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ESTABLISHMENT OF A CELL SUSPENSION CULTURE FROM *Calophyllum brasiliense* AND EVALUATION OF ITS ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY

ESTABLECIMIENTO DE UN CULTIVO DE CÉLULAS EN SUSPENSIÓN DE *Calophyllum brasiliense* Y EVALUACIÓN DE SU ACTIVIDAD ANTIOXIDANTE Y ANTI-INFLAMATORIA

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Abstract

In the present work, the establishment of a cell suspension culture (CSC) of *C. brasiliense* is reported. In addition, extracts were obtained from the CSC dry biomass and leaves of wild plant using organic solvents for the purpose of determining the total phenolic (FET) and total flavonoid (FLT) content. Furthermore, it was evaluated the antioxidant and anti-inflammatory activity of the obtained extracts. For the above, a CSC was established successfully, producing a maximum of 15.2 g L^{-1} of dry biomass at 12 days of culture. The production of FET, FLT and antioxidant activity (DPPH or ABTS) were related with culture growth and its maximum values were produced between 12 and 16 days of culture. The results showed that CSC yielded more FET, FLT and showed higher antioxidative activity than the wild plant. The methanolic and acetone extracts from wild plant showed inhibition of the edema induced by TPA in ear mice at 2 mg/ear, with 44.49 ± 10.75 % and 27.83 ± 9.59 % of inhibition, respectively, while the dichloromethane extract of CSC showed 39.41 ± 7.74 % of inhibition at 2 mg/ear and the positive control (indomethacin) 42.30 ± 4.46 % of inhibition at 0.5 mg/ear. It is the first time that a CSC establishment of *C. brasiliense* has been reported and its antioxidative and anti-inflammatory activity were demonstrated. The CSC reported here can be used as a bioresource to obtain extracts or bioactive compounds.

Keywords: plant growth regulator; *in vitro* culture, medicinal plant, extracts, total phenolics, total flavonoids.

Resumen

En el presente trabajo se reporta el establecimiento de un cultivo de células en suspensión (CSC) de *C. brasiliense*. Además, se realizó la obtención de extractos a partir de la biomasa seca de CSC y de las hojas de la planta silvestre usando solventes orgánicos con la finalidad de determinar el contenido de fenoles totales (FET) y flavonoides totales (FLT). Así mismo, se evaluó la actividad antioxidante y antiinflamatoria de los extractos obtenidos. Para lo anterior se estableció adecuadamente un CSC produciendo un máximo de 15.2 g L^{-1} de biomasa seca a los 12 días de cultivo. La producción de FET, FLT y actividad antioxidante (DPPH o ABTS) se relacionó con el crecimiento del cultivo y sus valores máximos ocurrieron entre los 12 y 16 días. Los resultados muestran que el CSC produjo más FET, FLT y presenta mayor actividad antioxidante que la planta silvestre. Los extractos metanólico y de acetona de la planta silvestre mostraron inhibición del edema inducido por TPA en oreja de ratón a la dosis de 2 mg/oreja, con porcentajes de inhibición del 44.49 ± 10.75 % y 27.83 ± 9.59 %, respectivamente, mientas que, el extracto de diclorometano de CSC mostró un porcentaje de inhibición del 39.41 ± 7.74 % a la dosis de 2 mg/oreja y el control positivo (indometacina) inhibió un 42.30 ± 4.46 % a la dosis de 0.5 mg/oreja. Es la primera vez que se reporta el establecimiento de un CSC de *C. brasiliense* y se demostró su actividad antioxidante y antiinflamatoria. El CSC reportado puede ser usado como una opción sustentable para obtener extractos o compuestos bioactivos.

Palabras clave: regulador de crecimiento vegetal; cultivo *in vitro*, planta medicinal, extractos, fenólicos totales, flavonoides totales.

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1 Introduction

Plants produce a wide variety of secondary metabolites, of which, flavonoids, alkaloids, glycosides, tannins, terpenoids and phenolics are the most important bioactive compounds (Indrakumar *et al*., 2012). *Calophyllum brasiliense* Cambess, which belongs to the family Calophyllaceae (APGIII, 2009), is a medicinal tree that produces phytochemicals such as xanthones, coumarins, chalcones, flavonoids and triterpenes with have a wide variety of biological activities (Bernabé-Antonio *et al*., 2014). One of the main importance of *C. brasiliense*, is because it produces coumarins, e.g., calanolides and GUT-70, which inhibit the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) (Huerta-Reyes *et al*., 2004a, 2004b; Kudo *et al*., 2013). It has also been shown that methanolic extracts and coumarins of *C. brasiliense* leaves are highly active against *Staphylococcus aureus*, *S. epidermidis* and *Bacillus subtilis* (Reyes-Chilpa *et al*., 2004; Yasunaka *et al*., 2005). Moreover, bark hydroethanolic extracts of *C. brasiliense* and the dichloromethanolic fraction exhibited inhibitory properties against *Helicobacter pylori*, both *in vitro* and *in vivo* (Souza *et al*., 2009). Other studies carried out by Brenzan *et al*. (2007, 2008, 2012) and Honda *et al*. (2010) demonstrated high antileishmanial activity of leaf extracts and isolated compounds of *C. brasiliense* on *Leishmania amazonensis*. The coumarins mammea A/BA, and mixtures of mammea A/BA + A/BB, mammea B/BA + B/BB and mammea C/OA + C/OB, were also highly active against human tumor cell lines (Reyes-Chilpa *et al*., 2004).

In other plants, phenolic compounds such as flavonoids, phenolic acids, tannins and diterpenes have been found to possess antioxidative or anti-inflammatory activity (Nieto-Trujillo *et al*., 2017; de la Mora-López *et al*., 2018; Gómez-Estrada *et al*., 2011; Attoumani *et al*., 2013; Proestos *et al*., 2013). In this regard, in terms of antioxidative compounds, only two reports pertaining to *C. brasiliense* have been carried out, in which, the aqueous, methanolic and ethanolic extracts of leaves exhibited 110.56, 99.17 and 99.57 % of inhibition, respectively, at 100 mg mL^{-1} (Flores *et al*., 2008). By other hand, has been demonstrated that the ethyl acetate and methanolic extracts of leaves or stem from *C. brasiliense* showed important antioxidative activity (Mesa-Venegas *et al*., 2010). However, only few plants have been shown to produce phenolic and flavonoid compounds using a plant cell culture system (Liu and Saxena, 2009; Krishnan *et al*., 2014; Lugato *et al*., 2014).

To date, most of reports on *C. brasiliense* have been carried out using the wild plants, and obtaining antioxidants or anti-inflammatory compounds such as phenolics, flavonoids or other type of compounds from wild plants is not an ecologically feasible option. In fact, in Mexico, this species is listed as a threatened species according to the Official Mexican Standard NOM-059-SEMARNAT-2010 (SEMARNAT, 2010) and few studies focused on its conservation and sustainable use have been made. Therefore, the use of cell cultures of *C. brasiliense* represents a potential option for obtaining bioactive compounds in a renewable way. We previously demonstrated that callus culture from *C. brasiliense* leaves can produce secondary metabolites such as calanolide B, calanolide C and apetalic acid, as well as a wide variety of fatty acids (Bernabé-Antonio *et al*., 2010, 2015).

To our knowledge, however, studies of antioxidant and anti-inflammatory activity with extracts from *C. brasiliense* cell suspension cultures have not been reported. The aim of this study was to establish a cell suspension culture from *C. brasiliense* Cambess leaves, determine the total phenolic and flavonoid content. We also evaluate the antioxidant and anti-inflammatory activity of cell culture extracts.

2 Materials and methods

2.1 Plant material

The leaves from wild plants of *C. brasiliense* were collected in November 2014, in San Andrés Tuxtla, Veracruz, Mexico, as previously reported (Bernabé-Antonio *et al*., 2015). We used these leaves to extraction, isolation and purification of some compounds.

2.2 Obtaining the callus and establishing the cell suspension cultures (CSC)

Callus cultures (Fig. 1a, 1b) from *C. brasiliense* Cambess leaves were previously established in MS (Murashige and Skoog, 1962) culture medium supplemented with sucrose 3 % (w/v), fructose 0.5 % (w/v), 24.84 μM of picloram (PIC) and 8.88 μM of 6-benzylaminopurine (BAP) (Bernabé-Antonio *et al*., 2015).

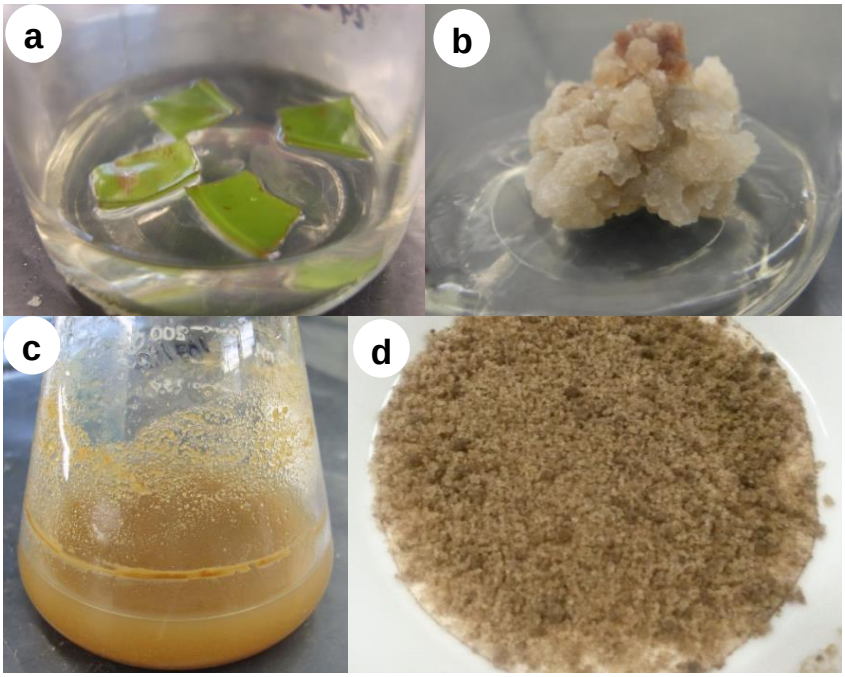

Fig. 1. Establishment of a cell suspension cultures from *Calophyllum brasiliense* leaves in MS medium containing PIC (24.84 μM), BAP (8.88 μM) and 0.5% fructose. a) leaf explants for callus induction; b) callus produced from leaf explants at 40 days of culture; c) cell suspension cultures at 12 days of culture; d) cell biomass obtained from "c)".

The cultures were incubated at 25 ± 2 °C with a photoperiod of 16 h with white fluorescent light (60 μmol m^{-2} s^{-1}). The callus was periodically subcultured (every 15 days) over the course of 6 months and they were used to establish the CSC. A portion of fresh and friable callus (1.5 g fresh weight; FW) was cultured in 125-mL Erlenmeyer flasks containing 25 mL of complemented MS liquid medium. The flasks were agitated at 115 rpm on an orbital rotary shaker and incubated at 25 ± 2 °C with a photoperiod of 16 h with white fluorescent light (60 μmol m^{-2} s^{-1}). The biomass production was increased by subsequent subcultures every 15 days for 6 months in 500 mL flasks containing 100 mL of the same liquid culture medium and 6 g fresh biomass.

2.3 Growth kinetics of the CSC

To determine the growth parameter of the *C. brasiliense* CSC, several 125-mL Erlenmeyer flasks containing 25 mL of culture medium were inoculated with 1.5 g fresh cell biomass (FW). Three flasks were harvested every three days, and the biomass was filtered and then dried in an oven at 50 °C for 24 h. Additionally, an aliquot (4 mL) of filtrate culture medium from each flask was obtained to determine the total sugar level during growth. The dried biomasses (dried weight; DW) were used to define the growth curve over of the course of 16 days. The specific cell growth rate (μ), defined as the increase in cell mass per unit time, was calculated by plotting the cell growth data in the form of a natural logarithm versus time. This methodology yielded a straight line over the exponential phase growth. The slope of the linear part of the plot corresponds to the specific cell growth rate and is defined as 1 per unit time. The time required for the biomass to double (the doubling time, dt) was computed from the μ experimental data. The dried biomasses of the growth kinetics were also used to determine the total phenolic content, the total flavonoid content, and the antioxidant and anti-inflammatory activities. The experiment was repeated twice.

2.4 Determination of the total sugar content

The filtrated culture medium of each flask from the kinetic growth was used to determine the total sugars. The total sugars of the culture medium were quantified

using the phenol-sulfuric acid method (DuBois *et al*., 1956). An aliquot (200 μL) of standard glucose solutions (10-100 mg L^{-1}) and culture media were mixed with 200 μL of an aqueous solution of phenol (5 %) in a test tube. Next, 1 mL of concentrated sulfuric acid was added rapidly to the mixture and the tubes could stand for 10 min. The tubes were then vortexed for 30 s and placed in a water bath for 20 min at room temperature to allow for color development. Light absorption at 490 nm was recorded on a CECIL 3000 series spectrophotometer (Cecil Instruments, United Kingdom). The entire procedure was repeated three times for each growth kinetic.

2.5 *Extraction of total phenol and flavonoids content*

The dried biomasses (50 mg DW) of CSC obtained from growth kinetics or wild plant leaves were finely milled and extracted with 5 mL of methanol in an ultrasonic bath Sonics Vibra-Cell VCX130 (Soni & Materials, Inc, USA) equipped with a CV 188 sonotrode for 10 min. Ice was added to the bath to keep the temperature below 35 °C. Then, vials with samples were centrifuged at 6000 rpm for 20 min and the methanolic extract (MeEx) were filtered. These methanolic extracts were subsequently used for the determination of total phenolic content, total flavonoid content and the antioxidant activity.

2.6 *Determination of total phenolic and flavonoid content*

The FET content was measured using the method reported by Yim *et al*. (2012), with slight modifications. In brief, 20 μL of methanolic extract was mixed with 100 μL of diluted Folin-Ciocalteau (1:1). After 3 min, 300 μL of Na_2CO_3 was added to the mixture and adjusted to 2 mL with deionized water. The mixture could stand in a dark environment for 90 min. Absorbance was measured against a blank reagent at 725 nm using a Spectronic GENESYS 2 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Gallic acid was used as a standard for the calibration curve with a concentration range of 80-1000 mg mL^{-1} (R^2 = 0.99) and analyzed as noted above. The results were expressed as mg of gallic acid equivalent (GAE) per g dry biomass (DW) (mg GAE g^{-1} DW).

The FLT content was estimated using the aluminum chloride method using quercetin as a standard to prepare the calibration curve (Liu and Saxena, 2009). Aliquots of methanolic extract (200 μL) were mixed with 1.25 mL of distilled water. Next, 75 μL of $NaNO_2$ (5 %) solution was added, mixed thoroughly and allowed to stand 6 min. Then, 150 μL of an $AlCl_3$ (10 %) solution was added and allowed to stand 5 min. Finally, 500 μL of NaOH (1M) was added and the volume of each reference sample was increased to 2.5 mL using distilled water. Absorbance was measured during the first 30 min at 510 nm and the results were expressed as mg of quercetin equivalent (QE) per g dried biomass (DW) (mg QE g^{-1} DW).

2.7 *DPPH radical scavenging activity assay*

Radical scavenging activity of CSC methanolic extract was evaluated using $DPPH^{\bullet+}$ radicals, as reported by Yim *et al*. (2012). A stock solution of DPPH (0.075 mM) in methanol was prepared daily. The $DPPH^{\bullet+}$ solution (3.8 mL) was mixed with 20 μL of CSC methanolic extract, and the mixture shaken vigorously for 1 min and left to stand at room temperature in the dark for 30 min. Absorbance was measured against a blank reagent at 517 nm. The Trolox equivalent antioxidant capacity (TEAC) was calculated from the calibration curve using a range of 0.5-80 mM of Trolox as an antioxidant standard. The data were reported as M TEAC per g of dry biomass (DW) (M TEAC g^{-1} DW).

2.8 *ABTS radical cation discoloration assay*

Assays were performed according to the procedure of Gong *et al*. (2012). A stock solution of ABTS (7 mM) plus 2.45 mM of potassium persulphate was prepared. The mixture could stand in the dark at room temperature for 12 h. The working solution of ABTS was obtained by diluting the stock solution with methanol until an absorbance of 0.70 ± 0.02 was obtained at 734 nm. Next, 2.0 mL of ABTS solution was mixed with 20 μL of methanolic extract, stirred vigorously and allowed to stand for 6 min. The absorbance was read at a wavelength of 734 nm. The results were expressed as mM TEAC per g dry biomass (DW) (mM TEAC g^{-1} DW) values by constructing a standard curve using Trolox (0.0-15.0 mM) as an antioxidant.

2.9 Compounds isolation from wild plant

The leaves from *C. brasiliense* Cambess were dried at 60 °C to a constant weight and then ground to a fine powder. A sample of 200 g of ground dry leaves was serially macerated with acetone and methanol. Three extraction cycles were performed for 24 h for each solvent. The extracts were filtered, and the solvents were removed using a rotary evaporator (Heidolph L3, Germany) to get 3.5 g of acetonic extract (AcEx) and 5 g of methanolic extract (MeEx). The extracts were checked by thin layer chromatography (TLC) (Silica gel 60 F_{254}, Merck), visualized by means of UV light, and sprayed with ceric ammonium sulfate. Because more compounds were visualized in the AcEx, this extract was subjected to normal-phase column chromatography (CC) (silica gel 60, 9858 Merck; Darmstadt, Germany) and eluted with a n-hexane/ethyl acetate gradient system of 1:0 to 5:5). As result, 40 fractions of 50 mL were collected and monitored by normal-phase TLC (Silica gel 60 F_{254}, Merck) and reverse-phase TLC (Silica gel 60 RP-18 F_{254S}, Merck). Then, fractions that showed TLC similarity were grouped to finally obtain 3 groups, R1 (0.95 g), R2 (30 mg) and R3 (2.4 g) and checked by normal-phase TLC. From R1 and R2 were isolated the known compounds canophyllol and apetalic acid from the acetone extract (AcEx) of leaves. The pure compounds were analyzed by nuclear magnetic resonance (NMR) in one (^{1}H, ^{13}C, DEPT) and two dimensions (COSY, HSQC and HMBC).

Canophyllol (28-hydroxy-3-friedelanone): Group R2 obtained from acetone extract by normal-phase CC with a *n*-hexane-ethyl acetate (80:20) elution system was recrystallize in methanol and was found to be canophyllol (18.2 mg). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.75 (3H, s, Me-24), 0.87 (3H, s, Me-25), 0.88 (3H, d, J = 6.7 Hz, Me-23), 0.91 (3H, s, Me-26), 0.98 (3H, s, Me-30), 0.99 (3H, s, Me-29), 1.13 (3H, s, Me-27), 1.69 (1H, ddd, J = 26.1, 13.1, 5.1 Hz, H-1a), 1.76 (1H, dt, J= 12.4, 2.7 Hz, H-6a), 1.88 (1H, dt, J= 18, 6.9 Hz, H-16a), 2.40 (1H, ddd, J = 13.7, 5.1, 1.9 Hz, H-2a), 3.63 (2H, br s, H-28). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 213.0 (C3), 67.9 (C28), 59.4 (C10), 58.1 (C4), 52.4 (C8), 42.0 (C5), 41.4 (C2), 41.1 (C6), 39.3 (C13), 39.3 (C18), 38.0 (C14), 37.4 (C9), 35.3 (C11), 35.1 (C17), 34.2 (C29), 34.4 (C19), 33.3 (C22), 32.8 (C30), 31.3 (C21), 31.2 (C15), 30.0 (C12), 29.0 (C16), 28.0 (C20), 22.2 (C1), 19.1 (C27), 19.0 (C26), 18.1 (C7), 18.0 (C25), 14.6 (C24), 6.7 (C23). The data obtained by NMR (^{1}H and ^{13}C) were compared with those described by Thuy *et al*. (2007) and Sangsuwon *et al*. (2013).

Apetalic acid: Group R1 was subjected to normal-phase CC with a *n*-hexane-ethyl acetate (1:0-5:5) obtained 36 fractions of 50 mL each one and the fractions that showed TLC similarity were grouped to finally obtain 7 groups, R1-1 to R1-7. Then, R1-7 (250 mg) was subjected to reverse-phase CC eluted with acetonitrile/water gradient system (95:05 - 00:100) to obtain 22 fractions of 20 mL each one, R-1-7-1 to R1-7-22. Fraction R-1-7-2 and R-1-7-3 obtained corresponded to apetalic acid (58.9 mg). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 12.38 (1H, 5-OH), 6.60 (1H, d, J = 10 Hz, H-6), 5.46 (1H, d, J = 10 Hz, H-7), 4.51 (1H, qd J=6.5, 3.3 Hz, H-2), 3.69 (1H, m, H-19), 2.83 (1H, dd, J= 15.1, 8.7 Hz, H-20a), 2.66 (1H, dd, J= 15.1, 6.8 Hz, H-20b), 2.55 (1H, qd, J=7.2, 3.3 Hz, H-3), 1.83 (1H, dddd, J = 13.1, 10.3, 8.5, 6.0 Hz, H-22a), 1.52 (1H, m, H-22b), 1.45 (3H, s, Me-18), 1.38 (3H, s, Me-17), 1.36 (3H, d, J = 6.6 Hz, Me-15), 1.18 (2H, m, H-23), 1.14 (3H, d, J = 7.3 Hz, Me-16) and 0.86 (3H, t, J = 7.4 Hz, Me-24). ^{13}C-NMR (125 MHz, $CDCl_3$): *delta* 201.0 (C4), 179.3 (C21), 160.5 (C14), 160.5 (C11), 157.2 (C5), 125.6 (C7), 115.6 (C6), 108.8 (C13), 102.5 (C10), 101.2 (C12), 78.1 (C2), 76.0 (C8), 44.1 (C3), 38.6 (C20), 35.4 (C22), 30.4 (C19), 28.4 (C18), 28.0 (C17), 20.7 (C23), 16.2 (C15), 13.9 (C24), 9.2 (C16). The data obtained by NMR (^{1}H and ^{13}C) were compared with those reported by Plattner *et al*. (1974) and Huerta-Reyes *et al*. (2004a).

2.10 Dichloromethane extract (DCMEx) obtention of CSC

The dry ground biomass of CSC (49.6 g) of 12-day growth (Fig. 2) was macerated three times with dichloromethane for 24 h each. The dichloromethane extract (DCMEx) was concentrated in a rotary evaporator to remove the dissolvent.

2.11 Evaluation of anti-inflammatory activity in mice

Male ICR mice (weight: 28 g) were used, and the experiments were performed according to the official Mexican Rule NOM-062-ZOO-1999 Guidelines (Technical Specifications for the Production, Care, and Use of Laboratory Animals) and international ethical guidelines for the care and use of experimental animals.

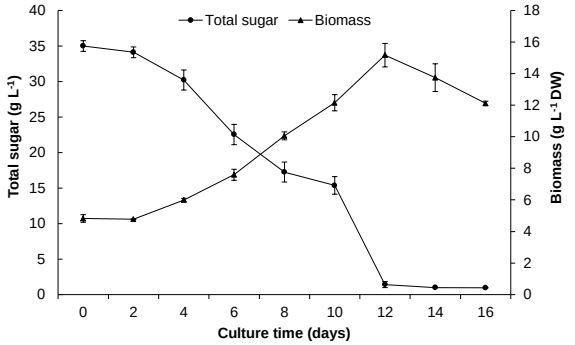

Fig. 2. Growth kinetic and consumption of total sugars of cell suspension culture from *Calophyllum brasiliense* leaves during 16 days of culture. Error bars represent standard deviation of three repeated measurements.

The mice were maintained at a temperature of 22 ± 3 °C and a humidity of 70 ± 5 % with 12-h light/dark cycles and food/water *ad libitum*. The mice were given at least three weeks to adapt to their laboratory environment prior to initiating the experiment. The mice were assigned to groups of seven individuals, and the phorbol ester 12-O-tetradecanoylphorbol-3-acetate (TPA) (2.5 μg dissolved in 20 μL acetone) was applied to the internal and external surfaces of each mouse's right ear to generate edema. Doses of 0.5 or 1.0 or 2.0 mg/ear of acetone extract (AcEx) and methanol extract (MeEx) from the leaves, canophyllol and apetalic acid isolated from wild plant, as well as the dichloromethane extract (DCMEx) from the CSCs or indomethacin (positive control) were dissolved in acetone and applied topically immediately after the administration of TPA. Four hours after administration of the inflammatory agent, the animals were sacrificed by cervical dislocation and circular sections of 6 mm in diameter were obtained from both the treated (t) and the non-treated (nt) ears, which were weighed to determine inflammation. The percentage of inhibition was calculated using the following expression: inhibition $\% = [(\Delta w\_treatment/\Delta w\_control)\times 100]-100$, where $\Delta w\_control$ represents the ear-weight difference of the group treated with TPA and $\Delta w\_treatment$ represents the ear-weight difference of the group treated with extracts, isolated compounds or indomethacin.

2.12 *Statistical analysis*

All data were subjected to variance analysis (ANOVA) followed by a Tukey's multiple range test ($p \leq 0.05$) for antioxidant activity, total phenol and total flavonoid contents. We used ANOVA followed by Dunnett´s multiple comparisons (means) test to evaluate the anti-inflammatory activity of the extracts. SAS 9.0 software (SAS Institute Inc., 2002) was used for the statistical analysis.

3 Results and discussion

3.1 *Cell suspension culture and growth kinetic*

In previously studies, we enhanced the growth of callus culture from leaf explants (Fig. 1b) of *C. brasiliense* Cambess (Bernabé-Antonio *et al*., 2015). Those friable callus (40 days old) were used in this work to establish the cell suspension cultures (CSC) (Fig. 1c) in MS medium using 24.84 μM PIC plus 8.88 μM BAP and 0.5 % (w/v) fructose. The growth kinetics of the CSC was maintained until 16 days of culture, over which it showed a typical growth curve (Fig. 2). The adaptation phase lasted 4 days and exponential growth was observed at 12 days of culture. Moreover, the maximum accumulation of dry biomass weight (DW) was 15.17 g L^{-1} DW and this occurred on the same day 12 (Fig. 2). The curve growth pattern was similar to that reported by Pawar and Thengane (2009) in CSC from *Calophyllum inophyllum*. However, the growth time of *C. inophyllum* was up to 60 days and a low fresh biomass weight (FW) (1.4 g L^{-1} FW) was produced compared with *C. brasiliense*. The CSC from *C. brasiliense* were slightly yellowish during the time of maximum biomass accumulation and then and light brown in the death phase (after 12 days). In terms of the consumption of total sugars, a decrease was observed in the sugar content of the culture medium as the biomass increased; the remnants of the total sugars were stable during the death phase (Fig. 2).

3.2 *Total phenolic and flavonoid content*

The production of total phenolic and flavonoids were quantified over the course of 16 days according to the growth kinetics of the CSC (Fig. 2). In general, a relation linking an increase in total phenols, total flavonoids and biomass production was observed; nevertheless, a slight decrease was observed on the 10th day (Fig. 3). A similar behavior was observed in the total flavonoid content (Fig. 3).

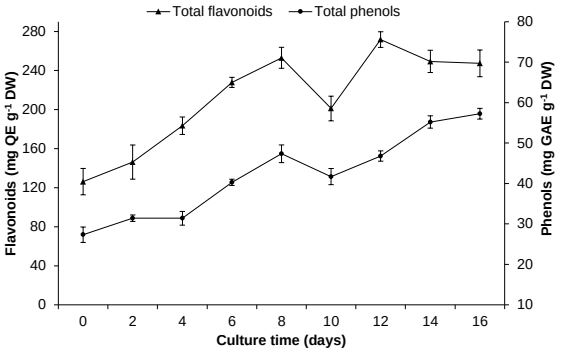

Fig. 3. Kinetic of production of total phenols and flavonoids content of a cell suspension culture from *C. brasiliense* leaves during 16 days of culture. Error bars represent standard deviation of 3 replicates.

It is likely that the decrease in total phenols and flavonoids of CSC from *C. brasiliense* during the exponential growth phase was since the cells could divert their metabolism to growth in biomass or to the biosynthesis of another group of secondary metabolites. Furthermore, it is known that in most plant cell cultures the largest amounts of secondary metabolites are accumulated in stationary-phase cultures (Ramawat and Mathur 2007).

In the present study, it was observed that during the growth kinetic of the CSC, the maximum production of total phenols (57.3 mg GAE g^{-1} DW) occurred at 16th day and the total flavonoids (271.8 mg QE g^{-1} DW) at 12th (Fig. 3); moreover, the production of these compounds was higher by 76.7 and 72.2%, respectively, compared to the leaves of wild plants (Table 1). This finding suggests the cell cultures of *C. brasiliense* retain the ability to produce secondary metabolites as reported in callus culture from *C. brasiliense* leaves (Bernabé-Antonio *et al*., 2015; 2010). Other studies have reported the biological activity of some flavonoids and phenolic compounds (e.g. catechin, epicatechin, gallic acid, protocatechuic acid) and extracts, but obtained from wild plants of *C. brasiliense*, which possess antibacterial, antiviral, anti-inflammatory, anticancer and antiallergic activities (Pretto *et al*., 2004; Souza *et al*., 2009). Similarly, in other species such as *Salvia hispanica* L. high amounts of total flavonoid content (268 mg QE/mg extract) have also been found. (Rivera-Cabrera *et al*., 2017).

3.3 Antioxidant activity of extracts

Phenolic acids and flavonoids are of special interest due to their excellent ability to scavenge free radicals (Bravo, 1998; Breyer *et al*., 2007).

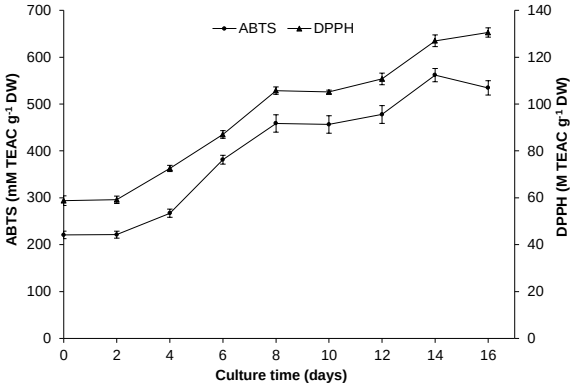

Fig. 4. Kinetic of antioxidant activity of cell suspension culture extracts from *C. brasiliense* during 16 days of culture. Error bars represent standard deviation of 3 replicates.

The antioxidant activities of MeEx from cell suspensions of *C. brasiliense* exhibited significant differences ($p \leq 0.05$) compared with those determined in wild plants. Moreover, the antioxidant activity of extracts from wild plants was lower to that detected in the cell cultures (Table 1). On the other hand, the antioxidant evaluation of cell suspension extracts demonstrated an increase in antioxidant activity on DPPH or ABTS radicals positively related with an increase in total phenols and/or total flavonoid contents produced during the growing time of the *C. brasiliense* cell suspension (Fig. 2, 3). Moreover, the maximum antioxidant activity of CSC extracts on ABTS (561.7 mM TEAC g^{-1} DW) and DPPH (130.5 M TEAC g^{-1} DW) radicals occurred at 14 and 16 days, respectively (Fig. 4). Other studies have reported antioxidant activities of leaf extracts from *C. brasiliense* as being 8,492 μM TEAC g^{-1} extract for ABTS and 2,651 μM TEAC g^{-1} extract for DPPH (Mesa-Venegas *et al*., 2010). Recently, it was demonstrated that extracts of *C. brasiliense* leaves obtained from a supercritical extraction had a higher antioxidant activity associated with a considerable amount of total phenolics extracted (Gonçalves *et al*., 2013). The production of these secondary metabolites can be enhanced for commercial purposes. In fact, Ali *et al*. (2013) established a CSC of *Artemisia absinthium* L. in which antioxidant compounds (i.e., gallic acid, caffeic acid and catechin) were produced; in CSC of *Marchantia linearis* Lehm & Lindenb flavonoids (quercetin, luteolin and apigenin) were also detected (Krishnan *et al*., 2014).

Table 1. Content of total phenols, total flavonoids and antioxidant activity of methanolic extracts from *Calophyllum Brasiliense* leaves.

Determinations	Plant resource	
	Cell suspension cultures*	Wild plants
Total phenols (mg GAE g^{-1} DW)	57.26 ± 1.3 a	32.4 ± 0.9 b
Total flavonoids (mg QE g^{-1} DW)	271.75 ± 8.0 a	157.8 ± 7.4 b
DPPH (M TEAC g^{-1} DW)	130.54 ± 1.9 a	96.3 ± 3.9 b
ABTS (mM TEAC g^{-1} DW)	534.53 ± 15.3 a	193.7 ± 3.4 b

Means ± standard deviation followed by the same letter within a row are statistically similar at p≤0.05 level according to Tukey's multiple range test. Values are means of triplicate determinations. *At 16 days of culture.

Table 2. Inhibitory activity of extracts, fractions and pure compounds from wild plants and CSC of *Calophyllum brasiliense*, on 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced mouse auricular edema.

Treatment	Sample	Dose (mg/ear)	Edema (mg)	Inhibition (%)	*p*
Control (TPA)	-	-	11.92 ± 1.54	-	-
Wild plant	MeEx	2	6.61 ± 1.28	44.49 ± 10.75	<0.0001
	AcEx	2	8.60 ± 1.14	27.83 ± 9.59	<0.001
	Canophyllol	1	8.72 ± 0.67	26.82 ± 5.67	<0.001
	Apetalic acid	1	7.94 ± 1.18	33.37 ± 9.97	<0.0001
CSC	DCMEx	2	7.22 ± 0.92	39.41 ± 7.74	<0.0001
Indomethacin	-	0.5	6.87 ± 0.53	42.30 ± 4.46	<0.0001

Values are means ± standard deviation of seven determinations. CSC: cell suspension cultures; MeEx: leaves methanolic extract; AcEx: leaves acetone extract; DCMEx: dichloromethanolic extract

3.4 Anti-inflammatory activity in the TPA model

In auricular edema, we employed the phorbol ester TPA (2.5 μg/ear) as a substance capable of causing local inflammation characterized by vasodilatation, cellular infiltration and erythema during the first 3 or 4 h after application of the toxic agent (Gábor, 2000). In the negative control group, the maximum level of edema as 11.92 ± 1.54 mg (100 %), which was evaluated as an increase in the weight of the auricular edema. Statistical analysis indicated that the treatments exhibited a significant reduction in the formation of edema compared with the negative control ($F_{0.05}$=100.73; <0.0001; Dunnet $test_{0.05}$=1.45). In this assay, indomethacin (positive control) showed 42.30 ± 4.46 % of inhibition at 0.5 mg/ear and the methanol (MeEx) and acetone (AcEx) extracts from leaves of *C. brasiliense* wild plants inhibited edema formation at a dose of 2.0 mg/ear; the largest effect was obtained with MeEx that showed 44.49 ± 10.75 % of inhibition while AcEx showed 27.83 ± 9.59 % of inhibition (Table 2). It has been reported that the juice of *C. inophyllum* fruits inhibits the activities of 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) enzymes, which produce leukotrienes and prostaglandins (inflammatory mediators), respectively, from arachidonic acid (Fylaktakidou *et al*., 2004; Zakaria *et al*., 2014). The inhibition of auricular edema by isolated compounds apetalic acid and canophyllol was significant with 33.37 ± 9.97 % and 26.82 ± 5.67 % of inhibition, respectively. Also, in other works the anti-inflammatory compound jacareubin y 6-desoxijacareubin were isolated from *C. brasiliense* and *C. inophyllum* (Gopalakrishnan *et al*., 1980). The anti-inflammatory activity of DCMEx (39.41 ± 7.74 % of inhibition at 2 mg/ear) from the cell suspensions is similar than that obtained from the leaf methanolic extract (MeEx) of wild plants (Table 2). In recent studies, extracts of cell suspension cultures and wild plant of *Buddleja cordata* exhibited values of 61.72 and 26.10 % antiinflammatory inhibition, respectively, at doses of 2 mg extract/ear, which showed statistically significant. (Gutiérrez-Rebolledo *et al*., 2018).

Conclusions

The establishment of a cell suspension culture of *C. brasiliense* is reported for the first time. MS culture medium supplemented with 24.84 μM PIC, 8.88 μM BAP and 0.5% fructose was suitable to establishment the cell culture. The production of phenolic compounds and total flavonoids is associated with the growth of culture, and these with the antioxidant activity. Cell culture extracts exhibited more FET, FLT and higher antioxidant activity than the wild plant. Regarding anti-inflammatory activity, the dichloromethane extract of CSC showed similar inhibition on auricular edema formation that the methanolic and acetone extracts of wild plant. Cell suspension cultures of *C. brasiliense* are the beginning for future studies to produce antioxidant and anti-inflammatory compounds.

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