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In vivo ANTI-INFLAMMATORY ACTIVITY AND ACUTE TOXICITY OF METHANOLIC EXTRACTS FROM WILD PLANT LEAVES AND CELL SUSPENSION CULTURES OF *Buddleja cordata* Kunth (Buddlejaceae)

ACTIVIDAD ANTI-INFLAMATORIA *in vivo* Y TOXICIDAD AGUDA DE EXTRACTOS METANÓLICOS DE HOJAS DE PLANTAS SILVESTRES Y CULTIVOS DE SUSPENSIONES CELULARES DE *Buddleja cordata* Kunth (Buddlejaceae)

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Abstract

Buddleja cordata is a species used by Mexican folk medicine for treating illnesses related to inflammation such as skin wounds and arthritis. It bio-synthesizes metabolites such as verbascoside that contributes to its ethno-therapeutic properties as anti-inflammatory remedy. HPLC analysis showed that the methanolic extract from cell suspension cultures (Bc-Cc) and wild plant leaves (Bc-Wp) contained verbascoside, but concentration was higher in Bc-Cc (87.48 mg/g) than in wild plant (47.34 mg/g). In the acute toxicity model, none of the extracts generated any lethality or adverse effects. In acute inflammation model induced with TPA, Bc-Cc extract showed a greater edema inhibition at 2 mg/ear (61.72%), as well for carrageenan model at 200 mg/kg (48.87%). Bc-Wp showed lesser anti-inflammatory effect in both acute inflammation models than Bc-Cc. For Adjuvant-induced arthritis both extracts at 250 mg/kg generated a moderate inhibition over edema (≈ 33%) at day 28, and they were statistically no different to phenilbutazone. The culture in suspension of *B. cordata* obtained by biotechnological process contains greater amount of verbascosido and showed better anti-inflammatory activity; thus, representing a source for obtaining this type of secondary metabolite of pharmacological interest.

Keywords: cell suspension culture, *Buddleja cordata*, verbascoside, anti-inflammatory activity, median lethal dose.

Resumen

Buddleja cordata es una planta medicinal usada en México para el tratamiento de enfermedades relacionadas con heridas en la piel y artritis. Esta especie bio-sintetiza metabolitos secundarios como el verbascósido, el cual contribuye a sus propiedades etno-medicinales. El análisis por HPLC mostró que el extracto metanólico del cultivo en suspensión celular (Bc-Cc) y de las hojas de la planta silvestre (Bc-Wp) contienen verbascósido, siendo su concentración mayor en el cultivo Bc-Cc (87.48 mg/g) que en la planta silvestre (47.34 mg/g). Ninguno de los extractos generó letalidad o efectos adversos en el ensayo de toxicidad aguda. En el modelo de inflamación tópica inducido con TPA, Bc-Cc mostró mayor actividad antiinflamatoria; a 2 mg/oreja generó 61.72% de inhibición y en el modelo de carragenina a 200 mg/kg generó 48.87%, mientras que Bc-Wp mostró menor actividad anti-inflamatoria en ambos modelos. En el modelo de artritis inducida con Adyuvante de Freud completo, ambos extractos a 250 mg/kg mostraron moderada actividad anti-inflamatoria, con inhibición de aprox. 33% al día 28. El cultivo en suspensión celular de *B. cordata* obtenido por proceso biotecnológico contiene mayor cantidad de verbascosido y mostró mejor actividad anti-inflamatoria; por lo que representa una fuente para la obtención de este tipo de metabolito secundario de interés farmacológico.

Palabras clave: cultivos de suspensión celular, *Buddleja cordata*, verbascósido, actividad anti-inflamatoria, dosis letal media.

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1 Introduction

During last two decades, study to support the ethno-medicinal use of plants as traditional remedies has increased. From this research, a growing number of new drugs have been developed through synthesis or semi-synthesis, employing, as structural prototype, Secondary Metabolites (SM) isolated from medicinal plants (Dakah *et al*., 2014).

In addition, novel investigation lines using biotechnology techniques have been established to develop *in vitro* cultures capable of producing SM, as well as to evaluate their pharmacological potential with a higher yield rate (Singh *et al*., 2009). In this context, there is great interest in discovering new drugs possessing beneficial pharmacological activities such as anti-inflammatory that those showed by commercialized allopathic drugs which have demonstrated significant secondary adverse side effects (Martínez-Vázquez *et al*., 1998; Pérez-Hernández *et al*., 2014).

Buddleja cordata Humb. Bonpl. & Kunth, commonly known as *Tepozán blanco*, has been used diversely in Mexican traditional medicine as a treatment for several health disorders, such as tumors, abscesses, sores, burn wounds, rheumatic pains, skin illness and inflammation related to arthritis. Today, these ethno-medicinal uses require support from scientific studies (Ávila-Acevedo *et al*., 2014).

Anti-inflammatory activity of some *Buddleja* species (Jensen, 2000; Pendota *et al*., 2013, Pendota *et al*., 2014) has been previously described; this biological activity was tested in preclinical murine models of acute inflammation induced by Carrageenan and skin wounds induced by surgical injury. The results showed that the aqueous and methanolic (MeOH) extracts obtained from whole wild plants (Wp) of *B. globosa* (Backhouse *et al*., 2008), *B. crispa* (Bukhari *et al*., 2016), and *B. polystachia* (Al-Ati *et al*., 2015) showed an important inhibitory effect. The inhibition was between 65 and 71%, on sub-plantar edema growth induced by Carrageenan in rats at doses of 50, 100, and 500 mg/kg administered by intraperitoneal (i.p.) route, as well as at doses of 300 and 600 mg/kg by intragastric (i.g.) route have a similar anti-inflammatory effect.

Additionally, aqueous extract from *B. cordata* exhibited anti-inflammatory activity on Carrageenan-induced edema in male Wistar rats at 10, 50, and 100 mg/kg when was administered via i.g. route, with an inhibitory effect on paw-edema development of 34.3, 42.4, and 50.5%, respectively (Martínez-Vázquez *et al*., 1998).

The SM produced by *B. cordata*, terpenoid- and phenolic-type, have been related with its medicinal uses, such as aucubin and methylcatalpol iridoids, sesquiterpenes, sitosterol steroids, linarin, flavones, hydroxycinnamic acids and verbascoside (Vb), with the knowledge that these SM have been reported as anti-inflammatory agents (Akdemir *et al*., 2011; Jensen, 2000; Martínez-Vázquez *et al*., 1998; Sánchez *et al*., 2013).

The *in vitro* cell suspension cultures obtained from roots, callus and leaves tissues of *B. cordata* produced linarin, some hydroxycinnamic acids (caffeic, p-coumaric, ferulic, and sinapic acids) and Vb, in higher concentration than Wp. The production of these SM depended on the type of *in vitro* cell culture; these *in vitro* cultures produced a high amount of Vb 116.36 mg/g dried weight (DW), while the contents of linarin and hydroxycinnamic acid were <3.01 mg/g DW (Estrada-Zúñiga *et al*., 2009). This is the first study that compares and quantifies the content of verbascoside between a wild plant and a sample of material produced by a biotechnological process (Wp vs. Cc). In addition, it is the first report that describes the comparison of the anti-inflammatory activity of Bc-Wp vs. and Bc-Cc.

To date there are no published reports related to the anti-inflammatory activity of extracts obtained from cell suspension cultures either of *B. cordata* or for the leaves of Wp in murine models of acute and chronic inflammation. The aim of this work was to evaluate the anti-inflammatory activity of MeOH extracts from Wp leaves and cell suspension cultures of *B. cordata* for comparison in order to evaluate the potential of a cell culture to produce anti-inflammatory bioactive metabolites, to determine their acute toxicity *in vivo*, and compare the concentration of their major SM by *in vitro* and HPLC techniques.

2 Materials and methods

2.1 Plant materials

Leaves from approximately 2-year-old plants were collected from Santa María Village in Apaxco, State of Mexico, Mexico, in January 2017. A sample was deposited at the Universidad Autónoma Metropolitana-Iztapalapa Campus (UAM-

I) Herbarium, identified as *Buddleja cordata* Kunth (Buddlejaceae), and was registered with specimen number 61170. Dried biomass from the cell culture of *B. cordata* was provided by the Centro de Investigación en Recursos Bióticos (CIRB)-Facultad de Ciencias de la Universidad Autónoma del Estado de México (UAEMex) and the UAM-I Biotechnology Laboratory. The cell culture has been previously established by Estrada-Zúñiga *et al*. (2009). Cells from culture were collected every 15 days after sub-cultivation due to this was established as the time in which *B. cordata* cell suspension reach its maximum production of verbascoside in liquid medium.

2.2 Preparation of extracts

Wild *B. cordata* leaves (250 g) were dried at room temperature and then were macerated with MeOH (5 L) for 2 weeks with constant shaking (three times). The MeOH extract was filtered and concentrated at 40°C using a rotary evaporator (Buchii RE-111) coupled to a vacuum system (BuchiiVacV-153) and a cooling system (ECO20). The Bc-Wp extract was maintained under conditions of darkness until its use. Dried biomass from cell suspension cultures (50 g) was macerated in MeOH (3 L) to obtain the final extract (Bc-Cc). Elaboration of the MeOH extract was the same as that described previously for wild plant.

2.3 High Performance Liquid Chromatography (HPLC) analysis

Prior to use, each MeOH extract and standard solution was filtered (0.45 μm, nylon filter) for HPLC analysis as described by Estrada-Zúñiga *et al*. (2009; 2016). Waters HPLC system with 717 plus Autosampler, 1525 Binary Pump (Waters, USA), 2847 Dual λ Absorbance Detector (Waters, USA) equipped with a Kromasil C18 column (250 mm x 4.6 mm, 5 μm; Supelco, Sigma Chemical Co., USA) was used. Mobile phase: 2% (v/v) acetic acid solution (solvent A) and acetonitrile (solvent B); a sample injection volume of 10 μL (samples of 1,500 μg/mL), a flow rate of 1.1 mL/min, and a wavelength of 330 nm for the detector. The system was run with a gradient program as follows: 2 min, 100% to 88% A; 5 min, 88% to 75% A; 5 min, 75% to 0% A, and 3 min, 0% to 100% A. Stock standard solutions (0.22 mg/mL) of Vb (Extrasynthese, France) were prepared to obtain the calibration curve (40, 75, 120, and 220 mg/mL; $y = 13570x - 361269$; $R^2 = 0.9947$). Standard and MeOH extracts were run in the same condition. CHEMSTATION chromatography software (Rev. A.08.03; Agilent Technologies) was utilized to acquire data from the detector. For detecting, identifying, and quantifying verbascoside (main metabolite) from samples, peak areas at the corresponding standard Retention time (*Rt*) were employed to determine their concentration from the prepared calibration curve. Each sample was injected three times ($n = 3$).

2.4 *in vitro techniques*

2.4.1 *Total phenolic content (TPC)*

This assay was determined using the Folin-Ciocalteu reagent with the method previously described by McDonald *et al*. (2001). Each experiment was carried out by triplicate at 800 μg/mL for each MeOH extract, absorbance results were interpolated on a standard curve using gallic acid as reference (range, 150 - 2,500 μg/mL), in the equation $y = 0.0227x + 0.1736$, where "y" is the absorbance values obtained from each sample and "x" is the value of the μg of gallic acid equivalents (GAE)/g of dried extract ($R^2 = 0.9927$).

2.4.2 *Total flavonoid content (TFC)*

The aluminum chloride colorimetric method used for flavonoid determination was described by Chang *et al*. (2002). Each experiment was carried out by triplicate at 2,400 μg/mL for each MeOH extract, while the absorbance results were interpolated on a standard curve using quercetin as reference (range, 150 - 2,500 μg/mL) in the equation $y = 0.001x + 0.0759$, where "y" is the absorbance value obtained from each sample and "x" is the value of μg of quercetin equivalents (QE)/g of the dried extract ($R^2 = 0.9969$).

2.5 *In vivo animal assays*

Adult Balb/C mice (25 ± 2 g) of both sexes were used for the preclinical acute toxicity and acute/chronic inflammation models. The mice were obtained from the Animal Vivarium of CMN-SXXI, IMSS, in Mexico City and were maintained in plastic cages during a 7-day conditioning period prior to the experiments under laboratory conditions (12h/12h light/dark cycles; temperature, 25 ± 2°C; humidity 55-80%) with Rodent Chow food and water *ad libitum*. Experiments were performed following the statutes of the International Committee for the Care and Use of Laboratory Animals (IACUC) and of Mexican Official Norm (NOM-062-ZOO-1999), revised in 2017.

2.5.1 *Acute toxicity*

This test was performed according to Procedure TG423 described by the OECD and Test Guidelines as described by Gutiérrez-Rebolledo *et al*. (2016). Groups of three animals (n = 3) were employed and the MeOH extracts of the Bc-Wp and Bc-Cc were administered by i.g. route after a fasting period of 12 h. Control received only the vehicle (Tween 80-:water, 1:9), and the treated groups received a single administration of MeOH extract at 2, 1, and 0.5 g/kg Body Weight (BW) doses, in a volume not exceeding 10 mL/kg BW. The animals were maintained under observation for 14 days and their BW gain was recorded on days 3, 7, 9, and 14. After that, animals were euthanized, and liver, stomach, spleen, and both kidneys were extracted, and macroscopic observation was conducted to find gross pathological lesions and relative weight changes. This experiment was performed in two independent tests for each group to establish the median lethal dose (LD_{50}), according to the parameters described by the Globally Harmonized Classification System for Chemical Substances and Mixtures categories in which this value is established as a range of doses related to lethality and toxicity signs in the laboratory animals: Category 1 (> 0 - 5 mg/kg), Category 2 (> 5 - 50 mg/kg), Category 3 (> 50 - 300 mg/kg), Category 4 (> 300 - 2000 mg/kg) and Category 5 (> 2000 - 5000 mg/kg), being 2000 mg/kg the dose limit admitted for this kind of preclinical studies.

2.5.2 *12-O-tetradecanoylphorbol-13-Acetate (TPA)-induced ear edema*

This assay was conducted as described by Gutierrez-Rebolledo *et al*. (2016). Control was treated with TPA (2.5 μg) in acetone on the right ear (Ws) and then the left ear received only 25 μL of acetone (Wo). The experimental groups (n = 7) received TPA and 30 min later, both MeOH extracts, or Indomethacin (0.5, 1, and 2 mg/ear), were applied into the right ear (Ws′), while their left ear received only 25 μL of acetone (Wo′). Treatments 1 (Bc-Cc) and 2 (Bc-Wp), were carried out in independent experiments with their respective controls. Anti-inflammatory activity was calculated according to the weight difference (mg) between the ear sections (6 mm) at 6 h, compared to control group, using the following formula:

$$\%Inhibition = \left[\frac{(Ws - Wo)\text{TPA control} - (Ws' - Wo')\text{TPA treated}}{(Ws' - Wo')\text{TPA control}}\right] \times 100 \quad (1)$$

2.5.3 *Carrageenan-induced sub-plantar edema in mice*

This model was performed as described by Gutiérrez-Rebolledo *et al*. (2016). Treated groups (n = 7) received, by i.g. route, Indomethacin (10 mg/kg), and both MeOH extracts (10, 25, 50, 100, and 200 mg/kg) 1 h prior to Carrageenan injection (20 μl, 2%). Treatments were solubilized in Tween 80:water (1:9), and the control received vehicle alone; all animals were administered in a volume not exceeding 10 mL/kg BW. Treatments 1 (Bc-Cc) and 2 (Bc-Wp) were carried out in independent experiments with their respective controls. Percentage of inhibition was calculated by comparing the measurement of paw edema at different times (1, 2, 3, 5, and 7 h) (Et), using a digital micrometer and the value of time zero (baseline) (E0) was determined in mm. The results were analyzed with the following formula:

$$\%Inhibition = \left[\frac{(Et - E0)\text{Carrageenan control} - (Et - E0)\text{Carrageenan treated}}{(Et - E0)\text{Carrageenan control}}\right] \times 100 \quad (2)$$

2.5.4 *Median Effective Dose (ED_{50})*

ED_{50} was determined by means of the data obtained from the previously above described experiments and their dose-response curves for TPA and Carrageenan (Gutiérrez-Rebolledo *et al*., 2016; Perazzo *et al*., 2013). This method was employed to evaluate the effectiveness of the MeOH extracts of *B. cordata* in relation to the inhibition of the inflammatory process by comparison with the Indomethacin groups.

2.5.5 *Complete Freund's Adjuvant (CFA)-induced arthritis*

This model was carried out according to Rasool *et al*. (2006), with modifications. All groups (n = 7)

were injected subcutaneously (s.c.) with 25 μL of CFA in the right hind paw on days zero and 14 (reinjection). Treatment groups were administered by i.g. route with phenilbutazone (PBZ, 100 mg/kg, reference drug), and the MeOH extracts of the Bc-Cc and Bc-Wp samples [250 mg/kg (ED_{50} value from acute inflammation induced by Carrageenan)], daily from day 7 to 27. A group of mice was not injected with CFA and was administered by i.g. route with vehicle only to obtain healthy animals. All treatments were solubilized in Tween 80:water (1:9), healthy-animal group and arthritic animals without treatment received vehicle alone. Paw edema was measured at different times (1, 4, 7, 14, 15, 21, and 28 days) (Et) using a digital micrometer (Mitutoyo model 293-831) and a value of day zero (E0) was determined in mm. Edema percentage of inhibition in each group was calculated from day 14 to day 28 and was compared with that of CFA group without treatment as follows:

$$\%Inhibition = \left[\frac{(Et - E0)\text{CFA control} - (Et - E0)\text{CFA treated}}{(Et - E0)\text{CFA control}}\right] \times 100 \quad (3)$$

BW gain was also registered on the same days. BW gain was calculated using each weight measurement at different times (1, 4, 7, 14, 15, 21, and 28 days) compared to baseline weight (day zero) prior to CFA injection. Then the differences (g) were employed to make a graph. Finally, on day 28 of the experiment, the mice were euthanized, and the edema tissue and popliteal ganglion nearest to the edema were extracted to calculate their relative weight. Relative weight (%) was calculated utilizing the total weight of both tissues (g) related to the total BW of the mice on day 28.

2.6 *Statistical analysis*

NCSS software was utilized for statistical analyses of the percentage of callus- or root-induced response, growth parameters, and phenylpropanoid concentration. One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple-media comparison tests were executed. A p value of <0.05 was assumed for significant differences. All of the experiments and measurements were done by triplicate. Sigma Plot ver.12.0 statistical software (2011-2012) was utilized for the analysis of the results. Data was presented as standard error of the mean (SEM). BW gain values in the acute toxicity test, BW gain and paw edema development during experimental arthritis were analyzed with bifactorial ANOVA, with a post-hoc Student-Newman-Keuls (SNK) test. On the other hand, organ relative weight was evaluated through ANOVA on ranks test, with a post-hoc SNK. Finally, for the development of paw edema in the Carrageenan model and ear-edema weight in TPA, one-way ANOVA was employed with a post-hoc SNK test. Results of p <0.05 were considered statistically significant.

3 Results and discussion

3.1 *Phenolic compound quantification: Verbascoside, and total phenol and flavonoid content*

The chemical profile of a medicinal plant is directly related to its traditional uses, biological activities, and pharmacological properties. In this context, the ethno-medicinal properties, such as anti-inflammatory, could be attributed to some SM bio-synthetized by *B. cordata*, such as Vb. Bc-Cc showed a Vb concentration of 20.48 ± 1.84 mg/g extract DW, while Bc-Wp exhibited 6.20 ± 0.77 mg/g extract DW. Additionally, the yield of resulting MeOH extracts was 43.26 and 7.06% (g of extract/g initial biomass DW), respectively. By HPLC analysis, it was demonstrated that the cell culture has the capacity to produce a greater amount of Vb than Wp (Figure 1A and 1B), and these results were related to total Vb concentration, which was higher for Bc-Cc (87.88 ±5.14 mg/g initial biomass DW) than for Bc-Wp (47.34 ±2.66 mg/g initial biomass DW). On the other hand, TPC test demonstrated that Bc-Wp had a greater amount of these compounds (45.47 ± 0.04 mg GAE/g DW of extract) than Bc-Cc extract (3.85 ± 0.01 mg GAE/g extract DW). This same behavior was observed for TFC test in which Bc-Wp exhibited higher values (937.85 ± 0.02 μg QE/g extract DW) compared to Bc-Cc results (20.35 ± 0.01 μg QE/g extract DW). These results revealed that, unless the leaves from Wp produced higher concentrations of SM, such as polyphenols and flavonoids than cell culture, this last one could bio-synthesize to nearly double the amount of Vb than Wp leaves, and at a high purity level which

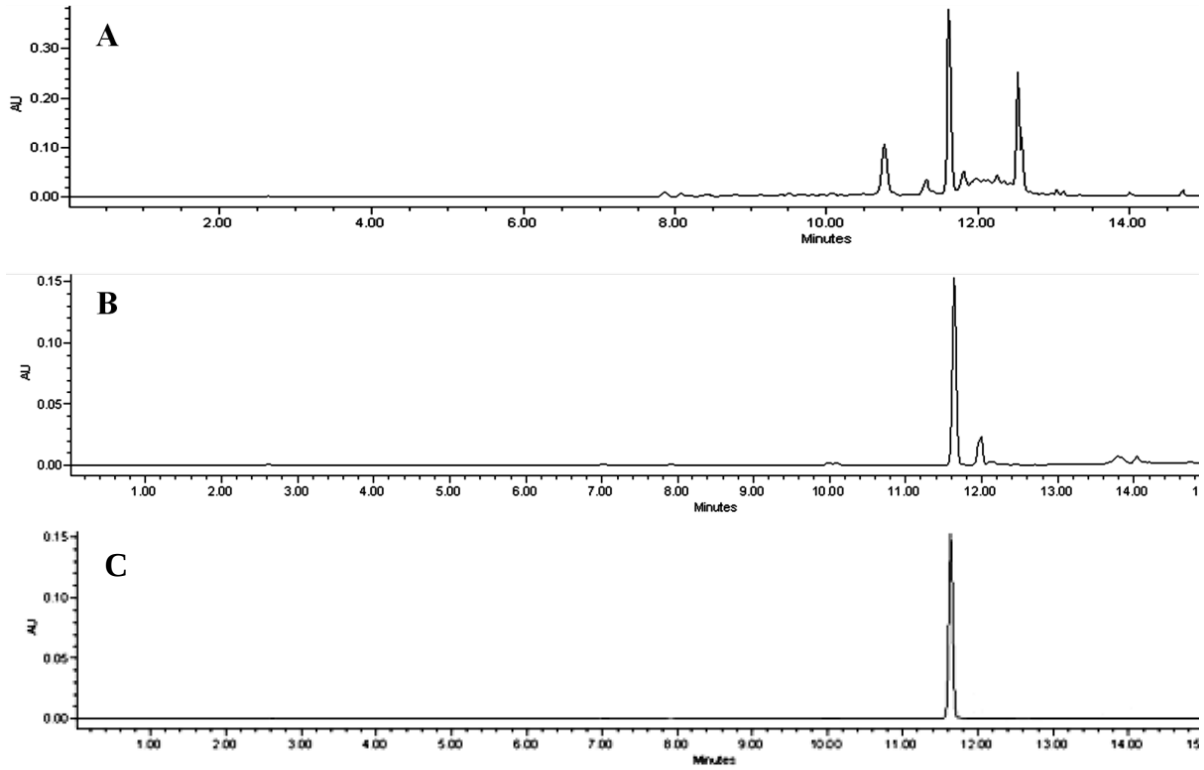

Fig. 1. HPLC Chromatograms of MeOH extracts from Wild plant *B. cordata* leaves (Bc-Wp) and its Cell suspension culture (Bc-Cc). A) Chromatogram of MeOH extract from Wild plant *B. cordata* leaves (Bc-Wp). B) Chromatogram of MeOH extract from *B. cordata* Cell suspension culture (Bc-Cc). C) Vb standard with Rt = 11.6 min.

could be obtained and isolated in MeOH extract, as illustrated in HPLC chromatograms (Figs. 1A and 1B).

From which is described previously, it appears that Bc-Cc underwent a decrease in both phenolic and flavonoid content compared to Bc-Wp. These results match those found by Estrada-Zúñiga *et al.* (2016), in which the authors compared TPC of MeOH extracts from Wp *B. cordata* aerial parts, greenhouse-cultivated plants, and micropropagated plantlets obtained biotechnologically *in vitro*. The results demonstrated that greenhouse plants exhibited values of 84.2 mg GAE/g, followed by Wp with 36.6 mg GAE/g, and finally, micropropagated plantlets 24.8 mg GAE/g of dried extract. The authors adjudicated these results to the decrease in TPC data and also a low antioxidant capacity, in terms of the different conditions under which each plant material was grew, such as environmental conditions and interactions, due to phenolic compounds are SM that the plant synthesized as a response to an external stimulus, such as an attack by microorganisms and nutrient competition with other plant species, which change their biochemical and physiological processes.

3.2 Acute toxicity

None of the tested doses (2, 1, and 0.5 g/kg) of Bc-Wp and Bc-Cc generated lethality or adverse effects on BW gain (data not shown), as well neither of both extracts modified behavior in mice during the 14-day observation period after a single i.g. administration. No macroscopic alterations were observed either in the main analyzed organs after euthanasia or in differences in the relative weights of these major organs among the groups (data not shown).

Although *in vitro* culture of cells obtained from medicinal plants is a line of investigation that is under constant development, to date there are scarce studies that evaluate toxicity of these cell suspensions and extracts made from them. In addition, there are few papers in which a biological activity *in vivo* of these cell suspensions extracts is tested (Pramanik and Chatterjee, 2009). Such studies are needed because, through biotechnological process, SM concentrations increase in plant tissues or in cell suspensions and these could generate toxic effects *in vivo*. As an example, LD_{50} value of MeOH extract from cell

suspension cultures obtained from leaves of *Pluchea indica* was 2.825 g/kg when administered i.g. in rats (Pramanik and Chatterjee, 2009), while LD_{50} in rats of aqueous and Ethanolic (EtOH) leaf extracts from Wp *P. indica* were 12.30 g/kg and 6.15 g/kg, respectively (Pramanik *et al*., 2007).

In this work, both Bc-Wp and Bc-Cc showed a LD_{50} value of >2 g/kg when administered by i.g. route in mice; thus, they are considered a Category 5 substance according to the Organization of Economic Co-operation and Development (OECD) Test Guideline 423 for compounds whose possible toxic or lethal effect in a single administration is between >2,000 and <5,000 mg/kg by i.g. route (Arthur *et al*., 2011). Nonetheless, further toxicological studies are needed to ensure their safety. It is noteworthy that LD_{50} value for main metabolite Vb produced by Wp and cell suspension cultures of *B. cordata*, was established in other studies as >5 g/kg in mice via i.p. administration (Etemad *et al*., 2015).

To date, there are few studies that support the safety of single administration of *Buddleja* species. One of this evaluated acute toxicity of EtOH extract from leaves and bark of wild *B. asiatica* Lour. in albino mice of either sex at doses of 500, 1,000, and 2,000 mg/kg of each extract by i.g. route. The authors found that, even at the highest dose, animal exhibited no lethal or adverse effects (Ullah *et al*., 2014). However, a greater number of subsequent toxicological studies are required to ensure the use of these suspension cell cultures obtained through biotechnology.

3.3 Anti-inflammatory and anti-arthritic evaluation

3.3.1 TPA model

One of the main uses of aerial parts of *Buddleja* species include aiding in wound healing (Jensen, 2000). In Mexican folk medicine, these aerial parts are also used to treated diseases and inflammations related to skin (Acevedo *et al*., 2000). Bc-Cc extract showed an important beneficial effect when applied topically that was dose-dependent. For 2-mg/ear dose, a value of 61.72% inhibition on ear-edema development was observed, statistically similar to that generated by Indomethacin at same dose (57.35%). However, the anti-inflammatory activity of Bc-Wp extract at same dose was lower (26.10%) than the effect of Indomethacin (58.25%), and it was not dose-dependent because all of the tested doses of this extract generated a similar statistical effect on ear-edema growth (Table 1). ED_{50} values for Bc-Cc and Bc-Wp were 1.26 and 3.93 mg/ear, respectively, while for Indomethacin in both experiments, ED_{50} was ≈1.47 mg/ear for a single topical administration in both experiments (Table 1).

These results are better than data reported for *B. globosa* aerial parts MeOH extract evaluated for TPA model, the anti-inflammatory effect was 56.7 % inhibition at 3 mg/ear (Backhouse *et al*., 2008). Also, main metabolite Vb, has been evaluated previously in mice TPA model, being isolated from ethyl acetate extract of *Castilleja tenuiflora*. Subsequently, it generated 49% inhibition on ear edema formation at 1.6 mg/ear dose (Sánchez *et al*., 2013), authors said that this effect was through the inhibition of important biochemical pathways such as Nuclear Factor-kappa B (NF-κB) expression. Compared to the results obtained in present work, only Bc-Cc MeOH extract exhibited a similar effect to that of *C. tenuiflora*-isolated Vb at a similar dose. From minor SM previously identified in *B. cordata* (Estrada-Zúñiga *et al*., 2009), linarin has shown to down-regulate the production of pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α), Interleukin (IL)-1β, and IL-6, as well as the release of Nitric Oxide (NO), all of which are involved in acute inflammation process (Kim *et al*., 2016b), related to the mechanism of action through which TPA generate edema formation.

3.3.2 Carrageenan model

In this experiment Bc-Cc generated a dose-dependent anti-inflammatory effect on paw-edema formation, with best response at dose of 200 mg/kg (48.87%), with no statistical differences compared to Indomethacin (54.11%). At hour 5 of the model, the same dose-dependent behavior was observed for Bc-Wp, however, this showed less anti-inflammatory activity compared to Bc-Cc, its major activity observed at 200 mg/kg dose (39.80%), nonetheless, this was lower than the inhibition achieved by Indomethacin (50.01%) (Table 2). ED_{50} values for Bc-Cc and Bc-Wp were 204.62 and 251.26 mg/kg, respectively, for hour 5 (Table 2). These results indicated that a lesser quantity of Bc-Cc extract generated better anti-inflammatory activity than Bc-Wp with an inhibition nearly to 50% over paw-edema development.

Backhouse *et al*. (2008), demonstrated that MeOH fraction obtained from MeOH extract of aerial parts from *B. globosa* Wp generated an inhibition of 61.40% on edema formation in third hour after its

Table 1. Anti-inflammatory effect of MeOH extract of leaves from *Buddleja cordata* Wild plant (Bc-Wp) and its Cell suspension culture (Bc-Cc) on the development of ear edema induced by TPA (mg).

Treatments 1	Doses (mg/ear)	Auricular edema formation (mg)	Inhibition percent (%)	ED_{50} (mg/ear)
TPA Control	-	16.88±0.76	-	-
Indomethacin	0.5	12.65±0.32[a]	25.05	1.47 (R^2 = 0.99)
	1	10.54±0.45•[a]	37.56	
	2	7.20±0.68•*[a]	57.35	
Bc-Cc MeOH extract	0.5	13.04±0.33[a]	22.15	1.26 (R^2 = 0.98)
	1	9.00±0.76•[ab]	46.68	
	2	6.46±0.38•*[ab]	61.72	
Treatments 2				
TPA Control	-	23.30±0.56	-	-
Indomethacin	0.5	16.93±0.32[a]	27.03	1.48 (R^2 = 0.99)
	1	14.06±0.34•[a]	39.41	
	2	9.69±0.60•*[a]	58.25	
Bc-Wp MeOH extract	0.5	17.87±0.56[a]	22.90	3.93 (R^2 = 0.95)
	1	17.29±0.67[ab]	25.49	
	2	17.14±0.42[ab]	26.10	

Data presented as mean (±) standard error (s.e.). The percent of inhibition edema is with respect to control group. Statistical analysis two ways ANOVA, post hoc SNK tests ($p \leq 0.05$). Each treatment (1 and 2) were carried out in independent experiments Treatments 1: · vs 0.5 mg; * vs 1 mg; a vs TPA control; b vs Indomethacin Treatments 2: · vs 0.5 mg; * vs 1 mg; a vs TPA control; b vs Indomethacin Bc-Cc, Cell suspension culture MeOH extract of *Buddleja cordata* Bc-Wp, *Buddleja cordata* Wild plant MeOH extract n = 7 per group.

Table 2. Anti-inflammatory effect of MeOH extract of leaves from *Buddleja cordata* Wild plant (Bc-Wp) and its Cell suspension culture (Bc-Cc) on the development of Carrageenan-induced acute inflammation (mm).

Treatments 1	Doses (mg/kg)	Paw edema formation (mm) T_{5h}	Inhibition percent (%)	ED_{50} (mg/kg)
Carrageenan Control	-	1.10±0.05	-	-
Indomethacin	10	0.50±0.01[a]	54.11	10
Bc-Cc MeOH extract	10	1.00±0.01[ab]	9.09	204.62 (R^2 = 0.98)
	25	0.92±0.01[abc]	16.36	
	50	0.69±0.06[abcd]	29.67	
	100	0.75±0.05[abcd]	23.76	
	200	0.56±0.02[abcdef]	48.87	
Treatments 2				
Carrageenan Control	-	1.00±0.05	-	-
Indomethacin	10	0.50±0.01[a]	50.01	10
Bc-Wp MeOH extract	10	0.91±0.02[ab]	9.00	251.26 (R^2 = 0.97)
	25	0.77±0.02[ab]	17.63	
	50	0.72±0.01[abc]	21.00	
	100	0.73±0.01[abc]	20.16	
	200	0.61±0.01[abcdef]	39.80	

Each group represents the mean (±) and standard error (s.e.). The application was by intragastric route. Statistical analysis two-way ANOVA, post hoc SNK test ($p \leq 0.05$). Each treatment (1 and 2) were carried out in independent experiments Treatments 1: [a] vs Carrageenan control. [b] vs Indomethacin. [c] vs Cc 10 mg/kg. [d] vs Cc 25 mg/kg. [e] vs Cc 50 mg/kg. [f] vs Cc 100 mg/kg Treatments 2: [a] vs Carrageenan control. [b] vs Indomethacin. [c] vs Cp 10 mg/kg. [d] vs Cp 25 mg/kg. [e] vs Cp 50 mg/kg. [f] vs Cp 100 mg/kg NE, no effect Bc-Cc, Cell suspension culture MeOH extract of *Buddleja cordata* Bc-Wp, *Buddleja cordata* Wild plant MeOH extract n=7 per group.

i.g. administration at dose of 600 mg/kg in Guinea pigs. Later, Bukhari *et al*. (2016), showed that, in hour 3 after i.p. administration of aqueous-MeOH extract from whole wild plant of *B. crispa*, paw-edema growth was inhibited by 65% at 200 mg/kg dose in rats. Finally, Al-Ati *et al*. (2015) found that aqueous fraction obtained from EtOH extract from Wp *B. polystachya* aerial parts inhibited paw edema formation by 60.70% in second hour after i.p. administration at 500 mg/kg dose in rats. Compared to the results reported by Bukhari *et al*. (2008), and Al-Ati *et al*. (2015), the anti-inflammatory effect generated by those tested extracts in Carrageenan murine model was lower than that of MeOH extract from Bc-Wp aerial parts (39.80%) at a 200 mg/kg dose, perhaps due to differences between plant species and their phytochemical profiles.

To explain through which possible mechanisms this systemic anti-inflammatory effect is taking place, we think is due to Vb because this major metabolite of *B. cordata* by itself generated inhibition in paw edema development of 21.50 and 27% at hours 4.5 and 6, respectively, after its administration by i.g. route in rats at 200 mg/kg (Akdemir *et al*., 2011). Also by linarin, a minor SM identified in *B. cordata*, which demonstrated in previous work an anti-inflammatory effect in Carrageenan model in mice with an ED_{50} value of 0.6 mg/kg administered i.g. The authors describe and establish that this beneficial effect is due to inhibition of inflammation-induced cyclooxygenase (COX-2), and arachidonic acid (AA) pathway (Martínez-Vázquez *et al*., 1998), both related to the paw edema-induction mechanism of Carrageenan.

3.3.3 CFA-induced arthritis model

Un-treated arthritic mice generated a maximal edema diameter of 1.59 ± 0.09 mm on day 15 (after CFA reinjection on day 14) and 1.38 ± 0.03 mm on day 28, compared to healthy animals which only received vehicle and that did not receive sub-plantar injection of CFA. This reinjection was performed in order to maintain the chronic inflammatory condition for a period of 28 days, while all the groups that received the treatments showed the following day (day 15) a percentage of inhibition of ≈16% compared to arthritic untreated group of animals, after being administered for two weeks since day 7.

This group was used as experimental arthritis control to determine the beneficial effect of treatments (PBZ, Bc-Cc and Bc-Wp MeOH extracts).

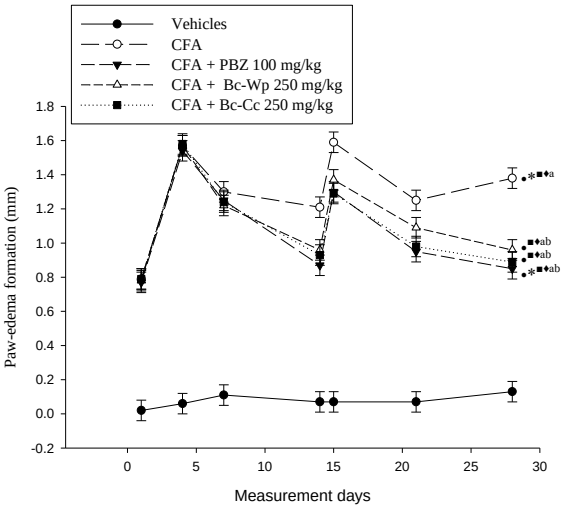

Fig. 2. Anti-inflammatory effect of MeOH extracts from Wild plant *B. cordata* leaves (Bc-Wp) and its Cell culture (Bc-Cc) on paw-edema development in male BALB/C mice during experimental arthritis. Data shown as mean ± Standard Error of the Mean (SEM). Treatments were administered daily by intragastric (i.g.) route from day 7 to day 28. Two-way ANalysis Of VAriance (ANOVA) of Repeated Measures (RM), post-hoc Student-Newman-Keuls (SNK), ($p \leq 0.05$) ^{a} vs. VEHicles (VEH); ^{b} vs. CFA control; ^{c} vs. CFA+PBZ; ^{d} vs. CFA+MeOH Bc-Wp 250 mg/kg ·vs. day 7; *vs. day 14; ■vs. day 15; ♦vs. day 21 CFA: Complete Freund's Adjuvant PBZ: PhenylButaZone Bc-Wp, *Buddleja cordata* Wild plant Bc-Cc, Cell suspension culture *Buddleja cordata* n= 7 per group.

Statistical analysis revealed that all three treatments generated moderate anti-arthritic effect on induced mice compared to the untreated arthritic group; however, there were no statistical differences between them. In this context, PBZ-administered mice showed inhibition in paw edema development of 38.42% at day 28, while Bc-Cc and Bc-Wp exhibited inhibition values of 30.26 and 35.46%, respectively, compared to un-treated CFA-induced group on day 28 (Fig. 2). None of the treatments were statistically similar to data shown by healthy animals at the end of the experiment.

CFA-induced experimental arthritis was previously described for mice, where authors sustained that edema development was related to an increase in neutrophils and lymphocyte $CD4^{++}$ infiltration, and to increased production of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, and interferon gamma [INF-γ]) (Rasool *et al*., 2006).

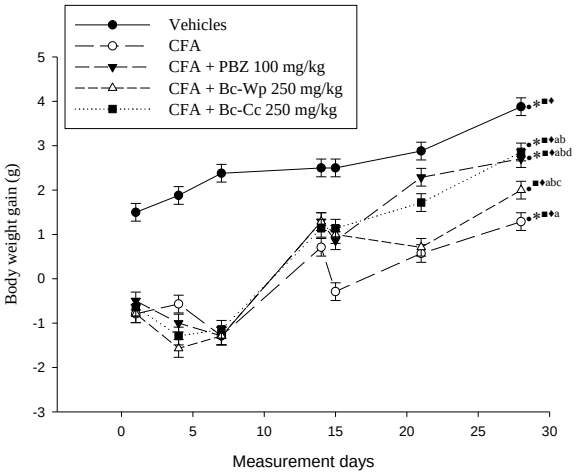

Fig. 3. Effect of MeOH extracts from Wild plant *B. cordata* leaves (Bc-Wp) and its Cell suspension culture (Bc-Cc) on body weight gain in male BALB/C mice during experimental arthritis. Data shown as mean ± Standard Error of the Mean (SEM). Treatments were administered daily by intragastric (i.g.) route from day 7 to day 28. Two-way ANalysis Of VAriance (ANOVA) of Repeated Measures (RM), post-hoc Student-Newman-Keuls (SNK), ($p \leq 0.05$) ^{a} vs. VEHicles (VEH); ^{b} vs. CFA control; ^{c} vs. CFA+PBZ; ^{d} vs. CFA+MeOH Bc-Wp 250 mg/kg ·vs. day 7; *vs. day 14; ■vs. day 15; ♦vs. day 21 CFA: Complete Freund's Adjuvant PBZ: PhenylButaZone Bc-Wp, *Buddleja cordata* Wild plant Bc-Cc, Cell suspension culture *Buddleja cordata* n= 7 per group.

Beneficial effect generated by both MeOH extracts prepared from Wp (Bc-Wp) and cell suspension cultures (Bc-Cc) of *B. cordata* may be due to their content of their main phenylpropanoid Vb. In an early work, Lenoir *et al*. (2011), found that aqueous extract of leaves from *Aloysia triphylla* (*Lemon verbena*) with a high content of Vb generated a decrease of chronic inflammation damage in a model of dextran sulfate sodium-induced colitis in Wistar rats at a dose of 5% of extract in their drinking water. It also reduced oxidative damage and favored regulation of antioxidant enzymes activity in intestinal tissue.

Another parameter that was measured during experimental arthritis was BW gain for each group, the differences between total weights of each day were compared to baseline weight before CFA paw injection. Results exhibited that un-treated arthritic mice showed lower BW gain (1.29 g) compared to that of the healthy group (3.88 g) at day 28.

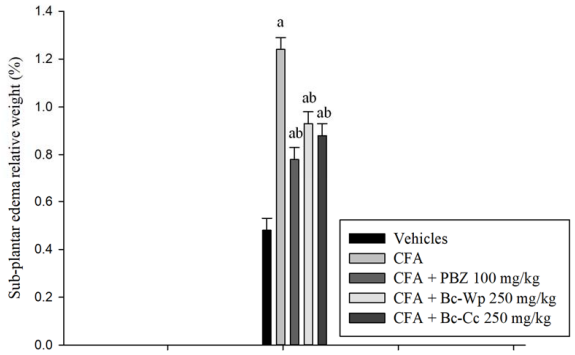

Fig. 4. Effect of MeOH extracts from Wild plant *B. cordata* leaves (Bc-Wp) and its Cell suspension culture (Bc-Cc) on relative weight of edema tissue of male BALB/C mice during experimental arthritis. Data shown as mean ± Standard Error of the Mean (SEM). Values in parentheses showed relative weight of each tissue related to the total animal body weight on day 28 of the experiment. ANOVA on ranks, Post-hoc SNK ($p \leq 0.05$) a vs Vehicles (VEH); bvs. CFA control; cvs. CFA+PBZ; dvs. CFA+ MeOH Bc-Wp 250 mg/kg CFA: Complete Freund's Adjuvant PBZ: PhenylButaZone Bc-Wp, *Buddleja cordata* Wild plant Bc-Cc, Cell suspension culture *Buddleja cordata* n = 7 per group.

All treatments (Bc-Cc, PBZ, and Bc-Wp) generated a statistical increase in BW gain of 121.71, 110.08, and 55.04%, respectively, in arthritic-treated mice compared to un-treated arthritic group; however, none of them reach similar values to those of the BW gain of healthy animals at day 28 (Fig. 3). It was previously described that an increment of pro-inflammatory cytokines such as TNF-α could act as an inhibitor of the neuropeptide Y/leptin axis, decreasing food intake and modifying BW gain during experimental arthritis (González-Hita *et al*., 2006) as well as, in this assay the animals cannot move because of the severe inflammation they develop.

At the end of experimental arthritis, relative weight of sub-plantar edema and popliteal ganglion tissues were calculated related to total weight of each mouse on day 28. Edema tissue of arthritic un-treated mice showed an increase of 1.84% compared to sub-plantar tissue from healthy animals (0.4%); however, arthritic mice treated with PBZ, or Bc-Wp, or Bc-Cc showed values of 0.78, 0.93, and 0.88%, respectively, with no statistical differences between them. Nonetheless, all of them generated a statistical decrease compared to the CFA un-treated group (Fig. 4).

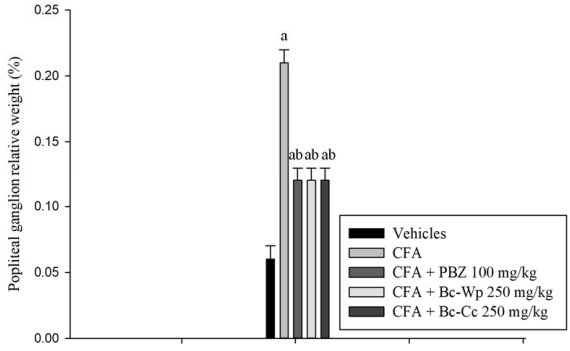

Fig. 5. Effect of MeOH extracts from Wild plant *B. cordata* leaves (Bc-Wp) and its Cell suspension culture (Bc-Cc) on relative weight of popliteal ganglion of male BALB/C mice during experimental arthritis. Data shown as mean ± Standard Error of the Mean (SEM). Values in parentheses showed relative weight of each tissue related to the total animal body weight on day 28 of the experiment. ANOVA on anks, Post-hoc SNK ($p \leq 0.05$) ^{a}vs. VEHicles (VEH); ^{b}vs. CFA control; ^{c}vs. CFA+PBZ; ^{d}vs. CFA+ MeOH Bc-Wp 250 mg/kg CFA: Complete Freund's Adjuvant PBZ: PhenylButaZone Bc-Wp, *Buddleja cordata* Wild plant Bc-Cc, Cell suspension culture *Buddleja cordata* n = 7 per group.

Finally, results for popliteal ganglion relative weight showed same behavior as those of sub-plantar edema, in which an increase was observed for un-treated arthritic mice (0.21%) compared to healthy group values (0.06 %); all other groups exhibited a statistical decrease of popliteal ganglion size (12%) in comparison to CFA un-treated group (Fig. 5). An increase in many organs or tissues size during experimental arthritis, such as popliteal ganglion or sub-plantar edema, is due to an increase of leukocyte infiltration and high production of pro-inflammatory mediators (Pesce *et al*., 2015). As in the acute toxicity model, no abnormal growth was observed in any of the organs extracted on day 28 of the study, mainly the spleen.

Reduction of follicular hyperplasia in ganglionic tissue observed in arthritic mice treated with Bc-Wp and Bc-Cc may be due to its main metabolite, Vb, which is a potent inhibitor of NF-kB pathway, related to leukocyte infiltration, prostaglandin bio-synthesis, and edema formation in tissues (Pesce *et al*., 2015). Finally, Kim *et al*. (2016a), described beneficial effect of *Chrysanthemum zawadskii var. Latilobum* EtOH extract in collagen-induced experimental arthritis in mice. These authors mentioned that this anti-arthritic activity may be due to the high linarin content found in the extract, being this compound one of the SM identified in both MeOH extracts of *Buddleja cordata*, but it compounds was not quantified in this work. Despite all the previously described results for this CFA-induced experimental arthritis model, more specific molecular biology tests are required as well as a greater number of doses tested, being this a first preliminary study through which the behavior of both MeOH extracts was observed using the ED_{50} obtained from the acute inflammation model of carrageenan-induced paw edema. Today the identification and quantification of this compound (linarin) is in process, for order to determine their concentration in both extracts (Bc-Cc and Bc-Wp).

Conclusions

Results showed that Wp *Buddleja cordata* (Bc-Wp) produces small quantities of polyphenols such as verbascoside and the *in vitro* cultures (Bc-Cc) of this plant bio-synthesize more Vb compared to Wp leaves and has high purity rate, and that this could be related to their anti-inflammatory effect in acute and chronic phase. Certainly, anti-inflammatory activity shown by MeOH extract of *B. cordata* cell culture (Bc-Cc) is similar to that reported for Vb per se, and allopathic reference drugs, highlighting the potential of cell suspension cultures for producing anti-inflammatory agents from medicinal plants tissue samples.

Nomenclature

AA	arachidonic acid
ANOVA	analysis of variance
Bc-Cc	*Buddleja cordata* cell suspension culture
Bc-Wp	*Buddleja cordata* wild plant
BW	body weight gain, g / body weight for doses, g or mg kg^{-1}
CFA	Complete Freund's Adjuvant
COX-2	inflammation-induced cyclooxygenase
DW	dry weight of extract and secondary metabolites, mg g^{-1}
ED_{50}	median effective dose, g or mg kg^{-1}
EtOH	ethanol
GAE	gallic acid equivalents, mg GAE g^{-1} DW of extract
HPLC	High-Performance Liquid Chromatography
i.g.	intragastric

IL	interleukin
INF-γ	interferon gamma
i.p.	intraperitoneal
LD_{50}	median lethal dose, mg or g kg^{-1}
MeOH	methanol
NF-κB,	nuclear factor-kappa B
NO	nitric oxide
PBZ	phenylbutazone
QE	quercetin equivalents, μg QE g^{-1} DW of extract
R_t	retention time
SEM	standard error of the mean
SM	secondary metabolites
SNK	Student-Newman-Keuls
TFC	total flavonoid content
TNF-α	tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TPC	total phenolic content
v/v	volume/volume
Vb	verbascoside
Wp	wild plant

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