^{-1}). A volume of 15 mL of culture medium and collected biomass at each time was stored at -20 ºC for the subsequent examination of intracellular and extracellular metabolites, respectively.

Phytochemical analysis

Initially, the samples (explants, callus and cells suspensions) were dried at 45 ºC for 24 hours; then they were pulverized in a mortar. Preliminary identification of secondary metabolites was done through staining and precipitation tests according to previously described protocols [20].

Extracts

Extracts were prepared in two types of solvents: ethyl acetate (EtOAc) and an aqueous ethanol solution at 50% (EtOH aq). An extraction of 0.5 g was done to each sample (dry and powdered) with 25 mL of solvent in an ultrasonic bath during 30 min at 30 ºC. Resulting homogenates were centrifuged at 3000 rpm for 15 min. Supernatants were retrieved and used to determine the metabolites.

Thin layer chromatography (TLC)

A volume of 10 mL of EtOH aq and EtOAc was concentrated in a rotary evaporator (IKA® HB10) under reduced pressure at 72 and 240 mbar, respectively. The remaining mixture was resuspended at 2 mL with the respective solvent. Subsequently, 5 µL of each extract were applied over TLC Silica gel 60 F_{254} (Merck, Darmstadt, Germany) plates, of dimensions 10 x 10 cm. The following solvent mixture was used as a mobile phase 1, ethyl acetate: methanol: water (100:13.5:10 v/v/v), and mobile phase 2, butanol: acetic acid: water (25:1:24, v/v/v). The plates were revealed with different staining reagents, following previously described protocols by [21,22].

Photo-colorimetric methods

The content of phenolic, flavonoid, cardiac glycosides compounds and the antioxidant activity were determined directly in the culture medium (extracellular metabolites) and the extracts (intracellular metabolites) as follows:

Total phenolic compounds (TPC)

These compounds were determined with the Folin Ciocalteu method [23]. Briefly, 2 mL of sample was mixed with 2.5 mL of the Folin & Ciocalteu's reagent (Sigma Chemical, St. Louis, MO, USA) at 10% (v/v). After 2 min, 2 mL of Na_2CO_3 at 7.5% (w/v) were added, following 10 min incubation at 50 ºC. The

absorbance of the reaction was measured at a wavelength of 765 nm in a Genesys 20 Spectronic spectrophotometer (Thermo Fisher Scientific). The TPC was expressed as milligrams of gallic acid equivalents per culture liter (mg GAE L^{-1}) or per gram of dry weight (mg GAE /g DW) based on a plotted standard curve (R^2 = 0.998) of gallic acid with concentrations of 80, 40, 20, 10 and 5 mg mL^{-1}.

Total flavonoid compounds (TFC)

These compounds were determined through the flavonoids-aluminum complexation method [24]. Briefly, 1 mL of sample was mixed with 0.3 mL of $NaNO_2$ at 5% (p/v). After 5 min of incubation in darkness, 0.5 mL of $AlCl_3$ at 2% (p/v) was added. The sample was stirred gently and neutralized 6 min later with 0.5 mL of 1N NaOH. After 10 min, the absorbance was registered at a 425 nm wavelength. The TFC were calculated based on a plotted standard curve (R^2 = 0.994) of quercetin with concentrations of 200, 100, 25, 12.5 and 6.25 mg mL^{-1}. Results were expressed as mg of quercetin equivalent per culture liter (mg QE L^{-1}) or per gram of dry weight (mg QE /g DW).

Total Cardiac Glycosides (TCG)

Cardiac glycosides were determined according to a previously described method [25], with some modifications. 750 µL of sample were mixed with 750 µL of recently prepared Baljet reagent (95 mL of picric acid at 1% + 5 mL of NaOH at 10%). The mixture was incubated for one hour in darkness and later diluted with 1.5 mL of distilled water. The absorbance of the reaction was measured at a wavelength of 495 nm. TCG were calculated based on a plotted standard curve (R^2 = 0.994) of peruvoside with concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 mg L^{-1}. Results were expressed as mg of peruvoside equivalents per culture liter (mg PE L^{-1}) or per gram of dry weight (mg PE /g DW).

Total Antioxidant Activity (TAA)

The TAA was determined using the ABTS radical cation decolorization assay [26]. Previously, an aqueous solution of 7 mM of ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] was prepared and the radical cation was obtained mixing equal volumes of the 7 mM ABTS solution and 2.45 mM of potassium persulfate. Three mL of the $ABTS^{\bullet+}$ diluted solution (0.70 ± 0.1 at 734 nm) were mixed with 100 µL of sample and then incubated for 10 min in the darkness. The absorbance was later measured at 734 nm using water as a blank. The TAA of each sample was calculated using a standard curve (R^2=0.99) of Trolox (Calbiochem®) with concentrations of 150, 75, 37.5, 18.8 and 9.4 mg mL^{-1}. Results were expressed as mg of Trolox equivalents per culture liter (mg TE L^{-1}) or per gram of dry weight (mg TE /g DW).

Phenolic compound analysis by HPLC-DAD

The HPLC profile of extracellular and intracellular phenolic/flavonoid compounds was determined during the exponential growth of cell suspension cultures of *T. peruviana*. With the aim of analyzing the content of intracellular aglycones, the extracts were subjected to acid hydrolysis according to a previously described procedure [27]. Briefly, 2 mL extract was mixed with 0.5 mL of an aqueous solution of 20% HCl. The mixture was heated at 85 ºC for 90 min and then allowed to cool. The volume was brought to 10 mL with EtOH aq, then filtered in 0.45 µm membranes and analyzed by HPLC. Intracellular and extracellular samples that were not hydrolyzed were also analyzed. In this case, 2 mL of each sample (extract and culture medium) were diluted up to 10 mL with EtOH aq, then filtered in 0.45 mm membranes and analyzed by HPLC.

Chromatographic analysis was carried out in Shimadzu Prominence HPLC equipment coupled to a diode array detector (SPD-M20 A) and LC Shimadzu Solution software, following a previously described protocol [19]. For data analysis, the 280 nm wavelength was selected. The retention times (t_R) and the UV–vis absorbance data of the peaks present in the samples and those obtained from analytical standards of flavonoid and phenolic compounds previously reported in *T. peruviana* [28] were compared.

Statistical analysis

All experiments were done in triplicate. Results are presented as values of the mean ± standard deviation (SD). The differences among samples were assessed through one-way ANOVA and a post hoc test (Tukey's honest significance test) with a significance level of 0.05, using the statistical software RStudio, version 1.1.383.

RESULTS

In vitro plant cells culture

The process of friable callus production lasted approximately 4 months. Cell suspensions were obtained from this biological material. The growth kinetic started with a 4-day old inoculum (after *lag* phase) and the exponential growth concluded on the 12th day reaching a maximum of biomass production of 14.26 ± 0.71 g L^{-1} (Figure 1). This result was consistent with the one reported previously for this suspension cell culture [15,17].

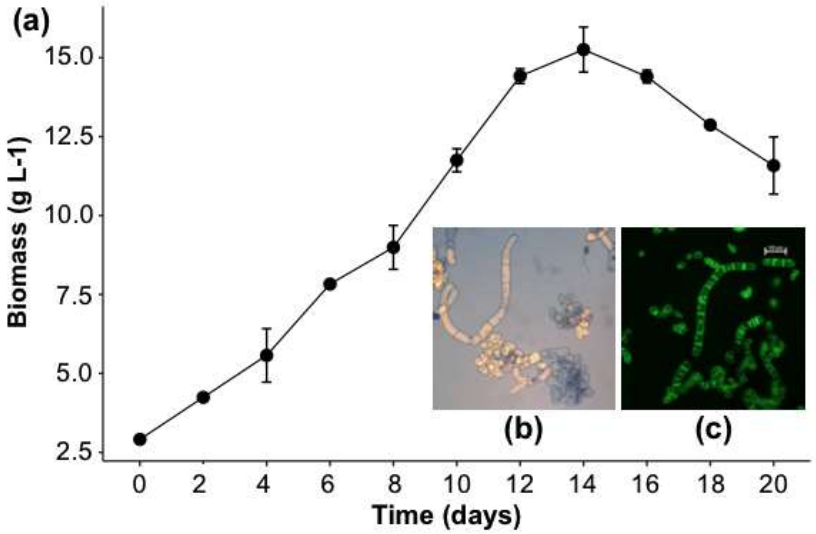


Figure 1. Growth curve (a) of plant cell suspension culture of T. peruviana. Cells stained with Evan's blue (b) and fluorescein acetate (c). Results are average ± SD of three individual experiments.

Phytochemical analysis

The qualitative determination of secondary metabolites was carried out for EtOH aq extracts of explants, callus and cell suspensions. Table 1 shows the main metabolite families detected. A high level of correspondence was observed among explants, callus cultures and cell suspensions results; there were only two families of metabolites - saponins and tannins - which were not detected in the cell cultures (callus and cell suspensions). Furthermore, coumarins were detected in cell cultures, but not in the explants.

Table 1. Preliminary phytochemical screening of explants, callus and cell suspensions of *T. peruviana*. Results are presented as presence (+) or lack (-) of metabolites.

Metabolite group	Assay	Explants	Callus	Suspension
Alkaloids	Dragendorff	+	+	+
	Mayer	+	+	+
Amino acids	Ninhydrin	+	+	+
Flavonoids	$AlCl_3$	+	+	+
Phenolics	$FeCl_3$	+	+	+
Saponins	Foam test	+	-	-
Triterpenes, sterols	Liebermann–Burchard	+	+	+
Cardiac glycosides	Baljet´s reagent	+	+	+
Coumarins	NaOH 5%	-	+	+
Sugar	Keller Killiani	+	+	+
Leucoanthocyanidins	HCl - Amyl alcohol	+	+	+
Tannins	Gelatin-NaCl	+	-	-

TLC

This analysis revealed intracellular triterpenes and steroids production mainly in the EtOAc extracts of all samples; while phenolic, flavonoid and antioxidant compounds were mostly detected in EtOH aq extracts. Cardiac glycosides were detected in both extracts. In addition, differences were observed in the TLC profile between explants and *in vitro* cultures of *T. peruviana*. Comparison of the retention factor (R_f) suggests the presence of peruvoside and two triterpenoids (oleanolic and ursolic acid) in all samples (*Supplementary file*).

Determination of TPC, TFC, TCG and TAA

Table 2 presents the results of metabolites production in EtOH aq extracts in explants, callus and cell suspension cultures. The TPC, TFC and TAA were higher in cell suspensions compared to explants and callus; on the contrary, the TCG content was up to three times higher in the explants. In turn, when cell suspensions were compared, non-significant differences were observed in the content of the compounds assessed (value $p > 0.05$) during exponential growth (day 0 to 12). After day 12, a slight increase in the content of metabolites occurred.

Figure 2 presents the results of the extracellular metabolites production for cell suspension cultures. The highest content of phenols and flavonoids occurred in day 8 (59.08 ± 4.4 mg GAE L^{-1}) and day 10 (128.25 ± 27.13 mg QE L^{-1}), respectively. The highest level of cardiac glycosides was produced in day 6 (449.26 ± 54.21 mg PE L^{-1}). The maximum antioxidant activity occurred in day 12 (267.65 ± 12.24 mg TE L^{-1}). These results reveal that during exponential growth the cells produce a higher concentration of phenols, flavonoids, cardiac glycosides and antioxidants at the extracellular level, which constitutes a benefit for subsequent separation and purification processes of relevant metabolites.

Table 2. Content of phenolic compounds (TPC), flavonoids (TFC), cardiac glycosides (TCG) and antioxidant activity (TAA) in ethanol extracts from explants, callus and cell suspensions (c.s) of *T. peruviana*. Results are average ± SD of three individual experiments.

Samples	**TPC** (mg GAE /g DW)	**TFC** (mg QE /g DW)	**TCG** (mg PE /g DW)	**TAA** (mg TE /g DW)
Explants	2,49 ± 0,44^{b}	0,48 ± 0,18^{d}	6,39 ± 0,29^{a}	3,58 ± 0,24^{b}
Callus	3,50 ± 0,34^{a}	1,51 ± 0,29^{cd}	1,49 ± 0,01^{cd}	7,29 ± 0,11^{ab}
0-day (c.s)	2,52 ± 0,01^{b}	1,41 ± 0,01^{bcd}	0,97 ± 0,01^{de}	7,29 ± 0,02^{ab}
2-day (c.s)	3,23 ± 0,48^{ab}	1,66 ± 0,30^{bcd}	0,91 ± 0,04^{e}	10,63 ± 4,14^{a}
4-day (c.s)	3,70 ± 0,11^{ab}	1,74 ± 0,07^{bcd}	1,20 ± 0,11^{cde}	10,11 ± 0,29^{a}
6-day (c.s)	3,38 ± 0,09^{ab}	2,04 ± 0,27^{bc}	1,59 ± 0,02^{e}	9,83 ± 0,87^{a}
8-day (c.s)	3,65 ± 0,31^{ab}	1,83 ± 0,08^{bcd}	1,33 ± 0,06^{cde}	10,24 ± 0,33^{a}
10-day (c.s)	3,66 ± 0,24^{ab}	1,95 ± 0,60^{bc}	1,29 ± 0,04^{cde}	10,85 ± 2,18^{a}
12-day (c.s)	3,30 ± 0,34^{ab}	2,33 ± 0,33^{abc}	1,20 ± 0,04^{cde}	10,27 ± 0,20^{a}
14-day (c.s)	4,20 ± 0,36^{a}	2,67 ± 0,22^{ab}	2,25 ± 0,10^{b}	10,79 ± 0,38^{a}
16-day (c.s)	4,57 ± 0,39^{a}	2,87 ± 0,34^{ab}	1,14 ± 0,03^{cde}	10,86 ± 1,80^{a}
18-day (c.s)	4,57 ± 1,31^{a}	3,58 ± 0,47^{a}	0,99 ± 0,05^{de}	11,18 ± 0,23^{a}
20-day (c.s)	4,85 ± 0,37^{a}	3,77 ± 0,13^{a}	0,91 ± 0,09^{e}	10,54 ± 0,67^{a}

Different letters indicate the statistically significant difference according to the Tukey's honest significantly test at $p < 0.05$.

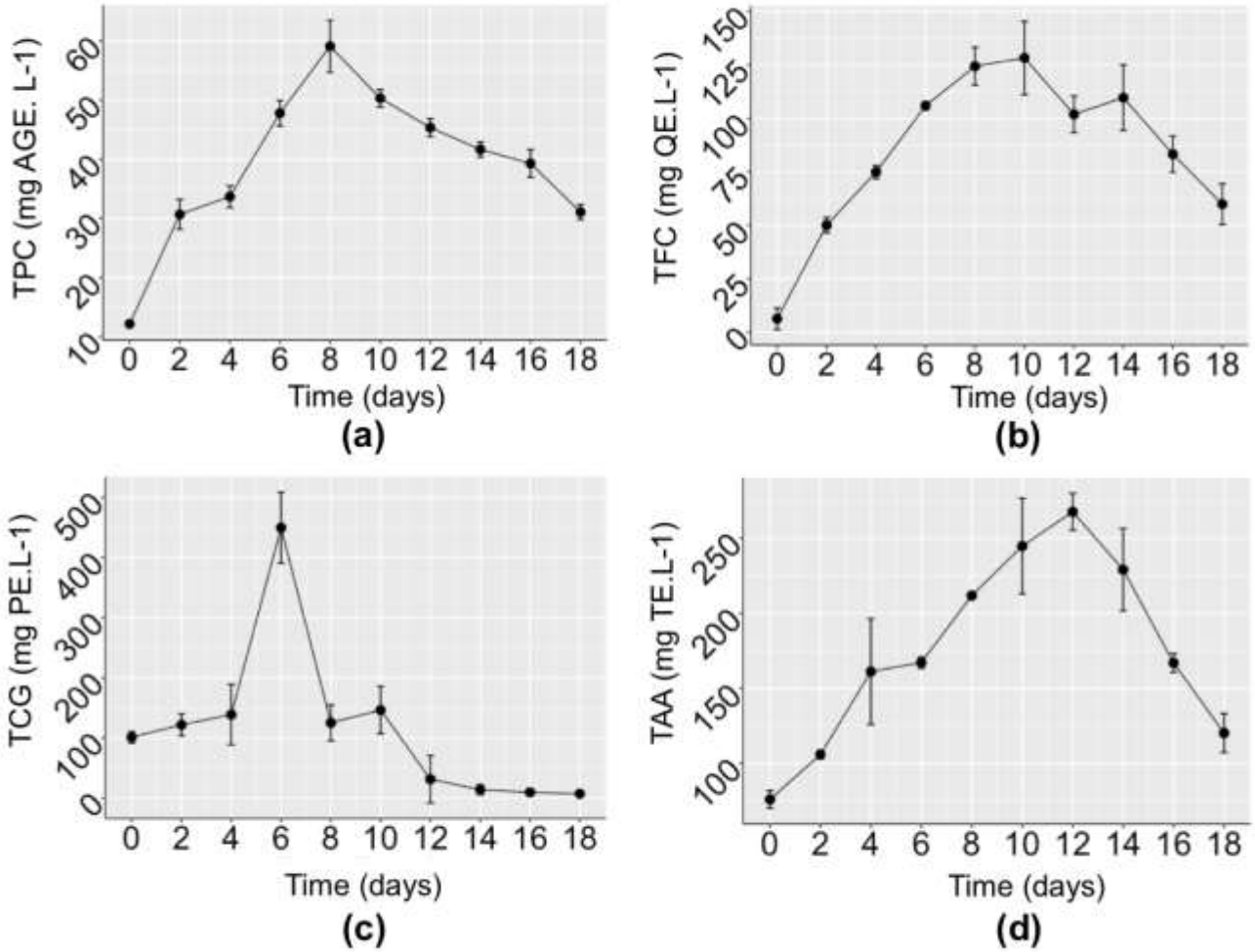


Figure 2. Extracellular content of total phenolic compounds (a), total flavonoids (b), total cardiac glycosides (c) and total antioxidant activity (c) in cell suspension culture of *T. peruviana*. The results are average ± SD of three individual experiments.

Phenolic compounds analysis by HPLC

The analysis by HPLC was used to recognize extracellular and intracellular phenolic/flavonoid compounds in cell suspensions of *T. peruviana.* Figure 3 shows the chromatograms of the samples between days 5 to 10 of exponential growth.

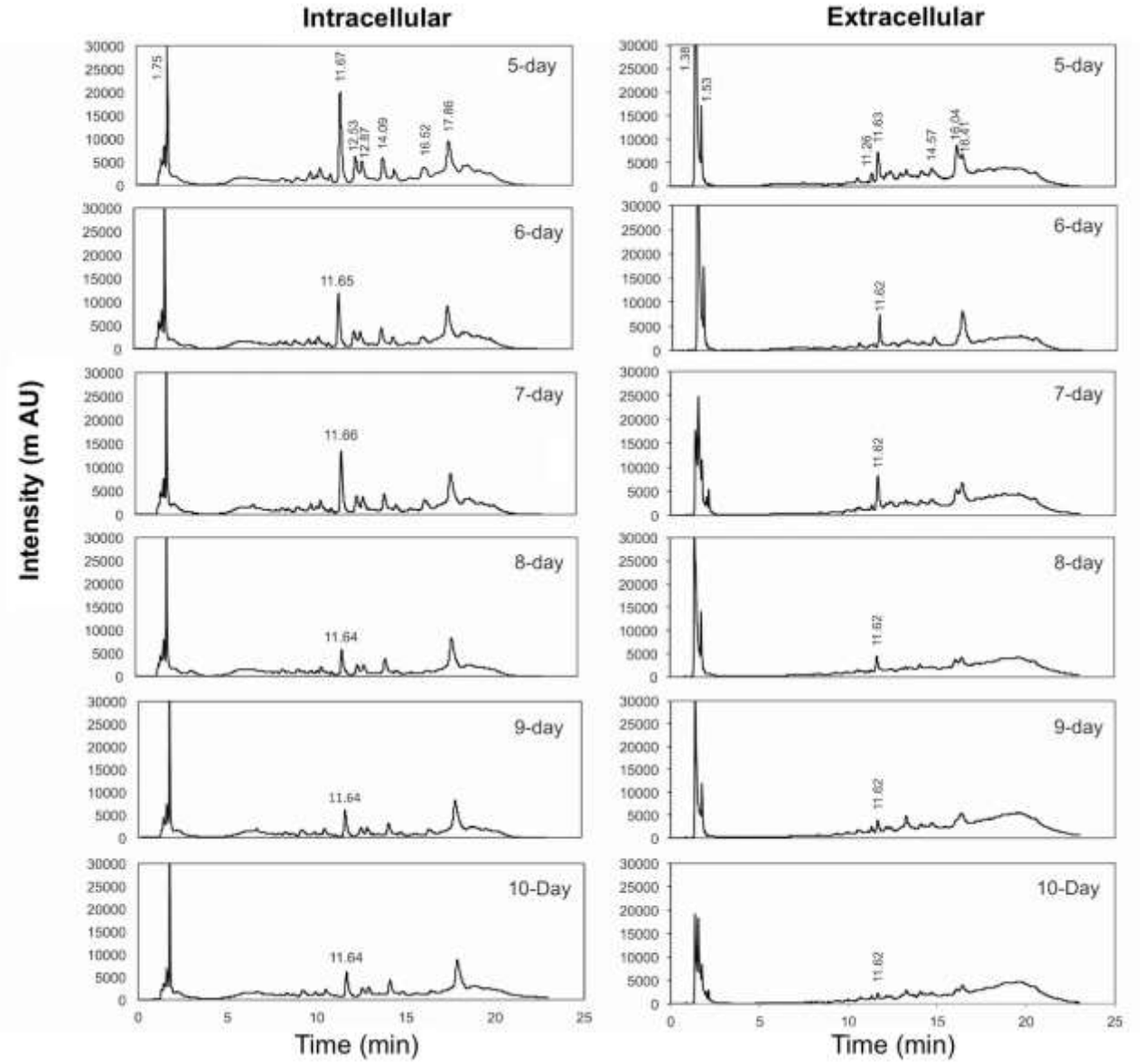


Figure 3. Chromatographic profile (HPLC) at 280 nm of phenolic compounds in intracellular extracts in EtOH aq and the culture medium (extracellular) of the cell suspensions of *T. peruviana*, during the exponential growth phase (day 5 to 10 of exponential grown).

There is clearly an evidence of differences in the chromatographic profile between intracellular and extracellular samples, observing only one common peak with t_R at 11.6 min. The intensity of this peak decreased progressively in the samples as the culture time passed. Comparison of t_R and maximum absorbance (Ab_{max}) of peaks detected in the samples and the standards, showed that this peak would possibly correspond to dihydroquercetin (t_R = 11.6 min; Ab_{max} = 220/237/280) (Figure 4). The other flavonoids previously reported in fruits and leaves of *T. peruviana* were not detected in the cell suspension cultures, possibly due to the low concentration of those compounds in the samples.

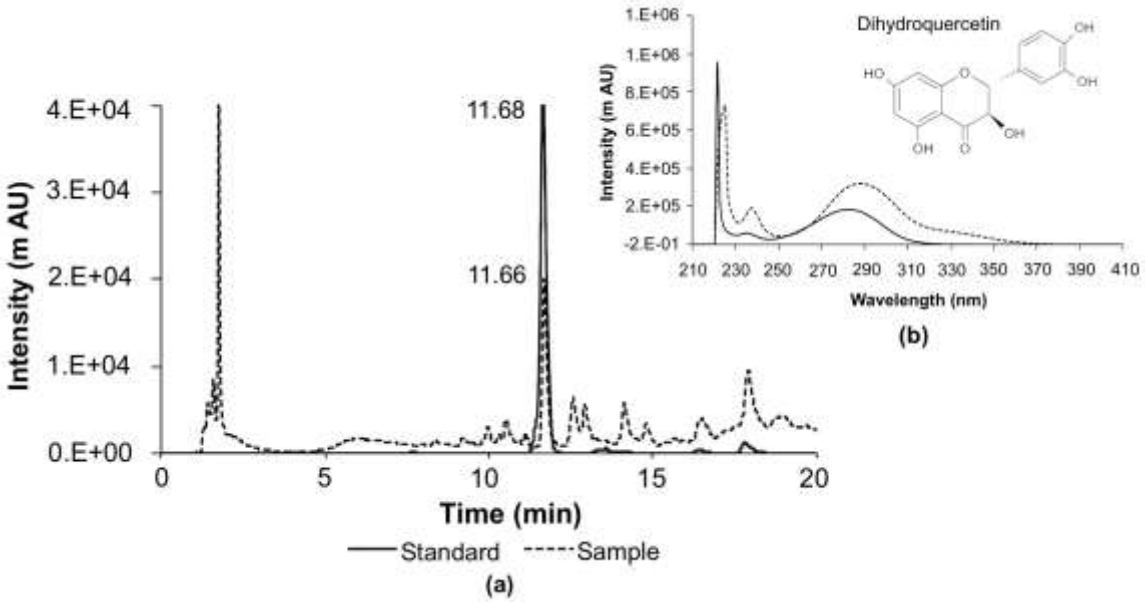


Figure 4. Comparison of the chromatogram at 280 nm (a) and the UV/vis absorption spectrum (b) of an EtOH aq extract of *T. peruviana* cells harvested on day 5 of culture and the dihydroquercetin standard.

The analysis of hydrolyzed intracellular extracts shows two major peaks of 280 nm; the first corresponds to an unidentified compound with t_R = 5.6 min and the second one to dihydroquercetin (t_R = 11.6 min). Moreover, four minor peaks were also detected which were not identified, with t_R 1.41, 1.76, 7.04 and 9.82 min (Figure 5).

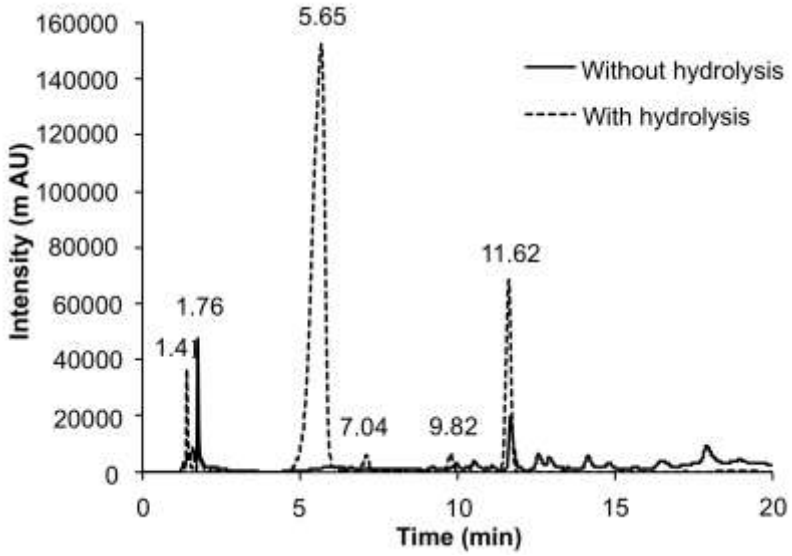


Figure 5. Comparison of chromatograms (before and after hydrolysis with HCl, 20%) of ethanol extracts of suspension cell of *Thevetia peruviana* at day 5 exponential grown. Retention times (t_R) of relevant peaks are shown.

Dihydroquercetin quantification

This compound was quantified by HPLC in the culture medium (extracellular) and the intracellular extracts (hydrolyzed and not hydrolyzed) of cell suspensions, based on a standard curve (R^2 = 0.999) of dihydroquercetin with concentrations from 6.25 - 100 µg mL^{-1}. Between day 5 and day 10 of exponential growth, 2.88 and 4.83 mg of dihydroquercetin total/100 mL in cell suspension was obtained. The conjugated dihydroquercetin was the main intracellular form of this compound in cell suspensions (Figure 6).

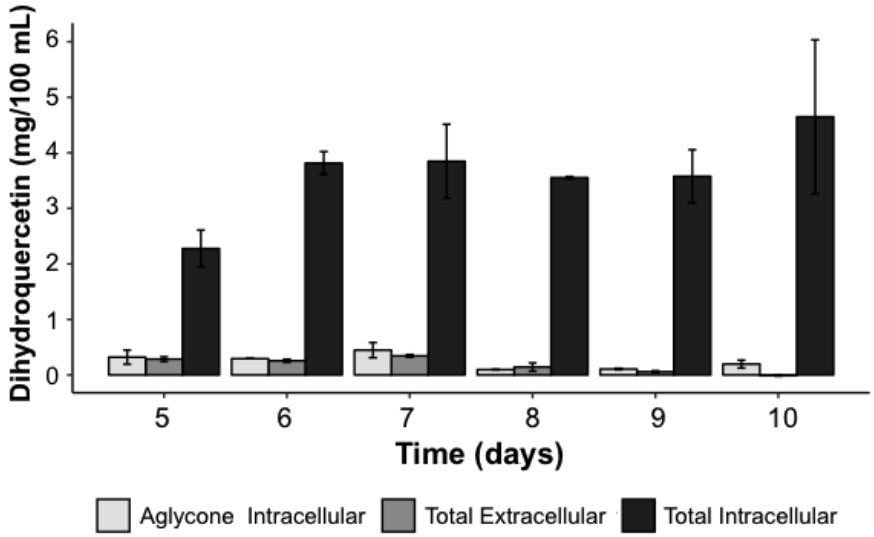


Figure 6. Amount of dihydroquercetin in suspension cell of *Thevetia peruviana.* Results are average ± SD of three individual experiments.

DISCUSSION

In vitro culture of plant cells is an attractive alternative for the production of secondary metabolites of high economic value. This study was able to establish that *T. peruviana* cell cultures (callus and cell suspensions) present a very similar phytochemical profile to naturally cultured plants [29]. Interestingly, secondary metabolites that represent a higher biomedical interest in *T. peruviana* (cardiac glycosides, phenols and flavonoids) were detected from callus and cell suspensions. Furthermore, presence of coumarins was identified in cell cultures, but not in explants (fruit pulp). These compounds, as well as phenols and flavonoids, are derived biogenically from shikimic acid [30], something that confirms the activation of this pathway is presented on *in vitro* cultures.

Triterpenoids are other metabolites family of *T. peruviana* with interesting biological properties. By using TLC analysis and specific standards the study was able to detect oleanolic and ursolic acids in the explants and cell cultures. These triterpenoids compounds are registered as significant antibacterial, antiviral, antiulcerative and anti-inflammatory agents [31], which is why their presence in *in vitro* cultures is of great relevance.

A production of cardiac glycosides was also observed; specifically, peruvoside, detected through TLC. This compound is one of the cardiac glycosides in *T. peruviana* that has the highest demand, due to its cardiovascular effects and to recent findings which suggest it acts as an anti-leukemic [32] and anti-tumor against triple negative and ER+ (estrogen receptor positive) breast cancer cells [33]. The detection of peruvoside in the cell line used in these experiments was previously reported [15], showing its biosynthesis pathway stability in the cultures.

Quantitative analysis of metabolites showed a significant increase in the intracellular content of phenolic compounds and flavonoids, in cell suspensions compared with explants. This increase could be attributed to cellular stress factors during *in vitro* culture, such as light, photoperiod, agitation and pH of the culture medium, which could trigger the phenylpropanoids biosynthesis pathway. Especially, plant phenolics are considered to have a key role as defense compounds against environmental stresses [34], being naturally synthesized when plants are cultured in *in vitro* conditions. In contrast, cell suspensions of *T. peruviana* showed a significantly lower intracellular cardiac glycosides content compared to explants; similar results were previously described in *Digitalis sp*, where low levels of cardiac glycosides were found in callus and suspension cultures without morphogenesis, even if potential precursors of these compounds were administered to the cultures [35]. Other studies suggest that the biosynthesis of cardiac glycosides in plant cell cultures requires the formation of morphological structures, such as embryoid cells (and probably non-embryoid green cells) [36]. Although the complete route of cardiac glycosides biosynthesis in plants is incomplete to date, a recently transcriptomic study in *Calotropis procera* (Asclepiadaceae), demonstrated that there is a specific tissue expression of the transcripts involved in the biosynthesis of this compounds [37], which would explain the need of morphological structures for production enhancement. At the same time, culture conditions such as light intensity and the absence of some minerals in the media (e.g. calcium

and magnesium) have been positively associated with increased accumulation of cardiac glycosides in callus culture of *Digitalis sp* [38]. According to the above, different strategies related to culture environment, media composition or embryoid cell induction could be explored to increase the cardiac glycosides accumulation in *T. peruviana* cell culture.

On the other hand, a high content of cardiac glycosides, phenolic and flavonoid compounds were observed at extracellular level in the cell suspensions. Several studies have shown that these metabolites groups may undergo biotransformation reactions in the cell cultures, which determine their transportation, storage and excretion. Cardiac glycosides [39] and phenolic/flavonoid compounds [13,40] may be hydroxylated, esterified and glycosylated after their biosynthesis, for their later storage in vacuoles. However, non-glycosylated forms (aglycones) are not stored in vacuoles hence they diffuse rapidly through the membrane, explaining their presence in the culture medium. In the case of cell suspensions of *T. peruviana*, it is clear that during exponential growth a progressive release of metabolites takes place in the culture medium, until reaching a maximum that varies depending on the type of compound. The subsequent decrease could be explained for any of the following events: 1) compounds are regained, bio-transformed and stored into cells; 2) their biosynthesis is reduced by precursors exhaustion; or 3) they are degraded in the medium of the culture.

Esterified, hydroxylated and glycosylated flavans and flavanols, such as apigenin-5-methylether [41], glycosylated dimethoxyflavanones [42], sinapoyl and feruloyl esters of kaempferol and quercetin [10] have been identified in *T. peruviana* plants. These compounds are antioxidants [43] and some of them retain inhibitory activity of reverse transcriptase and integrase enzymes of HIV-1 [10]. This study was not able to identify aglycones of these compounds possibly due to their low concentration in the samples. However, in suspension cultures a compound tentatively identified as dihydroquercetin was detected. Diydroquercetin is a dihydroxiflavonol with potent antioxidant activity and promising therapeutic properties in chronic grade inflammatory states such as cancer, cardiovascular and hepatic diseases [44-46]. This compound, also known as taxifolin, has been found in other species of the Apocynaceae family, such as *Trachelospermum jasminoides* [47,48]. Dihydroquercetin is a natural precursor of quercetin in plants. Quercetin biosynthesis is catalyzed by the flavonol synthase (FLS, EC 1.14.11.23), a non-hemic ferrous enzyme that belongs to the family of 2-oxoglutarate-dependent dioxygenases, which catalyzes the formation of a double bond between the C-2 and C-3 carbons of dihydroquercetin. FLS also exhibits flavanone 3-hydroxylase (F3H) activity, accepting flavanones as substrates for the dihydroxyflavonols biosynthesis, thus providing a connection route between flavanones and flavonols [49]. Therefore, identification of FLS/F3H (e.g. through transcriptomic studies) would be a continuation of the present study that would contribute to the knowledge of the dihydroquercetin metabolic pathway in *T. peruviana* cell cultures.

CONCLUSION

These results represent a step forward towards secondary metabolites screening produced through *in vitro* cultures of *T. peruviana,* particularly in cell suspensions. The study was able to prove that the metabolic pathways responsible for metabolites biosynthesis of pharmaceutical interest are active in cell suspension cultures. Future studies will aimed at screening, increase and stabilization of bioactive metabolite production present in cultures.

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