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Establishment of cell suspension culture with anti-inflammatory activity and the effect of salicylic acid on the production of callus from *Cnidoscolus chayamansa* Mc Vaugh

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ABSTRACT

***Cnidoscolus chayamansa* Mc Vaugh (Euphorbiaceae), known as Chaya, Mexican spinach or Mayan spinach, is of interest for its high nutraceutical value and broad medicinal uses. Previously, it has been described that *C. chayamansa* has several biological activities (antiprotozoal, antimycobacterial, gastroprotective, cardioprotective, antioxidant, hypoglycemic, anti-inflammatory and hepatoprotective) and from active extracts moretenol, moretenyl acetate, β-amirenone, β-amyrin acetate, moretenone, lupeol acetate, scopoletin, 3,7-dimethyl ether of kaempferol and 5-hydroxy-7-3', 4'-trimethoxyflavanone have been isolated. In this work, the biotechnology procedure to obtain callus and cell suspension using a combination of auxin [NAA (2.5 mg/L)], cytokinin [BAP (5 mg/L)] and sucrose (30 g/L) as a carbon source is described. In addition, high-resolution liquid chromatography (HPLC) analysis of the organic extracts from cell line showed that it contains scopoletin (3.68 mg/g dry extract); while by thin layer chromatography (TLC) analysis moretenol acetate (minor compound), β-sitosterol, *p*-cumaric acid, cinnamic acid and kaempferol were identified. The organic extract from cells was inactive in the topical inflammation (TPA) and systemic (carrageenan) models. Finally, elicitation with salicylic acid was performed, showing that callus formation was in a shorter time (28 days) than the crop with plant growth regulators (50 days) with a similar percentage of callogenesis of 77.77 and 75%, respectively. This research can be a pioneer for the production of metabolites of biological interest from this medicinal plant.**

INTRODUCTION

Cnidoscolus chayamansa Mc Vaugh (Euphorbiaceae) is known as chaya, Mexican spinach or Mayan spinach, and grows mainly in the Mayan region of Guatemala, Belize, in the Southeast of Mexico (Yucatan Peninsula) and some parts of Honduras (Ross-Ibarra, 2003). It is widely used as food in diet of some native communities for its high nutraceutical value. Chaya leaves have been reported to have a higher nutrient content than *Spinaceae oleraceae* (spinach), with high levels of proteins, fiber, minerals (calcium, potassium, iron), vitamin C and carotene (Kuti and Torres, 1996). In Mexico, it is used in traditional medicine for the treatment of several diseases, such as diabetes mellitus II,

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Table 1. Anti-inflammatory activity of *C. chayamansa* leaf extracts in the inflammation model induced with TPA and carrageenan.

Extract	Inhibition (%)	ED_{50}	Model	Reference
Hex EtOAc EtOH	30.76% (2 mg/ear) 32.04% (2 mg/ear) 31.90% (2 mg/ear)	>2 mg/ear	TPA/Mouse CD1	García-Rodríguez et al. (2014)
EtOAc	30.29% (5^a h)	>500 mg/kg	Carrageenan/ Mouse CD1	
$CHCl_3$:MeOH (1:1)		1.66 mg/ear and 1.58 mg/ear 467.73 and 351.53 mg/kg	TPA/Mouse BALB/c Carrageenan/Mouse BALB/c	Pérez-González et al. (2017, 2018a)

rheumatism, gastrointestinal disorders, weight control, and diseases related to inflammation and vaginal infections (García-Rodríguez et al., 2014, Pérez-González et al., 2016, 2017, 2018a).

Wild species showed antimycobacterial, gastro-protective, antioxidant, cardioprotective, hypoglycaemic, antiprotozoal, anti-inflammatory (in acute and chronic models) and hepatoprotective activites (García-Rodríguez et al., 2014; Mena et al., 2017; Pérez-González et al., 2017, 2018a, 2019a; Pillai et al., 2012; Valenzuela-Soto et al., 2015, 2019). The $CHCl_3$:MeOH extract (1:1) from aerial parts showed an important acute anti-inflammatory activity (TPA model) with an medium effective dose (ED_{50}), similar to the reference drug, Indomethacin -Ind- (Table 1). From the active extract, the main terpenes identified as moretenol, moretenil acetate, β-amyrenone, β-amyrin acetate, moretenone and lupeol acetate (LA) were isolated, together with other minor compounds. These pure compounds also showed significant anti-inflammatory activity in TPA and carrageenan models. Other isolated compounds were 3,7-dimethyl ether of kaempferol and 5-hydroxy-7-3',4'-trimethoxyflavanone; these compounds were also active in both models (Pérez-González et al., 2017; 2018a). In addition, this extract showed significant anti-inflammatory activity in a chronic inflammation model (Complete Freud's Adjuvant -FCA-); the extract at 200 mg/kg, was administered by intragastric (i.g.) via during 28 days and it inhibited 45.33% of inflammation process and phenylbutazone (reference drug) inhibited 37.49%; but the extract at higher dose (450 and 900 mg/kg) showed a moderate inhibition (~35%) (Pérez-González et al., 2018a). Also, the hepatoprotective effect has been described for *C. chayamansa* EtOH extract against the hepatic damage caused by isoniazid/rifampicin (INH/RIF) mixture (Pillai et al., 2012). Recently, it was reported that the $CHCl_3$:MeOH extract (1:1) at 200 and 400 mg/kg showed hepatoprotective effect against the damage induced with RIF/INH/pyrazinamide (PZA) mixture, when was administered by i.g. via for 39 days. This extract at both doses favored body weight gain with respect to the positive control (silymarin), and anti-TB group with liver damage (induced by INH/RIF/PZA); in addition, the animals that received the extract at 400 mg/kg showed no steatosis and the 200 mg/kg group showed moderate steatosis, similar effect to silymarin (Pérez-González et al., 2018a). On the other hand, in a recent paper, β-D-glucopyranoside,(1*R*)-O-isopropyl-6-O-(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-2,3,4-triacetate was reported for the first time; in addition, they reported the scopoletin quantification in crude extract (0.19 mg/g of dry extract); these last compounds were isolated from $CHCl_3$:MEOH extract of *C. chayamansa* stem. This last paper also described the hepatoprotective activity against damage induced with RIF//INH/PZA (50:50:100 mg/kg) administered daily during 85 days at 200 and 400 mg/kg, and silymarin (2.5 mg/kg) was used as reference. The extract (at both doses) favored body weight gain and decreased the SOD and CAT values with respect to the anti-TB group and this extract decreased steatosis in liver (Pérez-González et al., 2019a).

Pérez-González et al. (2018b) described the biotechnology conditions to obtain callus cultures from leaf explants of *C. chayamansa*, using different concentrations and combinations of plant growth regulators (PGR); in this case, 6-benzylaminopurine (BAP) or kinetin (KIN) and 2,4-dichlorophenoxyacetic acid (2,4-D) was used in culture medium Murashige and Skoog (MS) and sucrose as carbon source. The highest callus yield was observed when using the combination of BAP (5.0 mg/L) and 2,4-D (2.5 or 5.0 mg/L) in MS medium with sucrose. This same author, González et al. (2019b) reported that, from immature leaves of *C. chayamansa* in MS culture medium with D-glucose (as a carbon source), and using different combinations of auxin [2,4-D; α-naphthalene-acetic acid (NAA); Indole-3-acetic acid (IAA)] and two cytokinins (BAP and KIN), it was found that callus induction was 25 and 100%. NAA (2.5 mg/L) + BAP (5 mg/L) were the best combination; with this PGR combination a cell suspension was obtained with the best phenotypic characteristics (friability, color and conditions), cell growth kinetics was performed and

at the same time, the production of Lupeol acetate (LA, main compound) was quantified by high-resolution liquid chromatography (HPLC). LA quantification was carried out in the $CHCl_3$:MeOH extract (1:1) from suspension culture cells. The authors reported that the highest production of LA was obtained at day 40 of culture, with a content of 38.1 mg of LA/g of dried cell extract. The content of LA in vegetal material obtained by biotechnological process was greater with respect to the wild material (being 2.9 mg of LA/g of dry extract). In addition, the $CHCl_3$:MeOH extract (from wild material) showed moderate antioxidant activity with medium effective concentration (EC_{50}) = 16.60 and 4.95 mg/mL by DPPH and ABTS methods, respectively, and showed scarce activity against *Staphylococcus aureus, Listeria monocytogenes* and *Staphylococcus coagulase* (evaluated by the Kirby-Bauer method) (Pérez-González et al., 2019b). Other compounds detected in this extract from wild and biotechnological material of these cells from the suspension culture were scopoletin and β-sitosterol. Plant cell culture is an efficient technology for secondary metabolite production; however, there are certain problems in their production, such as a delay in the response time in cell formation, although an alternative to this problem is elicitation (Khan et al., 2019). Elicitation is an efficient biotechnological method to promote the defense mechanism in plants and to improve the quantity and quality of secondary metabolites in *in vitro* cultures (Taghizadeh et al., 2019).

Continuing with this research, in this manuscript we describe the anti-inflammatory activity of the $CHCl_3$:MeOH extract (1:1) from suspension cell culture obtained by biotechnological processes using the combination of NAA (2.5mg/L) + BAP (5.0 mg/L), in MS medium plus sucrose as a carbon source, and their metabolic content determined by HPLC and thin layer chromatography (TLC). Elicitation with salicylic acid in leaf explants for callus formation is also described, monitoring its time of response, morphology and identification of major metabolites present in the callus extract ($CHCl_3$:MeOH).

MATERIALS AND METHODS

Plant material

C. chayamansa was collected in the Miguel Hidalgo Delegation, Mexico City, Mexico, on January 16, 2016. A voucher (16252) was deposited in the Herbarium of the Mexican Institute of Social Security (IMSSM). Additionally, three plants (0.6 m in height) that were kept in the greenhouse of the Autonomous Metropolitan University Iztapalapa (UAM-I) were used. From these plants, leaf explants were obtained for the *in vitro* culture experiments.

Leaf explant obtention in aseptic condition

The immature leaves were cut into segments of 1 cm^2, later the methodology described by Pérez-González et al. (2018b) was used. With this methodology, 100% asepsis of the explants was achieved, which were used for callus formation.

Induction of callus and incubation conditions

The aseptic segments of the leaf were placed in culture tubes containing 15 mL of MS culture medium (Murashige and Skoog, 1962) supplied with sucrose (30 g/L) and two PGRs to promote the callus induction: an auxin (NAA, at 2.5 mg/L) and a cytokinin (BAP, at 5 mg/L). The pH of the culture medium was adjusted to 5.7 ± 0.1, Phytagel (2 g/L) was added, 20 mL this medium was poured into culture tubes (150 × 20 mm) and sterilized at 121°C and 15 psi during 18 min. Once the aseptic leaf segments were inoculated in the culture medium, they were incubated at 26 ± 2°C under fluorescent white light (50 µmol * m/s), in photoperiod of 16 h of light/8 h of darkness. After 40 days of incubation, the percentage of induction of callus was obtained with respect to the total explants used.

Callus that showed better friability and were visually healthy were selected to be sub-cultured every 40 days in fresh medium. The maintenance stage was carried out in six cycles of subcultures (each 40 days), observing that the phenotypic characteristics (color and friability) were similar. When the phenotypic characteristics of the callus were constant, it was considered as a suitable cell line and was named with the key ANB.

Establishment of cell suspension cultures

The fresh biomass (3 g) from ANB cell line was transferred to 250 mL Erlenmeyer flasks containing 50 mL of MS liquid medium, supplemented with sucrose (30 g/L), NAA (2.5 mg/L) and BAP (5 g). mg/L) and were incubated under the conditions described above, with stirring of 110 rpm on a rotary shaker (Prendo AGO 60-40). In addition, these suspension cultures were sub-cultured every 30 days and five cycles for the proliferation of biomass were carried out. During these cycles, the phenotypic characteristics were monitored.

Preparation of organic extract phytochemical profile

From 1.2 g of dry biomass (ANB cell), the extract $CHCl_3$:MeOH (1:1) was prepared by maceration process at room temp. From this process, 346.3 mg of crude extract was obtained, with a yield of 28.85%, key ANB,

and was stored at ambient temp. From this extract, the phytochemical profile for the detection of polyphenols, terpenes and sterols was carried out using the methodology previously described (Pérez-González et al., 2017; 2018b). In addition, the phytochemical profile was analyzed by HPLC, using the conditions to detect terpenes, scopoletin and flavonoids described by Pérez-González et al. (2019b).

Determination of anti-inflammatory activity *in vivo*

Male Balb/c mice (23 ± 2 g) were used, which were kept under standard laboratory conditions (25°C, 12/12 h light/dark, 50% relative humidity) according to the Official Mexican Standard (NOM-062-ZOO-1999) revised in 2016. Food and water were provided *ad libitum*.

Determination of anti-inflammatory activity *in vivo*

12-O-tetradecanoylphorbol-13-Acetate (TPA) induced ear edema

All test groups (n = 5) received 2.5 µg of TPA dissolved in acetone in the right ear, while in the left ear only acetone was applied; 30 min later, the Ind (drug reference) and the ANB extract were applied to the right ear at the dose of 2 mg/ear (dissolved in acetone). The anti-inflammatory activity was calculated according to the weight difference of ear sections (6 mm) at 6 h, and the inhibition percentage was calculated according to the methodology described by Gutiérrez-Rebolledo et al. (2018).

Acute systemic inflammation induced with carrageenan

Paw edema was induced by subcutaneous injection (s.c.) of 20 µL of 2% carrageenan in saline solution. One hour before the carrageenan injection, the treated groups (n = 5) were administered with Ind (10 mg/kg, reference drug) and the ANB extract (200 mg/kg) i.g. The samples were solubilized in Tween 80:H_2O (1:9) and the control group received only the vehicle. The percentage of inhibition was calculated with the formula described by Pérez-González et al. (2017).

Preparation of elicitor

The salicylic acid (SA) (1 mM) was dissolved in 1 L distilled water, followed by continuous stirring for 1 h for proper mixing of SA (Kang et al., 2019).

Callus establishment whit elicitor (SA)

Explants of approximately 1 cm^2 were used and were kept aseptic according to the methodology described by (Pérez-González et al., 2019b), placed subsequently in MS medium containing 30 g/L sucrose, 3 g/L agar, and fortified with several SA concentrations (25, 50, 100 µM) (Kang et al., 2019). The cultures were put to growth room and maintained properly at temp 26 ± 2°C under fluorescent white light (50 µmol × m/s), in photoperiod of 16/18 h of light/darkness. They were recorded on days 8, 14 and 28 of incubation, the percentage of induction of callus was obtained with respect to the total explants used, observing that the phenotypic characteristics (color and friability).

Statistical analysis

All callus induction experiments were performed in triplicate with four experimental units (n = 12). The Sigma Plot 12.0 software was used for the statistical analyzes of each experiment. A one-way ANOVA analysis was performed followed by the SNK post hoc test for the TPA experiment, while two-way ANOVA was used for the carrageenan model followed by the SNK post hoc test.

RESULTS AND DISCUSSION

Callus induction

With NAA (2.5 mg/L) and BAP (5.0 mg/L) 75% of callogenesis was obtained, whose phenotypic characteristics (greenish color, rough and friable texture) were ideal (Figure 1) to establish a cell line in suspension. PGRs are essential for the growth and development of plants and for biotechnological processes such as suspension cell cultures. The type and concentration of PGR have an important effect on cell differentiation, biomass and the production of plant-secondary metabolites (Isah et al., 2018). For the medicinal species *C. chayamansa,* there is only one report that describes the effect of PGRs on callus formation (Pérez-González et al., 2018b), where they used 24 combinations of 2,4-D and several concentrations of BAP (0.0 to 5 mg/L) and it was reported that combinations of 2,4-D (2.5 mg/L) + BAP (5 mg/L) and 2,4-D (5 mg/L) + BAP (2.5 mg/L) allowed 100% callogenesis, while with the combinations of 2,4-D (0.5 mg/L) + BAP (0.5 mg/L) and 2,4-D (2.5 mg/L) + BAP (0.5 mg/L) there was only 75% callus formation. It should be noted that they used sucrose as a carbon source. It is well known that callus formation depends mainly on the cellular sensitivity of plant species against the

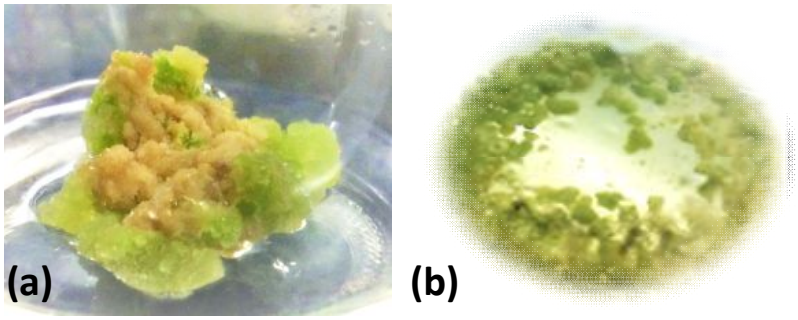

Figure 1. *C. chayamansa* callus produced with the combination of NAA (2.5 mg/L) + BAP (5.0 mg/L) (a), culture of suspension cells from *C. chayamansa* with NAA (2.5 mg/L) + BAP (5.0 mg/L) obtained at day 30 (b).

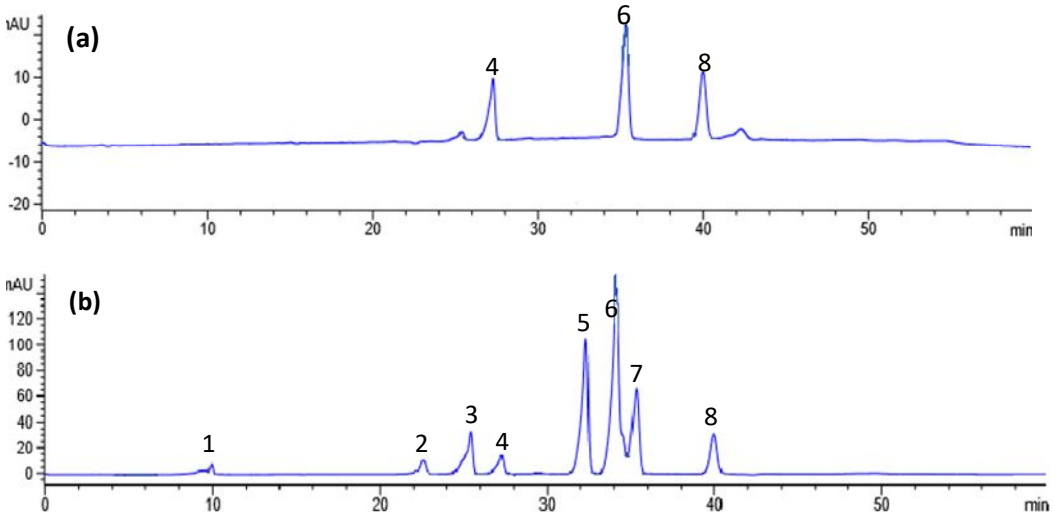

Figure 2. HPLC chromatogram for phenolic identification in ANB extract. (a) Compared with commercial standards of the phenols (b): Chlorogenic acid (1), Caffeic acid (2), Ferulic acid (3), *p*-Coumaric acid (4), Rutine (5), Cinnamic acid (6), Quercetin (7), Kaempferol (8).

concentrations of PGR and their specific receptors that are present in different plant tissues (Modarres et al., 2018).

Preparation of the organic extract and phytochemical profile

The yield of the extract from ANB cell line was 28.85%; this yield was higher compared to wild plant material, being 10.68% (Pérez-González et al., 2017). By TLC analysis, scopoletin was detected (R_f = 0.36 in $CHCl_3$ elution system with 8 drops of MeOH), while moretenol acetate and β-sitosterol were also detected, and these were compared to previously isolated standards from wild plant (Pérez-González et al., 2017; 2018a). The HPLC analysis of the ANB extract (Figure 2a) showed the presence of *p*-cumaric acid (4; T_R = 27.27 min), cinnamic acid (6; T_R = 35.32 min) and kaempferol (8: T_R = 39.98 min), detected at a wavelength of 300 nm (Figure 3); the Rt of these compounds were compared with commercial standards -Sigma- (Figure 2b). The conditions used for this analysis were previously reported (Pérez-González et al., 2019b).

In addition, scopoletin was quantified in the ANB extract by HPLC analysis. Commercial scopoletin standard (Sigma) showed Rt = 11.53 min at a wavelength of 330 nm (Figure 4a). The quantification of this compound was performed with the standard curve using the equation y = 14354x + 583.88 R^2 = 0.99889. Through this process, it was determined that the concentration of scopoletin in the ANB extract was 3.68 mg/g of dry

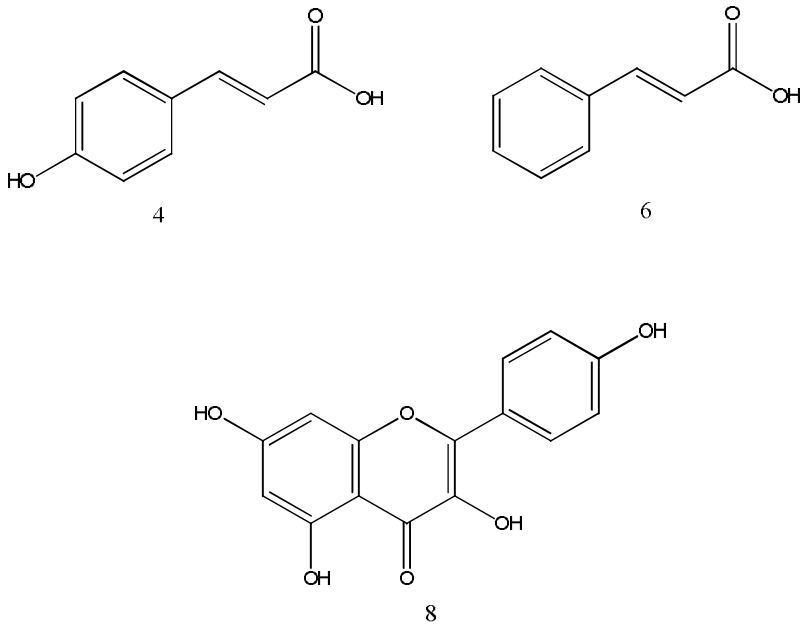

Figure 3. Chemical structures of phenolic compounds: p-Coumaric acid (4); Cinnamic acid (6) and Kaempferol (8) identified in the ANB extract.

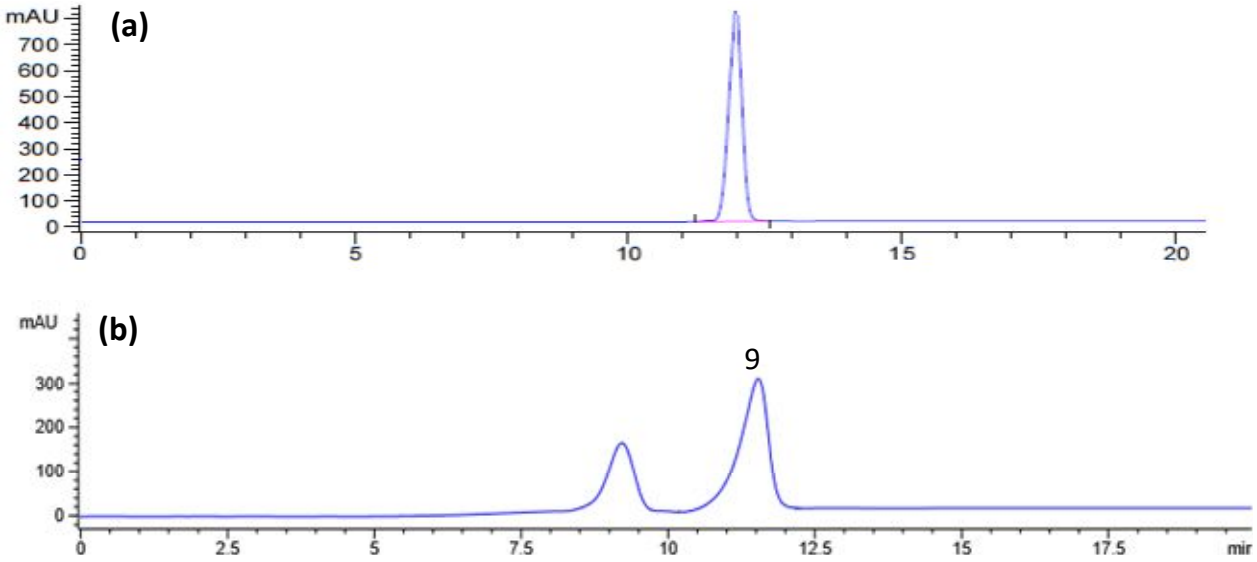

Figure 4. HPLC chromatogram of scopoletin standard (a) and the ANB extract (b), with Rt = 11.53 min.

extract (Figure 4b).

Scopoletin (Figure 5) is a coumarin that has been reported in the EtOH extract from *C. texanus* aerial parts (Yuan et al., 2007; de Oliveira et al., 2018), and in $CHCl_3$:MeOH extract from *C. chayamansa* leaves (wild material); it extract contained 0.19 mg of scopoletin/g of dry extract and organic extracts from cell line contain 3.68 mg of scopoletin/g dry extract (Pérez-González et al., 2019a; 2019b). Scopoletin shows different biological activities, such as anti-inflammatory, hypotensive, anticonvulsive, antispasmodic, antipyretic, hypoglycemic, hepatoprotective and antiarthritic (Pérez-Hernández et al., 2014; Ding et al., 2008; Moon et al., 2007; Kim et al., 2004; Pérez-González et al., 2019a). In addition, by

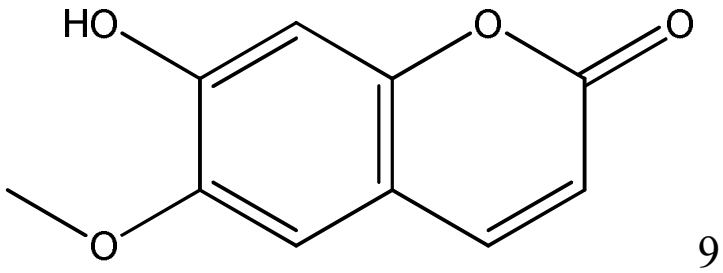

Figure 5. Chemical structure of scopoletin.

HPLC analysis, we found that the ANB extract contained three phenolic compounds (*p*-cumaric acid, cinnamic acid and kaemperol) and scopoletin as main compounds. By TLC, moretenol acetate (small account) and β-sitosterol were detected. The conditions described in this manuscript, allowed obtaining the culture of cells in suspension by biotechnological process and are a very viable procedure for the large-scale production of metabolites of biological importance. It is worth mentioning that it is the first time that the presence of scopoletin, *p*-cumaric acid, cinnamic acid in *C. chayamansa* has been observed.

There are reports of other plants that were obtained through a biotechnological process and it producing secondary metabolites with biological activity. An example of this, is the cell suspension cultures of *Calophyllum brasiliense,* where the dichloromethane extract showed anti-inflammatory and antioxidant activities (Cisneros-Tórres et al., 2020); the root cultures and plantlets from *Tanacetum parthenium* obtained by biotechnology proccess produce phenolic compound and parthenolide, these compounds was detected in MeOH extract and showed anticancer, antidiabetic activities, and cardiovascular and neurodegenerative protection (Nieto-Trujillo et al., 2017). The micropropagation of *Buddleja cordata* showed antioxidant activity due to the presence of verbascoside and other phenolic compounds (Estrada-Zúñiga et al., 2016).

Pérez-González et al. (2019b) reported that the cells in suspension from *C. chayamansa* obtained by biotechnological process using regulators: NAA (2.5 mg/L) + BAP (5 mg/L) and glucose as a carbon source, it was found that at day 40, this suspension culture has the maximum production of LA (38.1 mg of LA/g of dry cell extract). The LA content was higher than that found in the wild plant (2.9 mg of LA/g dry extract); in that case, the same PGRs were used but with different carbon sources (sucrose), and it was observed that the extract obtained from the cells in suspension did not contain LA, so it should be noted that the carbon source is decisive on the production of this secondary metabolite of biological interest (Pérez-Gonzalez et al., 2019a).

Acute anti-inflammatory activity

The results obtained on the TPA model are described in Table 2; the ANB extract at 2 mg/ear showed scarce anti-inflammatory activity, being 32.51% and Ind (reference drug) showed 58.10% at the same doses. In a previous paper, it was reported that the $CHCl_3$:MeOH (1:1) extract from *C. chayamansa* leaves (wild plant), administered topically, showed an ED_{50} = 1.66 mg/ear using TPA model (Pérez-González et al., 2017). Another report with the same inflammation model, using CD1 mice, reported that Hex, AcOEt and EtOH extracts from the leaves of *C. chayamansa* at dose of 2 mg/ear inhibited an average of 31% (García-Rodríguez et al., 2014). It should be noted that the ANB extract showed a similar percentage of inhibition to that reported for the Hex, AcOEt and EtOH extracts; however, it was less active than the $CHCl_3$:MeOH extract from the wild plant. According to TLC and HPLC analyses, the ANB extract showed a different profile with respect to wild material. For example, the presence of LA, a main compound in wild plant extract, was not detected; only scopoletin was detected (R_f = 0.36 in $CHCl_3$ elution system with 8 drops of MEOH), and this coumarin was detected as a minor compound in the wild plant extract. In addition, β-sitosterol and moretenol acetate were detected in both extracts. The conditions used to obtain the cells in suspension, which allowed to obtain the ANB extract, are a source of obtaining scopoletin, moretenol acetate and β-sitosterol.

In carrageenan model, the ANB extract showed a poor percentage of inhibition on the inflammatory process with respect to Ind; the organic extract at 3, 5 and 7 h showed 1.41, 4.65 and 2.39% inhibition, respectively. Results are shown in Table 3. This result showed that the extract at 200 mg/kg shows scarce anti-inflammatory effect, since the behavior of the ANB extract in the systemic inflammation model is very low respect to Ind. Pérez-González et al. (2017) reported that the anti-inflammatory activity for the $CHCl_3$:MeOH extract from the wild plant showed an ED_{50} = 467.72 mg/kg at 5th h.

The anti-inflammatory effect shown by the wild plant is

Table 2. Topical anti-inflammatory effect (TPA model) of ANB extract.

Treatment	Dose (mg/ear)	T(5h) mg	inhibition %
TPA		6.52 ±0.06	0
Ind	2	3.04±0.06[ac]	58.10
ANB	2	4.4±0.04[ab]	32.51

ANOVA statistical analysis one way post hoc SNK test (p≤0.05). [a]vs TPA Control; [b]vs Ind; [c]vs ANB 2 mg/ear n = 5.

Table 3. Systemic anti-inflammatory effect (carrageenan model) of ANB extract.

Time (h)	Treatments		
	Carrageenan	Indomethacin (10 mg/kg)	ANB (200 mg/kg)
1	0.35 ± 0.02	0.29 ± 0.03 [a](16.85%)	0.46 ± 0.03 [ab](NE)
3	0.51 ± 0.07	0.37 ± 0.08 [a•](27.30%)	0.51 ± 0.03 [ab•](1.41%)
5	0.8 ± 0.17	0.54 ± 0.06 [a](31.91%)	0.76 ± 0.06 [ab•*](4.65%)
7	0.64 ± 0.1	0.54 ± 0.09 [a•*] (16.06 %)	0.62 ± 0.06 [ab•*](2.39%)

Each group is represented as the mean (±) and its standard error (s.e.). Values in parentheses indicate the percentage of edema inhibition with respect to the control group. Bifactorial ANOVA statistical analysis, post hoc SNK test (p≤0.05); [a]vs carrageenan; [b]vs Ind; [c]vs ANB 200 mg/kg. •vs 1 h; *vs 3 h. •vs 5 h:NE; no effect; n = 5.

moderate; using this model, it contains LA as the main compound (Pérez-González et al., 2018a); however, this compound was not detected in the ANB extract. This manuscript describes an additional biotechnological process to induce some active secondary metabolite such scopoletin, *p*-cumaric acid, cinnamic acid and kaemperol from *C. chayamansa*. To date, this is the second report on the use of biotechnological processes to obtain cell line from *C. chayamansa,* this plants is widely used in traditional medicine and with nutritional importance.

SA effect on callus formation

In the elicitation process using SA, callus formation and morphology could be monitored, and the response to SA was favorable, since a concentration of 25 µM showed a percentage of 16.66 ± 0.60 (8 days), 19.44 ± 0.54 (14 days) and 38.88 ± 2.27 (28 days). On the other hand, in a major concentration of SA (50 µM), the percentage was also effective but the percentage was lower, being 0.0 (8 days), 5.55 ± 1.7 (14 days) and 30.55 ± 0.77 (28 days); finally, at concentration of 100 µM of SA, these percentage of callus formation was better: 13.88 ± 1.20 (8 days), 41.66 ± 2.93 (14 days) and 77.77 ± 2.78% (28 days). The percentages on callus formation at different concentrations of SA are shown in Figure 6.

The callus formation response at concentrations 25 and 100 µM was time dependent; however, at the concentration of 50 µM, the maximum effect of callus formation at day 28 was very low, with 30.55 ± 0.77%, and similar effect was observed at 25 µM (38.88 ± 2.27%). The best concentration of SA was 100 µM, which showed a percentage of callus formation similar to that shown by the culture with PGR (75%, 40 days). This response by callus formation was in a shorter time (28 days). On day 28, the three concentrations showed calluses with different morphologies that are described in Table 4.

Elicitation is an efficient strategy to promote defense mechanism in plants, due to this, there are more secondary metabolites. On the other hand, this technique also favors the response time to callus formation (Kang et al., 2019). At the moment, there is no report of elicitation for *C. chayamansa*; however, there are reports of elicitation with SA for the Euphorbiaceae family, such as for the species *Jathopha curcas,* using the SA for cultures of suspended cells, reporting the increase in compounds as alkanes and fatty acids (Mahalakshmi et al., 2013). Therefore, elicitation with SA is an alternative to reduce callus production times and increase the production of secondary metabolites (phenolic and coumarins); however, it is essential to perform other experiments to confirm the presence of secondary metabolites with anti-inflammatory effect and other biological activities.

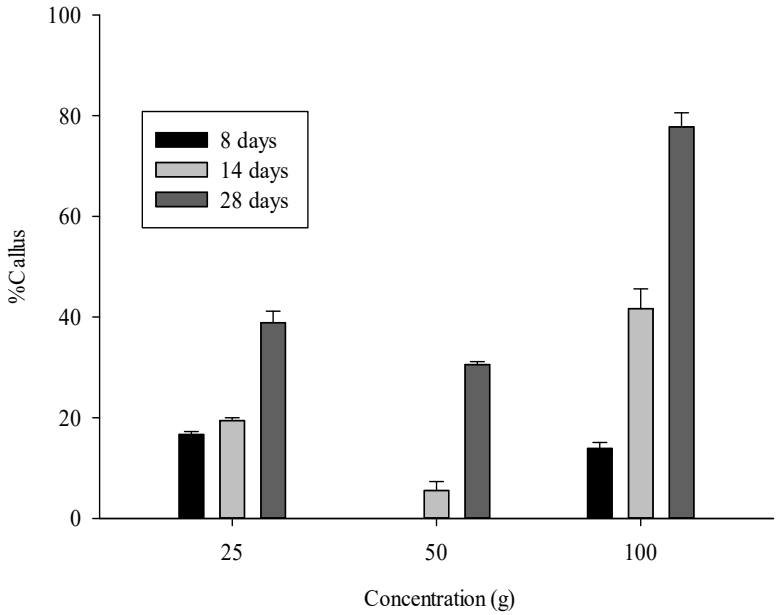

Figure 6. Effect of SA on callus formation. Each group is represented as the mean (±) and its standard error (s.e.).

Table 4. SA effect for callus formation *C. chayamansa* on day 28.

Concentration (µM)	Callus Dimension (mm)	Callus formation (%)	Characteristics/Morphology		Imagen
			Color	Texture	
25	16	38.88 ± 2.27	LB	F	
50	8	30.55 ± 0.77	GB	C	
100	22	77.77 ± 2.78	LB	F	

GB, Brown green; LB, light brown; G greenish; C, compact; F, friable. Each group is represented as the mean (±) and its standard error (s.e.).

Conclusion

The suspension cells of *C. chayamansa* were obtained with the mixture of PGR [NAA (2.5 mg/L) and BAP (5 mg/L)] and sucrose as a source of biosynthetic carbon. In this suspension cell, scopoletin was the main compound; in addition, moretenol acetate (in small quantities), β-sitosterol, *p*-cumaric acid, cinnamic acid and kaemperol

were identified. The cell extract showed moderate topical and poor systemic anti-inflammatory effect. Elicitation with SA at 100 µM reduced the response time for callus formation, reaching 77.77% on day 28.

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Disclosure

All authors have read and approved the final version of the manuscript and declare that they have no conflict of interest.

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