Original article

A screening of plants used in Colombian traditional medicine revealed the anti-inflammatory potential of *Physalis angulata* calyces

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ABSTRACT

The use of natural products by communities from the Colombian Caribbean region to treat health issues, together with biodiversity and geographical features, constitute a great scenery to develop new therapies based on ethnopharmacological heritage. Here, we investigated the anti-inflammatory potential of 10 commonly used plants in Colombian folk medicine, evaluating their effect on nitric oxide (NO) production by LPS-stimulated RAW 264.7 macrophages. The most active plant was evaluated *in vivo* using 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced mouse ear edema, along with its effect on the production of pro-inflammatory mediators *in vitro*. The extract of *Physalis angulata* L. calyces showed the highest activity. This extract was fractionated and its dichloromethane fraction (DF) was the most active *in vitro*, inhibiting the production of NO, prostaglandin E_2 (PGE_2), interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1 (CCL2). *In vivo*, DF showed a significant inhibition of ear edema and myeloperoxidase (MPO) activity, with evident reduction of the leukocyte infiltration into tissue. Our results support the ethnopharmacological use of the selected plants in folk medicine. *P. angulata* dichloromethane fraction represents a promising source of pharmacological compounds with great potential therapeutic use to treat inflammatory illness.

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1. Introduction

Inflammatory mediators are key signaling molecules implicated in the activation and regulation of inflammatory processes and are produced by various hematopoietic and non-hematopoietic cell types (Harden et al., 2013; Sofroniew, 2013). One of the most important mediators of the immune response is the nitric oxide (NO) radical produced by the inducible nitric oxide synthase (iNOS). This small molecule is not only essential for host innate immune response but also has a pivotal physiological role in the regulation of vasodilatation, neuronal transmission, cardiac

contraction, stem cell differentiation and proliferation (Lei et al., 2013). However, when iNOS activity is aberrant it has detrimental consequences and promotes multiple human diseases (Pautz et al., 2010). Thus, NO is a fundamental molecule for inflammation development and resolution. Given the simplicity of the NO measure trough the Griess reaction, the employment of activated immune cells (i.e. macrophages) has been established as a fast, economic and high-throughput alternative, not only to measure the anti-inflammatory potential of a molecule, but as a great criteria for bioassay-guided fractionation (Ye et al., 2014).

Chronic anti-inflammatory therapy carries several unwanted secondary effects and poses burden on the patients, reducing therapeutic adherence and compromising the effectiveness of the treatment (Tabas and Glass, 2013). To address this, drug discovery research has focused on synthetic and computational methods, leaving aside bioprospecting as an alternative to identify new chemical entities (Macilwain, 1998; Rishton, 2008). Despite this, medicinal plants have been a major source of bioactive molecules for years and continue to be an essential alternative when it comes to health care in many places around the world, especially the rural areas of developing countries, where socio-economic factors difficult the access to modern drugs and health care systems (Cragg

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and Newman, 2013). In countries like Colombia, with more than 10% of the global biodiversity, folk medicine is used as a primary health care, by communities with knowledge and regular access to medicinal plants (Gómez-Estrada et al., 2011). In this context, ethnopharmacological research and bio-guided isolation of active secondary metabolites remain a crucial strategy in the discovery of safer and more effective drugs (Mishra and Tiwari, 2011).

The Colombian Caribbean region has unique geographical and topographical features that had molded not only an extremely diverse flora and fauna, but also a great cultural diversity based on a wide ethnopharmacological knowledge centered on natural resources (Gómez-Estrada et al., 2011). Despite Colombian government efforts to protect natural biodiversity and traditions related to medicinal plants, factors like discrimination to minorities, forced migrations and lack of policies, have affected the preservation of unique traditions to Colombian culture (Gómez-Estrada et al., 2011; Regalado, 2013; Velásquez-Tibatá et al., 2013).

In an effort to document and increase the attention to biodiversity conservation and ethnopharmacology, our research group started a bioprospecting program based on traditional knowledge of anti-inflammatory plants from the Colombia Caribbean region. The aim of this work was to assess the effect of 10 plants frequently used in the folk medicine to treat inflammation related diseases and that have been previously reported for its traditional uses (Table 1.). Anti-inflammatory potential was assessed using the NO production in lipopolysaccharide (LPS)-stimulated macrophages, the most active plant was fractionated and evaluated using 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced mouse ear edema, measuring histopathological parameters and myeloperoxidase activity (MPO). Complementarily, the most active plant was further studied regarding its effects on the production of prostaglandin (PG) E_2, cytokines and chemokines by LPS-stimulated macrophages. Additionally, the free radical scavenging potential was evaluated using NO, ABTS and DPPH scavenging assays.

2. Materials and methods

2.1. Chemicals

12-O-tetradecanoyl-phorbol-13-acetate (TPA), indomethacin, ethylenediaminetetraacetic acid (EDTA), O-dianisidine, dulbecco's modified eagle medium (DMEM), penicillin–streptomycin, trypan blue, lipopolysaccharide (LPS), N-([3-(aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride (1400W), sodium nitrite, N-(1,1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, hematoxylin-eosin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dichloromethane, dimethylsulfoxide, ethanol, and petroleum ether were obtained from Merck (Kenilworth, NJ, USA) or Carlo Erba (Val-de-Reuil, France). Hexadecyltrimethylammonium (HTAB), hydrogen peroxide ($H2O2$), bromide of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Calbiochem® (San Diego, CA, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Gaithersburg, MD, USA). All other chemicals and reagents used were of analytical grade.

2.2. Plant material and preparation of the extracts

The plant material was collected in the Caribbean coast of Colombia and plants were identified by a botanist at the Herbarium of the University of Antioquia (HUA, Medellin, Antioquia, Colombia) and the Botanical Garden Guillermo Piñeres (JBC, Túrbaco, Bolivar, Colombia) where voucher specimens were deposited (Table 2).

Except for *Dracontium dubium*, all plant material were dried at room temperature, powdered and extracted by maceration with ethanol at room temperature until exhaustion. The crude ethanolic extract was filtered and the solvent evaporated under reduced pressure at $40 \pm 5\,°C$ using a rotary evaporator (Heidolph VV 2000, Heidolph, Kelheim, Germany). Tubers of *D. dubium* were washed, peeled and crushed in a minimum amount of water, then lyophilized (FreeZone® Dry 2.5, Labconco, Kansas, MO, USA). The powder obtained was finally extracted by maceration as described before.

Physalis angulata extract was fractionated through liquid-liquid partition with petroleum ether, dichloromethane and methanol-water (9:1) to obtain a petroleum ether (EF), dichloromethane (DF) and methanol fraction (MF).

Test extracts/fractions were dissolved in dimethyl sulfoxide (DMSO) or ethanol to obtain a stock solution, and stored at -20 °C. The final percentage of vehicle was adjusted to 0.1% (v/v) in cell culture experiments.

2.3. Phytochemical screening, flavonoids and polyphenols quantification

The phytochemical screening for presence of terpenes, steroids, quinones, glycosides, alkaloids and tannins was performed as reported by Herrera et al. (2014). Polyphenols were quantified using the Folín-Ciocalteu method with some modifications (Del-Toro-Sánchez et al., 2014). Briefly, the sample was mixed with Folín-Ciocalteu reagent (0.1 M), and 10 min later a solution of sodium carbonate (7.5%) was added. After incubating the mixture for 2 h at room temperature (RT), the Optic Density at 760 nm (OD_{760}) was determined using a microplate reader (Multiskan EX Thermo, Waltham, MA, USA). Results are expressed as mg of gallic acid equivalents (GAE) per gram of extract.

Flavonoids were determined by the aluminum trichloride method with some modifications (Adebayo et al., 2015). Samples were mixed with sodium nitrite (1.5%) and stirred for 10 min at RT, aluminum trichloride (3%) was then added and the mixture was now stirred for 5 min, before finally adding sodium hydroxide (1N). After 10 min, the OD_{450} was measured. Results are expressed as mg of catechin equivalents (QE) per gram of extract.

2.4. In vitro anti-inflammatory activity

2.4.1. Cell culture

RAW 264.7 (TIB-71™), murine macrophages were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% FBS and penicillin (10 UI/mL)/streptomycin (10 μg/mL) at 37 °C and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80–90% confluence.

2.4.2. Cell viability

The reduction of MTT to formazan was used to assess the effects of test extracts/fractions on cell viability following the protocol described by Mosmann, 1983. Cells (2×10^5 cells/mL) were seeded in 96-well microplates and treated with vehicle (DMSO/ethanol) or extracts/fractions (100–6.25 μg/mL) for 30 min, then stimulated with LPS (10 μg/mL). After 24 h, cells were incubated with MTT solution (0.25 mg/mL), 4 h later the formazan was dissolved in DMSO and the OD_{550} was measured. Triton X-100 (2%) was used as positive control. Percentages of cell survival relative to control group were calculated, as well as the lethal concentration 50% (LC_{50}).

Table 1
List of selected medicinal plant species employed in Colombian Caribbean Region to treat inflammatory related diseases including their ethno-pharmacological characteristics.

Scientific name	Local name	ethnopharmacological use	Part used	Mode of preparation/administration route	Reference
Calotropis procera (Aiton) Dryand. (Apocynaceae)	Algodón de seda	Headache Rheumatism	Roots Leaves	Exudate/Oral Cataplasm/Topic	Lal and Yadav (1983) Kumar Vijay et al. (2011)
Ceratopteris pteridoides (Hook.) Hieron. (Pteridaceae)	Cola de caballo	Respiratory Conditions Diuretic and Cholelithiasic	Leaves	Infusion/Oral	Alviz et al. (2013) Beltrán Villanueva et al. (2013)
Cordia alba (Jacq.) Roem, & Schult. (Boraginaceae)	Sauce sabanero	Bronchitis	Bark, Buds	Oil Decoction, Infusion/Oral	Waizel-Bucay (2005)
Croton malambo H.Karst. (Euphorbiaceae)	Croton	Rheumatism Pain Inflammation	Bark	Infusion/Oral	Salatino et al. (2007) Suárez et al. (2003)
Cryptostegia grandiflora Roxb. ex R. Br. (Apocynaceae)	Flor de muerto	Inflammation Infection	Leaves	Fresh plant in maceration/Cataplasm	Castro et al. (2014) Rivera et al. (2015)
Dracontium dubium Kunth (Araceae)	Chupadera	Snakebite Inflammation	Root	Poultice/Topic	Lévi-Strauss (1952)
Maclura tinctoria (L.) D. Don. ex Steud. (Moraceae)	Palomora	Inflammation Toothache Rheumatism	Bark	Latex Drops/Topic Decoction, /Oral	Beltrán Villanueva et al. (2013)
Merremia umbellata (L.) Hallier f. (Convolvulaceae)	Juan de Dios	Inflammation and Infections Antiulcer Rheumatism	Leaves Whole Plant	Decoction/Oral	Castro-Guerrero et al. (2013) Rivera et al. (2015)
Physalis angulata L. (Solanaceae)	Topo toropo	Inflammation and Infections Abdominal Cramps Asthma, Dermatitis, Rheumatism	Calyces Aerial Parts Whole Plant	Poultice/Topic Infusion/Oral Infusion/Oral	Rivera et al. (2015) Soares et al. (2003)
Tabebuia rosea (Bertol.) Bertero ex A.DC. (Bignoniaceae)	Palo de rosa	Inflammation Fever	Bark Stem, Bark	Decoction/Oral	Gómez-Estrada et al. (2011)

Table 2
Plant information: list of evaluated plants, collection sites, vouchers, organs used, extraction solvent and yields.

Scientific name	Collection site	Voucher number	Used part	Extract yields (%)
Calotropis procera	Galerazamba, Bolívar (10°47′52″N 75°15′41″W)	HUA175327	Leaves	9,73
Ceratopteris pteridoides	San Bernardo del Viento, Cordoba (9°21′18″N 75°57′16″W)	HUA166134	Whole Plant	6,79
Cordia alba	Pueblo Nuevo, Bolívar (10°44′26″N 75°15′43″W)	HUA175329	Flowers	11,59
Croton malambo	Galerazamba, Bolívar (10°47′22″N 75°15′36″W)	JBC 12,008	Bark	8,82
Cryptostegia grandiflora	Pueblo Nuevo, Bolívar (10°44′33″N 75°15′28″W)	HUA175331	Leaves	6,92
Dracontium dubium	La Unión, Sucre (8°51′37″N 75°16′48″W)	HUA 189,121	Root	N.D.
Maclura tinctoria	Pueblo Nuevo, Bolívar (10°44′42″N 75°15′48″W)	JBC 12,013	Bark	5,33
Merremia umbellata	Pueblo Nuevo, Bolívar (10°44′22″N 75°15′35″W)	HUA175331	Leaves	14,44
Physalis angulata	Pueblo Nuevo, Bolívar (10°44′N 75°15′W)	HUA175328	Calyces	16,05
Tabebuia rosea	Túrbaco, Bolívar (10°19′55″N 75°24′51″W)	HUA196871	Bark	9,20

All extracts were obtained by maceration with ethanol 96% and concentrated in a rotaryevaporator. The yields were calculated as: [Dry Concentrated Extract (g)/Dry Material (g)] $\times$ 100. N.D.: Not determined.

2.4.3. Pro-inflammatory mediators assessment

Cells (2×10^5 cells/mL) were seeded in 24-well microplates and treated with vehicle, test extracts/fractions (100–6.25 μg/mL), 1400 W (2.50 μg/mL), dexamethasone (7.85 μg/mL), or rofecoxib (6.29 μg/mL) for 30 min, then stimulated with LPS (10 μg/mL). After 24 h, culture supernatants were collected and kept at -20 °C until further analysis.

NO release was determined using the Griess reaction (Green et al., 1982). Equal volumes of supernatants and Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) were mixed and incubated at RT for 5 min. The OD_{550} was measured and compared with a sodium nitrite standard curve (1–200 μM). Percentage of inhibition was calculated using control cells and the IC_{50} was determined. To corroborate that NO depletion was caused by biological inhibition, instead of a scavenging effect of the extracts, the scavenging activity was measured using the method by Franco et al., 2014.

Production of PGE_2, IL-1β, IL-6, TNF-α and CCL2 was measured using ELISA following the instructions of the manufacturer (eBiosciences, San Diego, CA, USA).

2.5. In vivo anti-edema activity

2.5.1. Experimental animals

Female ICR mice (20–25 g) provided by the Instituto Nacional de Salud (Bogotá, Colombia) were allowed to acclimatize for at least two weeks before use and fed standard rodent food and water *ad libitum*. They were housed in filtered-capped polycarbonate cages and kept in a controlled environment (22 ± 3 °C and relative humidity: 65 to 75%), under a 12 h light/darkness cycle. All experiments were designed and conducted in accordance with the European Union regulations (CEC council 86/809) and approved by the Ethics Committee of the University of Cartagena (Minutes No. 32 August 4, 2011).

2.5.2. TPA-induced acute ear edema

The edema was induced by topical application of TPA (2.5 μg/ear) in acetone, according to the method described by De Young et al., 1989. Groups (*n* = 6) were treated on the inner and outer surfaces of the right ear with the acetone dissolved treatment (1 or 05 mg in 20 μL of vehicle) or indomethacin (0.5 mg in 20 μL of

vehicle), as reference drug. Immediately after, TPA was applied on both faces of the right ear. The left ear received only acetone, as a control. After 4 h, the animals were sacrificed, and a disk (6 mm diameter) removed from both ears (treated and untreated) was individually weighed on an analytical scale (Ohaus Pioneer™, Parsippany, NJ, USA). Edema was indicated by the weight increase of the right ear disk over the left, and the anti-edema activity was expressed as inhibition percentage of edema.

2.5.3. Histopathology

Tissue samples were preserved in buffered formalin, embedded in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin (H&E). The presence of edema, epidermal hyperplasia/hypertrophy, infiltration of mononuclear and polymorphonuclear cells, connective tissue disruption and dermal fibrosis was assessed by a blind pathologist using light microscopy (Axio Lab A1, Zeiss, Oberkochen, Germany). Parameters were scored from 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe).

2.5.4. Myeloperoxidase (MPO) assay

MPO activity was measured according to the method described by Bradley et al. (1982). Ear tissue was weighed and homogenized in phosphate-buffered saline (PBS), pH 7.4. The homogenate was centrifuged at 2576g for 30 min at 4 °C. The pellet was again homogenized PBS, pH 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). This suspension was subjected to cycles of freezing/thawing and brief periods of sonication (15 s) and centrifuged at 2576g for 10 min at 4 °C. Supernatants were mixed with O-dianisidine dihydrochloride (0.067%) and hydrogen peroxide (0.003%), and incubated for 5 min before measuring the OD_{450}. Enzyme activity was expressed as enzyme activity units, where 1 activity unit is defined as the amount of enzyme capable of metabolizing 1 μmol of hydrogen peroxide in 1 min at 25 °C.

2.6. Radical scavenging activity

The ABTS-scavenging effect was determined using the method described by Re et al. (1999), with modifications. ABTS (3.5 mM; in ethanol) and $K_2S_2O_8$ (1.25 mM) were mixed with ascorbic acid (positive control) or test extracts/fractions and incubated at RT for 5 min. The scavenging of the ABTS radical was determined by measuring the OD_{405}. The DPPH-scavenging effect was determined using the method described by Brand-Williams et al. (1995), with some modifications. In brief, DPPH (100 μg/mL), was mixed with ascorbic acid or test extracts/fractions, and then incubated at RT for 30 min. The scavenging of the DPPH radical was determined by measuring the OD_{550}. Vehicles were used as negative controls.

2.7. Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M) of at least two independent experiments. IC_{50} and LC_{50} were calculated employing non-linear regression and expressed as mean and its 95% confidence interval. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's and Tukey's post hoc tests. Values of $P < 0.05$ were considered significant.

3. Results and discussion

Ethnopharmacological studies allow not only to document traditional knowledge of plants, but to engage policy-makers in designing appropriate strategies for the conservation of biodiversity and cultural knowledge related to medicinal plants. We con-

tribute to this endeavor through our bioprospecting program, focused on isolation of bioactive compounds or therapeutically useful extracts/fractions from plants of the Colombian Caribbean region. In the first part of our work the anti-inflammatory potential of 10 plants traditionally used to alleviate inflammation (Tables 1 and 2) were evaluated based on their capacity to inhibit NO production by LPS-stimulated RAW 264.7 macrophages. In the second part, we focused on the fractionation and evaluation of the most active extract, as well as some insights into the mechanism of action.

3.1. Effect of the plant extracts on the NO production by LPS-stimulated RAW 264.7 macrophages

Stimulation of RAW 264.7 macrophages with LPS (10 μg/mL) elicited a significant accumulation of nitrite in the medium (41.3 6 ± 0.63 μM). As can be seen in Table 3, NO production was reduced by test extracts at different degrees. We employed the NO inhibition rates (IR) to classify plants among four categories: inactive (−), when the IR was below 20%; weakly active (+), when between 20 and 40%; moderately active (++), with IRs between 40 and 60%; and highly active (+++) when the IR was higher than 60%. Extracts were evaluated at non-toxic concentrations (cell viability above 85%) as determined by the MTT assay (Table 3), and did not show any significant NO-scavenging effect (data not shown). Therefore, the NO inhibition presented by the extracts is due to an effect on the cellular processes and not an *in vitro* scavenging effect or cytotoxicity.

At the evaluated concentrations, *D. dubium* and *T. rosea* showed the lowest activity with IRs below 10%. Although previous studies demonstrated the anti-inflammatory activity of *T. rosea* (Franco Ospina et al., 2013) and the anti-edema properties of *Dracontium* species (Gomes et al., 2010), our results are in agreement with several studies that demonstrated that NO production is not inhibited by extracts from plants belonging to the *Tabebuia* genus at concentrations below 200 μg/mL (Byeon et al., 2008). This suggests that the inhibition of NO production might not be a decisive target involved in the effect of these plants. On the other hand, the plants classified as weakly active, *C. pteridoides* and *C. alba*; have no reports of anti-inflammatory activity. However, there are reports of compounds isolated from other plants of the *Cordia* genus with this biological activity (Al-Musayeib et al., 2011; Geller et al., 2010). In this sense, their potential as treatment for inflammation could not be denied, as we employed a stringent top test concentration (100 μg/mL) and focused only on NO production.

To our knowledge, the anti-inflammatory effect of *M. tinctoria* (moderately active) has not been previously reported, positioning our study as the first precedent of the potential of this plant to treat inflammation. On the other hand, the results for *M. umbellata*, also classified as moderately active, are consistent with our previous report that determined its effectiveness reducing the edema and MPO activity in the TPA-induced mouse ear edema model; and supporting the traditional use given to this species (Castro-Guerrero et al., 2013). Likewise, *C. procera* leaves have also been reported to reduce edema and itching on different *in vivo* models (Saba et al., 2013), our results corroborate these findings and place the inhibition of the NO production as a possible target, partially responsible for *M. umbellata* and *C. procera* leaves anti-inflammatory activity.

The extracts of *C. grandiflora*, *C malambo* and *P. angulata* were catalogued as highly active. *C. grandiflora* extract inhibited NO production by 70%, in accordance with our previous findings that proved its capacity to reduce edema, MPO activity and leukocyte infiltration (Castro et al., 2014). Due to the cytotoxic effect of the *C. malambo* and *P. angulata* extracts, they were evaluated at the lower concentrations. Despite this, both extracts were highly

Table 3
Evaluation of the cell viability and NO inhibition potential of extracts obtained from Colombian Caribbean Coast plants on LPS-stimulated RAW 264.7 macrophages.

	Plant	Concentration (μg/mL)	% Cell viability	% Nitrite inhibition	Rank
1	*Calotropis procera*	100	100 ± 6.70[**]	45.11 ± 4.50	++
2	*Ceratopteris pteridoides*	100	99.10 ± 1.23[***]	33.48 ± 2.29	+
3	*Cordia alba*	100	100 ± 8.91[***]	33.15 ± 2.69	+
4	*Croton malambo*	50	86.56 ± 1.66[***]	97.05 ± 2.15	+++
5	*Cryptostegia grandiflora*	100	98.48 ± 0.63[***]	70.47 ± 0.81	+++
6	*Dracontium dubium*	100	96.45 ± 0.65[***]	7.23 ± 1.52	−
7	*Maclura tinctoria*	100	102.11 ± 1.61[***]	41.07 ± 2.12	++
8	*Merremia umbellata*	100	100 ± 0.81[***]	56.06 ± 1.74	++
9	*Physalis angulata*	25	93.84 ± 1.41[***]	91.94 ± 1.97	+++
10	*Tabebuia rosea*	100	96.65 ± 0.60[***]	9.23 ± 1.89	−
11	1400W	2.50	100 ± 1.59[***]	83.88 ± 1.07	+++

The cell viability was measured using the MTT assay and the NO· inhibition was assessed with the Griess assay as described in Methods. Extracts were classified by their activity as highly active (IR ≥ 60%, +++), moderately active (60% > IR ≥ 40%, ++), weakly active (40% > IR ≥ 20%, +), and inactive (IR ≤ 20%, −). 1400 W: N-(3-(aminomethyl) benzyl)acetamidine. Each value represents mean ± SEM (n = 8) from at least two independent experiments. [*]P < 0.05, [**]P < 0.01, [***]P < 0.001 compared with control group (One way ANOVA).

active, with IRs above 90%, making them the most promising extracts in the group. The efficacy of NO inhibition was estimated with the Selectivity Index (SI), defined as LC_{50}/IC_{50}, and used as criteria to choose the best fractionation candidate. As seen in Table 4, *C. malambo* and *P. angulata* had the lowest IC_{50} values. Nevertheless, the SI for *P. angulata* (32.15) doubled the SI for *C. malambo* (16.05), suggesting the potential of *P. angulata* as a safer anti-inflammatory agent and a better candidate for fractionation.

The anti-inflammatory activity of *P. angulata* has been widely studied, proving its capacity to reduce inflammation and modulate NO production by extracts from roots (Bastos et al., 2008) and aerial parts (Choi and Hwang, 2003; Sun et al., 2011) as well as by different isolated compounds (Pinto et al., 2010; Soares et al., 2006; Sun et al., 2010). However, our results constitute the first report of anti-inflammatory potential of its calyces. The distinctive calyx of the *Physalis* genus is an important structure formed by sepals as a source of carbohydrates for development and protection of the fruit against environmental threats (Puente et al., 2011). Hence, it represents a rich source of novel bioactive metabolites with a vast pharmacological potential, this is supported by other recent reports (Franco et al., 2014; Takimoto et al., 2014).

3.2. Assessment of the anti-inflammatory effect of the primary fractions obtained from P. angulata calyces

The total ethanolic extract of the calyces of *P. angulata* was fractionated through liquid-liquid partition, obtaining three primary fractions, a petroleum ether (EF), dichloromethane (DF) and methanol fraction (MF). These fractions were evaluated for its potential to inhibit NO production on RAW 264.7 macrophages.

EF and MF were evaluated both at 75 μg/mL whereas DF was evaluated at 25 μg/mL, in accordance with the MTT cell viability test (data not shown). Despite the difference in concentration, DF had a significantly higher NO IR (97%), compared to EF and MF, with IR below 50% (Table 5), indicating that the bioactive metabolites were concentrated in the medium polarity fraction. Moreover, DF displayed a significant concentration-dependent inhibition with IC_{50} of 2.48 (2.23–2.77) μg/mL, showing a comparable activity than the iNOS selective inhibitor 1400 W (IC_{50} of 3.72 (2.98–4.57) μg/mL). Moreover, none of the fractions displayed NO scavenging effects (data not shown).

The extract and fractions were also evaluated on the TPA-induced ear edema model. TPA is an irritating agent that activates protein kinase C (PKC), increasing the levels of pro-inflammatory mediators such as PGE_2, TNF-α, IL-1β, and IL-6; which are accompanied by edema, epidermal hyperplasia and immune cell infiltration (Gábor, 2003; Murakawa et al., 2006). TPA application

increased the ear thickness after 4 h (Fig. 1A), ear edema was significantly ameliorated by treatment with extract and fractions from *P. angulata* calyces. DF showed a dose-dependent reduction of the edema, with the dose of 1 mg/ear inhibiting up to 60% (P < 0.001), an effect comparable to the produced by indomethacin (0.5 mg/ear). None of the tested extract/fractions produced evident local or systemic adverse effects, suggesting that their topical administration is well tolerated.

The neutrophil accumulation is a characteristic feature of TPA-induced edema (Sánchez and Moreno, 1999) and is measured indirectly by the increase of MPO activity (P < 0.001). As seen in Fig. 1B, treatment with extract and fractions from *P. angulata* significantly decreased the MPO activity relative to the TPA group (P < 0.001). The highest activity was exerted by both MF and DF, which inhibited the enzymatic activity by over 71% at 1 mg/ear (P < 0.001). Additionally, DF also showed MPO inhibition at 0.5 mg/ear (39% inhibition P < 0.001) indicating a dose-dependent effect.

The anti-edema effect of the test extract and fractions was confirmed by histological examination of the ear tissue (Fig. 2). The edema on the TPA group (score: 16), was accompanied by increased ear thickness, disruption of tissue structure, immune cell infiltration, hyperplasia and hypertrophy of dermis, and epidermis (Fig. 2B). Administration of extract and fractions reduced the tissue damage induced by TPA (Fig. 2D–H), DF showed the highest anti-edema activity, restoring tissue architecture and diminishing the edema, cell infiltration which is reflected in the reduced lower histological score (Score: 4) (Fig. 2G and H) similar to the effect produced by indomethacin with a histological score of 2 (Fig. 2C).

Taken together, observations at macroscopic and histopathological level reveal that DF has the highest anti-inflammatory potential and represents a prominent source of bioactive metabolites. These *in vivo* results support the use of the NO determination by the Griess assay as a suitable method for a bio-guided fractionation of potentially anti-inflammatory plant extracts, in accordance with Dirsch et al. (1998).

3.3. Effect of DF on the production of pro-inflammatory mediators by LPS-stimulated RAW 264.7 macrophages

Macrophages are critical effectors and regulators of inflammation and the immune response. When stimulated with LPS, macrophages undergo classical M1 activation through activation of Toll-like receptor 4 (TLR4) (Kayagaki et al., 2013). TLR4 modulation by LPS promotes activation effector functions, NO production and reactive oxygen intermediates (ROI), PGE_2, cytokines (IFN-β, IL-1β, IL-6, IL-12, and TNF-α) and chemokines like the monocyte chemotactic protein-1 (MCP-1, CCL2), as well as other important

Table 4
Assessment of the LC_{50}, IC_{50} (NO), and SI of the extracts with the highest NO inhibition potential among the tested plants group.

	Plant	LC_{50} ± confidence intervals (μg/mL)	IC_{50} ± confidence intervals (μg/mL)	SI
1	*Croton malambo*	113.907 (97.537–131.361)	7.097 (6.229–8.199)	16.05
2	*Cryptostegia grandiflora*	>100	43.442 (41.748–45.136)	>2.30
4	*Physalis angulata*	130.628 (114.505–144.937)	4.063 (3.541–4.665)	32.15

The cell viability was measured using the MTT assay and the NO inhibition was assessed with the Griess assay as described in Methods. IC_{50} and LC_{50} were calculated with non-linear regression and expressed as mean and its 95% confidence interval ($n = 8$) from at least two independent experiments.

Table 5
Evaluation of the NO inhibition potential of fractions obtained from *Physalis angulata* calyces on LPS-stimulated RAW 264.7.

	P. angulata fractions	Concentration (μg/mL)	% Nitrite inhibition	Rank
1	Ether (EF)	75	42.897 ± 1.791	++
2	Dichloromethane (DF)	25	97.079 ± 0.494	+++
3	Methanol (MF)	75	49.232 ± 1.138	+

The cell viability was measured using the MTT assay and the NO inhibition was assessed with the Griess assay. Fractions were classified by their activity as highly active (IR ≥ 60%, +++), moderately active (60%>IR ≥ 40%, ++), weakly active (40%>IR ≥ 20%, +), and inactive (IR ≤ 20%, −). Each value represents mean ± SEM ($n = 8$) from at least two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group (One way ANOVA)
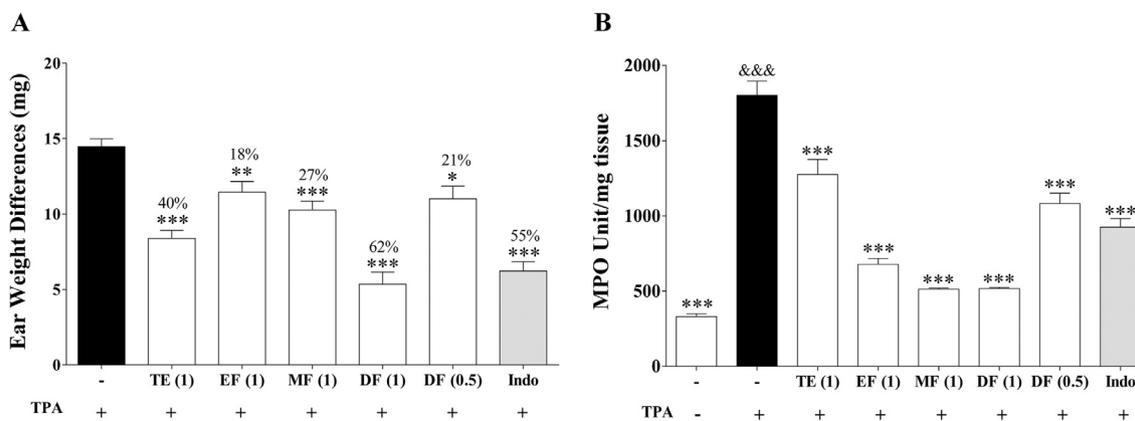
Fig. 1. *In vivo* evaluation of the anti-inflammatory effect of the extract and fractions obtained from the calyces of *P. angulata* in the TPA-induced mice ear edema. **TE(1):** Total extract (1 mg/ear), **EF(1):** Ether fraction (1 mg/ear), **MF(1):** Methanol fraction (1 mg/ear), **DF(1):** Dichloromethane fraction (1 mg/ear), **DF(0,5):** Dichloromethane fraction (0,5 mg/ear), **Indo:** Indomethacin. **(A):** Ear weight difference (mg) and inflammation inhibition percentage, circular 6 mm diameter sections of both treated and non-treated ear were weighted. **(B):** Enzymatic activity measured in ear tissue homogenized expressed as MPO units/mg tissue. Each value represents mean ± S.E.M. ($n = 6$) from at least two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with TPA group (One way ANOVA). &&&$P < 0,001$ compared with Control group (One way ANOVA).
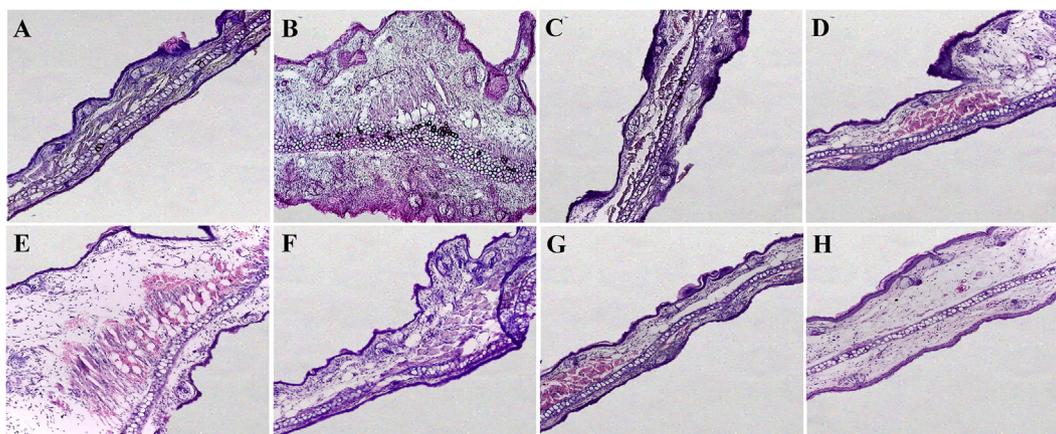
Fig. 2. Histology samples from the TPA-induced ear edema test, ear tissue was dehydrated, embedded in paraffin and stained with Hematoxylin-Eosin. All pictures were taken in an Axio Lab A1 microscope (Zeiss, Oberkochen, Germany) and correspond to the 10X zoom. **(A)** Control group, **(B)** TPA group, **(C)** Indomethacin (0,5 mg/ear), **(D)** TE (1 mg/ear), **(E)** EF (1 mg/ear), **(F)** MF (1 mg/ear), **(G)** DF (1 mg/ear) and **(H)** DF (0,5 mg/ear).

mediators of the inflammatory response (Bode et al., 2012). M1 macrophages and high NO levels positively correlate with many inflammatory diseases (Gupta et al., 2014). CCL2 provokes mast cell, eosinophil, and macrophage recruitment and activation in rat skin (Sun et al., 2013) and has a critical role in the onset of inflammation and fibrosis (Yamamoto, 2008).
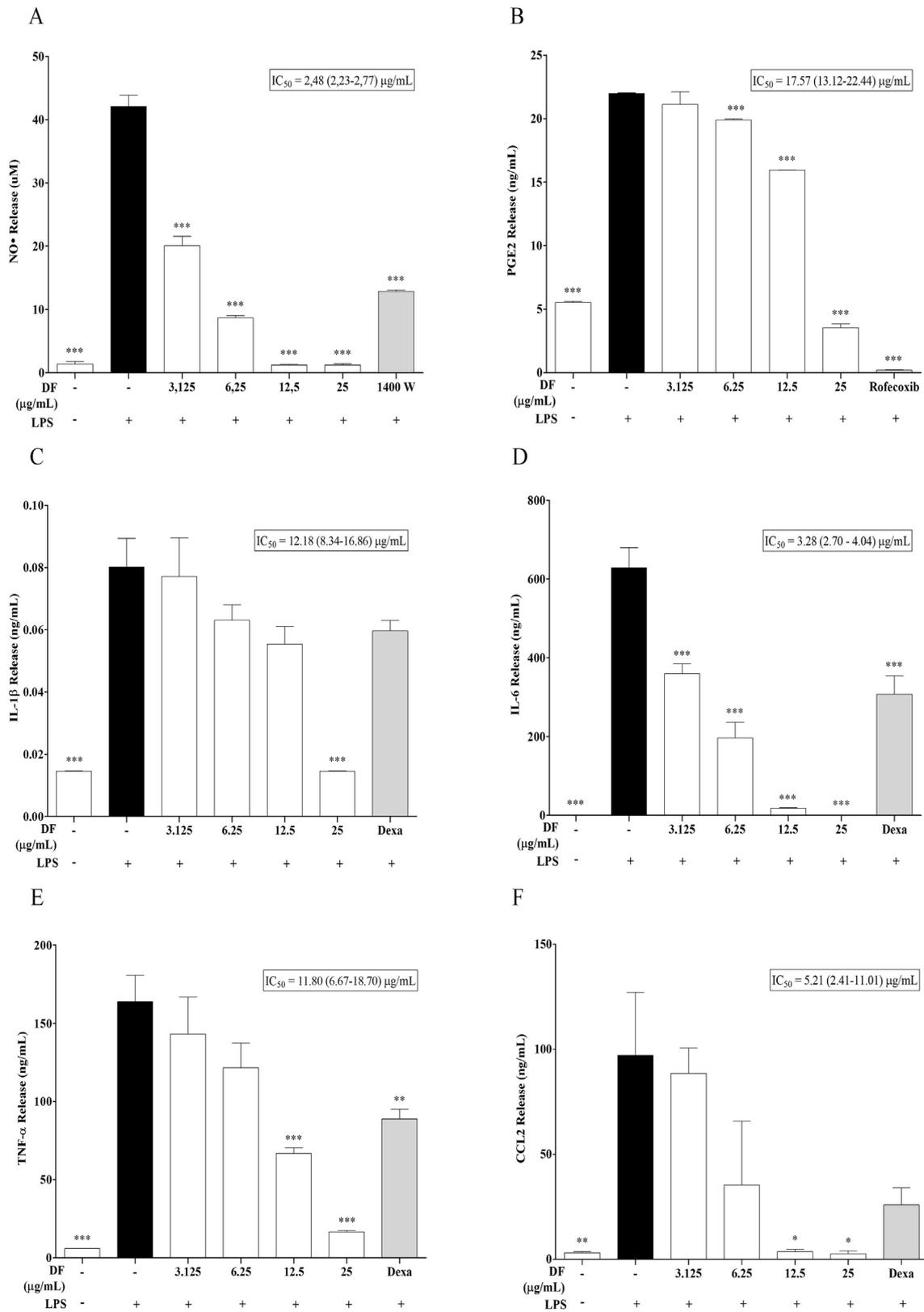
Fig. 3. Effect of the dichloromethane fraction (DF) on the production of pro-inflammatory mediators by LPS-stimulated RAW 264.7 macrophages. **(A):** NO•, **(B):** PGE2, **(C):** IL-1 β, **(D):** IL-6, **(E):** TNF-α, **(F):** MCP-1. Cells were incubated for 24 hours after stimulation and treatment with DF. Then supernatants were collected and mediators were measured through ELISA kits, except the NO• which was measured with the Griess reaction, as described in Methods. Each value represents mean ± SEM (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 compared with LPS group (One way ANOVA).

Table 6
Phytochemical characterization of the dichloromethane fraction obtained from the total ethanolic extract of the calyces of *Physalis angulata*.

Metabolite	*P. angulata* DF
Flavonoids	−
Terpenes and/or steroids	++
Quinones	−
Glycosides	+++
Steroidal core	++
Alkaloids	−
Tannins	−

(−) Not detected, (+) Weak presence, (++) Moderate presence and (+++) Strong presence.

Considering the results showed by DF *in vivo*, we evaluated its effect on pro-inflammatory mediators using LPS-stimulated RAW 264.7 macrophages. As shown in Fig. 3A–F, high concentration of NO, PGE$_2$, IL-1β, IL-6, TNF-α, and CCL2 was observed after LPS stimulation, with at least a 4-fold increase ($P < 0.001$). In the absence of LPS, incubation with DF did not modify the release of pro-inflammatory mediators (data not shown). DF significantly inhibited the LPS-induced release of all the evaluated pro-inflammatory mediators in a concentration-dependent manner (Fig. 3A–F), with IC$_{50}$ values below 20 μg/mL, suggesting a potential immunomodulatory effect.

Collectively, we have demonstrated that calyces of *P. angulata* promote an anti-inflammatory state of macrophages, decreasing the release of pro-inflammatory mediators. Such effects are, consistent with previous *in vitro* and *in vivo* reports of extracts and compounds obtained from other parts of the *P. angulata* plant, which inhibited NO, PGE$_2$, IL-6, IL-1β, IL-12, and TNF-α production (Bastos et al., 2008; Sun et al., 2010); or increased IL-4 and IL-10 levels (Sun et al., 2011; Yu et al., 2010), distinctive cytokines of M2 macrophages. We hypothesized that the effects of DF could be related with the blockage of the TLR4 signaling pathway or a profile transition from M1 to M2 macrophages (Martinez and Gordon, 2014), whichever the case might be, further research is needed to determine the actual mechanisms.

Compounds isolated from the calyces of species of *Physalis* genus as physalins (*Physalis alkekengi* L.), withanolides (*Physalis pruinosa* L.) and sucrose esters (*Physalis peruviana* L.), have also show anti-inflammatory potential by the modulation of stimulated macrophages and expression of NO, PGE$_2$, IL-6, CCL2 or TNF-α, through the inhibition of NF-κB activation (Franco et al., 2014; Ji et al., 2012; Takimoto et al., 2014). Although, we did not study in detail the chemical composition of DF, the phytochemical screening of the fraction showed presence of glycosides, steroids and terpenes (Table 6) and colorimetric quantification revealed a low quantity of polyphenols (8.78 ± 0.165 mg Gallic acid/g extract) whereas flavonods were not detectable. Due to the low concentration of these pharmacologically important secondary metabolites, we think that the biological effects of the fraction may be more influenced by glycosides as they appear to be the most abundant.

As reactive oxygen species are commonly known as triggers of inflammatory processes and its production is considered a potential target of anti-inflammatory drugs (Debnath et al., 2013), we evaluated the potential scavenging effect of *P. angulata* calyces, using DPPH and ABTS assays. The results showed that the total extract and fractions have no radical scavenging effect (100 μg/mL), suggesting that the effects observed, are not related to antioxidant activity (data not shown).

4. Conclusions

In short, our results confirm and support the ethnopharmacological employment of the selected plants in the Colombian folk medicine, constituting the first scientific report of anti-inflammatory potential for *Ceratopteris pteridoides, Cordia alba,* and *Maclura tinctoria*. The extract obtained from calyces of *Physalis angulata* had the highest anti-inflammatory potential according to the *in vitro* results, and the dichloromethane fraction inhibited the production of inflammatory mediators (NO, PGE$_2$, IL-6, IL-1β, TNF-α, and CCL2) by LPS-induced macrophages, as well as reducing the edema, cell infiltration and MPO activity in a TPA-induced mice ear edema assay. To our knowledge, this is the first scientific report of the anti-inflammatory potential of the calyces of *P. angulata*, placing them as promising source of bioactive metabolites. Nevertheless, a bio-guided isolation of bioactive compounds is still necessary.

Author contributions

D. Rivera and Y. Ocampo, performed the experiments, analyzed data, and wrote the first draft of the manuscript. J. Castro collaborated with plant collection, extract preparation, experiments and data analysis. L. Barrios oversaw tissue processing and histological analysis. F. Diaz, performed plant collection and extract preparation. L. Franco, principal investigator, coordinated the project, conceived and designed the experiments, assisted in data analysis, revised the manuscript and approved the final draft.

Conflict of interest

The authors declare no conflict of interest.

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