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Anti-inflammatory screening of plant species from the Colombian Caribbean Coast

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ABSTRACT

Chronic inflammation has been recognized as an underlying pathophysiological mechanism in the initiation and progression of noncommunicable diseases, representing a significant morbidity and mortality cause worldwide. The prolonged use of anti-inflammatory drugs has been associated with different adverse effects, so there is a permanent need to develop new drugs to treat these pathologies. The anti-inflammatory potential of 37 extracts coming from 31 plant species from the Colombian Caribbean coast, was evaluated determining their ability to inhibit nitric oxide (NO) production using lipopolysaccharide (LPS) activated macrophages. The most active extracts were evaluated for their effect on the production of tumor necrosis factor (TNF-α) and interleukins 1β and 6 (IL-1β and IL-6) in macrophages. Fifteen extracts showed potent inhibitory activity of the production of NO, being the extracts of *Ambrosia cumanensis*, *Trichilia hirta*, *Hyptis capitata* (leaves and seeds), *Mammea americana*, and *Crateva tapia,* the most active extracts. The extracts of *A. cumanensis* and *M. americana* were considered promising, which significantly decreased the production of all proinflammatory cytokine evaluated. The species of *A. cumanensis* and *M. americana* are a promising source of molecules with anti-inflammatory activity. They should be evaluated in *in vivo* models of inflammation, as well as perform their fractionation to identify the compounds responsible for the activity.

INTRODUCTION

Inflammation is a fundamental component of the host defense mechanism against noxious stimuli. This process induces the proliferation and interaction of several types of cells and the release of various chemical mediators, including prostaglandins, leukotrienes, cytokines, chemokines, and nitric oxide (NO). Although the inflammatory response is necessary for the host's defense, when it occurs in an exacerbated manner, it constitutes a triggering factor for various chronic noncommunicable diseases (Calder, 2015).

Nonsteroidal anti-inflammatories and corticosteroids are the most commonly used groups of drugs for the treatment of inflammatory processes. Still, they also have numerous adverse effects, including heartburns, gastric and duodenal ulcers, cardiovascular diseases, kidney failure, osteoporosis, increase risk of susceptibility to infections, hyperglycemia, and obesity. This represents a problem for patients' health, causing alterations that sometimes are more serious than the initial condition, in addition to increasing the costs of long-term treatments. All these reasons justify the enormous efforts made worldwide to discover new therapeutic alternatives for the treatment of inflammatory processes (Manson *et al*., 2009; Shi *et al*., 2003), being natural products one of the main objectives because they have been, and still are a fundamental source in the discovery of drugs (Killeen *et al*., 2014). Consequently, many investigations have been mainly directed toward the screening of plant species as a strategy in search for metabolites with anti-inflammatory activity. This search for anti-inflammatories coming from natural sources is gaining more importance today due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) pandemic since the end of 2019, a virus that induces an exacerbated inflammatory process if adequate anti-inflammatory treatments are not received at the beginning of the infection. Colombia is one of the most mega-

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diverse countries in the world since it has a unique combination of geographic and topographical attributes that have allowed it to have an extraordinary diversity of flora (Gómez-Estrada *et al*., 2011). However, many of the medicinal properties of a high percentage of the plant species that make up the Colombian flora are still unknown.

Macrophages are one of the most important effector cells in the inflammatory response; however, during chronic inflammatory processes, they play an essential role in tissue damage in a large number of diseases that occur with inflammatory processes such as arthritis, atherosclerosis, obesity, cancer, diabetes, and inflammatory bowel disease (Kühl *et al*., 2015; Na *et al*., 2018; Treuter *et al*., 2017). Active macrophages produce various molecules, including the so-called reactive oxygen species and reactive nitrogen species (RNS), among which is NO, produced by the different isoforms of the enzyme nitric oxide synthase. The inducible isoform of nitric oxide synthase (iNOS) is responsible for the production of NO during the inflammatory response. The NO reacts with the superoxide radical anion ($\bullet O_2^-$) to produce peroxynitrite ion ($ONOO^-$), which plays an essential role in the host's defense. Peroxynitrite is a potent oxidizing agent that exerts its effects through the mutagenesis of microbial DNA, the inactivation of virulence factors, and the metabolic blockade in invading microorganisms, but due to its lack of specificity at high concentrations or when the synthesis occurs continuously, it produces tissue damage; this process being particularly well described in cardiovascular diseases especially in atherosclerosis (Bogdan, 2015; Lugrin *et al*., 2014). *In vitro* quantification of NO is inexpensive and quick in comparison with other mediators. Hence, its use to identified plant extracts with anti-inflammatory potential constitutes an important strategy that must be investigated. With the aim of exploring the ethnobotanical richness of our Caribbean region in search of new therapeutic agents with anti-inflammatory potential, in this work, we evaluated 31 plant species used in the traditional medicine of the Colombian Caribbean coast to treat various diseases related to inflammatory processes, determining their capacity to inhibit the production of the inflammatory mediator NO, in macrophages. In addition, the anti-inflammatory activity of the most active species (nitrite inhibition > 95%) was confirmed, determining their effects on the production of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in LPS-activated RAW 264.7 cells.

MATERIALS AND METHODS

Reagents

Macrophages RAW 264.7 were acquired from the American Type Culture Collection (Manassas, VA). Penicillin-streptomycin, trypan blue, and lipopolysaccharide from *Escherichia coli* (LPS), N′-[[3-(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride (1400W), sodium nitrite, N-[1,1-naphthyl] ethylenediamine dihydrochloride, 4-aminobenzenesulfonamide, sodium nitroprusside (SNP), dexamethasone, rofecoxib, Folin–Ciocalteu, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma Aldrich (St Louis, MO). Bromide of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) was obtained from Calbiochem® (San Diego, CA). Ethanol, dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Pittsburgh, PA). ELISA kits from eBiosciences.

Collection and identification of plant material

Plant species were collected in the Caribbean coast of Colombia. The taxonomic identification was carried out in the Guillermo Piñeres Botanical Garden of Cartagena and the Colombian National Herbarium of the National Science Institute of the National University of Colombia; voucher specimens of each species were kept in the corresponding institution.

Preparation of extracts and preliminary phytochemical analysis

The previously dried and milled plant material was subjected to extraction by maceration with 96% ethanol at room temperature (25°C ± 3°C). The obtained extract was filtered and concentrated in a rotary evaporator under reduced pressure and controlled temperature (40°C–45°C) and stored at –20°C. The following secondary metabolites' presence was determined qualitatively: alkaloids, coumarins, tannins, cardiotonic glycosides, flavonoids, saponins, triterpenes/steroids, and quinones, using a previously reported methodology (Herrera *et al*., 2014). Each analysis was carried out in triplicate.

Effects on the production of NO and cytotoxicity in macrophages RAW 264.7

RAW 264.7 macrophages were maintained in DMEM, enriched with 10% inactivated FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml), at 37°C and 5% CO_2. The viability of macrophages RAW 264.7, treated with different concentrations of the extracts, was determined using the method proposed by Ferrari *et al*. (1990), with some modifications (Ferrari *et al*., 1990).

The RAW 264.7 macrophages were seeded in 96-well plates (2×10^4 cells/well) and incubated for 48 hours. Subsequently, the cells were washed with phosphate buffered saline (PBS) and treated with the extracts under study at different concentrations; Triton X-100 (20%) was used as a positive cytotoxicity control. Thirty minutes later, the cells were stimulated with LPS (1 µg/ml) and incubated for 24 hours. After this time, a 70 µl aliquot of each well was preserved for subsequent determination of nitrite levels, and the cells were rewashed with PBS, and 100 µl of the MTT solution (250 µg/ml) was added and incubated again for 4 hours. Finally, the supernatant was removed and the formazan crystals were dissolved with 100 µl of DMSO and the absorbance was determined in a microplate reader (Multiskan GO, Thermo Scientific) at DO_{550}. The evaluated extracts were considered cytotoxic when the percentage of cell survival was less than 80% (Ospina *et al*., 2013). The extracts' ability to inhibit NO production was determined by quantifying nitrite levels in supernatants from cell culture using the Griess reaction (Green *et al*., 1982). 70 µl of Griess reagent was added to 70 µl of the supernatant from the previously stored cell culture medium. The samples' absorbance was determined in a Multiskan GO microplate reader at $DO_{550,}$ and the concentration was calculated using a standard $NaNO_2$ curve (1–200 µM). The positive control was 1400 W (10 µM), a selective inhibitor of iNOS.

NO scavenging activity

The scavenging activity of the most active extracts on NO was determined using the method described by Sreejayan and Rao (1997), with some modifications (Sreejayan and Rao, 1997). NO was generated spontaneously by SNP in an aqueous solution with physiological pH, which in turn produces nitrite when interacting with oxygen, which can be easily determined using Griess reagent. The radical scavengers compete for the NO• radicals with the oxygen, with the consequent reduction in nitrite production. In summary, 1 ml of 10 mM SNP solution in PBS was mixed with 10 µl of the extracts under study at the maximum concentration at which they were evaluated against RAW 264.7 macrophages in the model to determine their ability to inhibit NO mediator production. The mixture was left in incubation for 4 hours at room temperature, after which 100 µl of this mixture was transferred to a 96-well plate, and 100 µl of the Griess reagent was added. The OD_{550} was determined in a microplate reader (Multiskan GO, Thermo) and compared with a standard curve of sodium nitrite to calculate nitrite concentration.

Quantification of phenolic compounds

Folin–Ciocalteu's method was used for the quantification of phenolic compounds (Del-Toro-Sánchez *et al*., 2014). Briefly, 30 µl of different concentrations of the extracts under study was taken and added to 150 µl of a Folin–Ciocalteu solution (0.1 M); the mixture was incubated at room temperature for 10 minutes, after which 120 µl of a sodium carbonate solution (7.5%) was added and incubated again for 2 hours. The DO_{760} was determined in a microplate reader (Multiskan GO, Thermo). The results are presented as mg of gallic acid per gram of dry extract.

Determination of the antioxidant potential

DPPH radical scavenging activity

The DPPH• free radical scavenging capacity was determined using a standard method (Brand-Williams *et al*., 1995) with some modifications (Castro *et al*., 2019). In a 96-well microplate, 75 µl of different concentrations of the extracts were mixed with 150 µl of the DPPH methanolic solution (100 µg/ml). The mixture was incubated at room temperature for 30 minutes, after which the disappearance of the DPPH• radical at DO_{550} was determined spectrophotometrically. The results were expressed as micromoles of Trolox per gram of dry extract.

ABTS radical scavenging activity

The $ABTS^{\bullet+}$ radical scavenging activity was determined using a standard method (Re *et al*., 1999) with some modifications (Castro *et al*., 2019). The $ABTS^{\bullet+}$ radical originates by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) incubated at 4°C in the dark for 16 hours. The solution of the $ABTS^{\bullet+}$ radical was diluted with ethanol to obtain an absorbance value of 0.70 ± 0.01 at 734 nm; 180 µl of this solution was mixed with 20 µl of the solutions with different concentrations of the extracts under study. The mixture was incubated at room temperature for 30 minutes, after which the disappearance of the $ABTS^{\bullet+}$ radical to DO_{734} was determined spectrophotometrically. The results are presented as micromoles of Trolox per gram of dry extract.

Quantification of cytokines and PGE2

Macrophages RAW 264.7 were seeded in 24-well plates (2×10^5 cells/well) and incubated for 24 hours. After that time, cells were treated with the extracts and incubated for 30 minutes, activated with LPS (1 µg/ml), and incubated again for 24 hours to produce the inflammatory mediators (IL-1β, IL-6, TNF-α, and PGE2). The culture supernatants were collected and kept at −20°C until further analysis. The production of IL-1β, IL-6, TNF-α, and PGE2 was measured using a standard sandwich ELISA procedure following the manufacturer's instructions of kits from eBiosciences (San Diego, CA). Dexamethasone and rofecoxib were used as positive controls and evaluated under the same conditions.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test. Values of $p < 0.05$ were considered significant.

RESULT AND DISCUSSION

Colombia has an enormous biological diversity and is considered one of the 17 "mega-diverse countries" in the world, according to the United Nations Environment Program. Large amounts of data about Colombia's ecosystems are being collected in the "Colombia BIO" expeditions, including novel biodiversity in previously unexplored regions due to the internal conflict. Unfortunately, for a high percentage of these species, there is sparse knowledge, which has avoided taking advantage of the benefits in the food, medicine, and industry (De Vega *et al*., 2020). An alternative way to take advantage of these biological resources is the practice of bioprospecting, which implies the interaction between different types of knowledge, especially ancestral knowledge that local and indigenous communities in South American countries have, with the scientific knowledge contributed by the academic and industrial sectors, especially the pharmaceutical industry (Duarte and Velho, 2008). To contribute to the bioprospecting of our natural resources and in accordance with the permanent need to develop new drugs with anti-inflammatory properties, in this work, we selected 31 plant species used in the popular medicine of the Colombian Caribbean coast to assess its anti-inflammatory potential.

Collection, identification of plant material, and extract preparation

Information on the collected species, including common names, the part of the plant evaluated, the collection site, the species' registration codes, and the yields of the extracts obtained, is presented in Table 1 and their preliminary phytochemical analysis is presented in Table 2.

Effect on RAW 264.7 cell viability

As shown in Figure 1, only the extracts of *Momordica charantia*, *Inga vera*, *Ambrosia cumanensis* (seeds and leaves), *Trichilia hirta*, *Tabernaemontana cymosa* (seeds), and *Anacardium occidentale* showed toxicity (cell viability under 80%) on RAW 264.7 macrophages at 100 µg/ml. Therefore, these extracts were

Table 1. Information on vegetable species in the study.

Scientific name	Family	Local name	Part used	Voucher number	Extract yields (%)	Collection site
Crotalaria retusa L.	Fabaceae	Cascabelito	Seeds	JBC 12007	8.7	Galerazamba, Bolívar.
Heliotropium indicum L.	Boraginaceae	Rabo de alacran	Seeds	JBC 3691	6.8	(10°47′22″N, 75°15′35″W)
Mammea americana L.	Calophyllaceae	Mamey	Leaves	JBC 467	7.3	
Murraya exotica L.	Rutaceae	Azahar de la india	Leaves	COL 538418	6.3	
Pedilanthus tithymaloides (L.) Poit.	Euforbiaceae	Pitamorreal	Leaves	JBC 1018	8.1	
Momordica charantia L.	Cucurbitaceae	Balsamina	Leaves	JBC 793	9.8	San Basilio de Palenque, Bolívar. (10°6′12″N, 75°11′56″W)
Ambrosia cumanensis Kunth.	Asteraceae	Artemisa	Leaves	COL 538448	5.8	San Bernardo del viento, Córdoba. (9°21′18″N, 75°57′16″W)
			Seeds	COL 538448	9.8	
Anacardium occidentale L.	Anacardiaceae	Marañon	Seeds	JBC 4431	7.5	
Annona squamosa L.	Annonaceae	Guanabana	Seeds	JBC 4431	8.0	
Bursera graveolens Kunth.	Burseraceae	Caraña	Bark	JBC 5115	7.9	
Bursera simaruba (L.) Sarg	Burseraceae	Almácigo	Bark	JBC 4458	6.5	
Caesalpinia coriaria (Jacq.) Willd.	Fabaceae	Dividivi	Fruit	COL 538422	8.2	
Capparis odoratissima (Jacq.) Hutch.	Capparaceae	Olivo	Leaves	JBC 1492	7.6	
Chenopodium ambrosioides L.	Amaranthaceae	Paico	Leaves	JBC 4005	9.4	
Cecropia peltata L.	Urticaceae	Yarumo	Leaves	JBC 1383	7.8	
			Bark	JBC 1383	7.8	
Crateva tapia L.	Capparaceae	Naranjuelo	Leaves	JBC 12017	6.4	
Diospyros inconstans Jacq.	Ebenaceae	Caimitillo	Bark	JBC 1438	10.2	
Eryngium foetidum L.	Apiaceae	Culantro	Leaves	COL 538419	8.8	
Gustavia superba (Kunth) O.Berg.	Lecythidaceae	Membrillo	Leaves	JBC 1382	6.8	
Hippomane mancinella L.	Euphorbiaceae	Manzanillo	Leaves	JBC 2478	6.7	
			Fruit	JBC 2478	5.4	
			Seeds	JBC 2478	8.7	
Hura crepitans L.	Euphorbiaceae	Ceiba blanca	Bark	JBC 788	7.2	
Hyptis capitata Jacq.	Lamiaceae	Botón negro	Leaves	JBC 2478	10.4	
			Seeds	JBC 2478	9.8	
Inga vera Willd.	Fabaceae	Guama	Seeds	JBC 17149	8.1	
Piper peltatum L.	Piperaceae	Santa María	Leaves	JBC 1438	4.2	
Ruellia tuberosa L.	Acanthaceae	Campana	Leaves	JBC 3932	6.8	
Sarcostemma clausum (Jacq.) Schult.	Apocynaceae	Bejuco de sapo	Leaves	JBC 2502	7.3	
Sterculia apetala (Jacq.) H. Karst.	Malvaceae	Camajuro	Seeds	COL 538417	7.8	
Tabebuia ochracea (Cham.) Standl.	Bignoniaceae	Polvillo	Bark	JBC 5153	6.8	
Tabernaemontana cymosa Jacq.	Apocynaceae	Bola de puerco	Seeds	JBC 3243	9.6	
			Bark	JBC 3243	7.3	
Thevetia peruviana (Pers.) Merr.	Apocynaceae	Cavalonga	Flowers	JBC 66	7.4	
Trichilia hirta L.	Meliaceae	Jobo macho	Seeds	JBC 4330	7.9	

The taxonomic identification was carried out in the Guillermo Piñeres Botanical Garden of Cartagena (Voucher JBC) and the Colombian National Herbarium of the National Science Institute of the National University of Colombia (Voucher COL). The yields were calculated as follows: [Dry Concentrated Extract (g)/Dry Material(g)] × 100.

evaluated to determine their highest nontoxic concentration and their anti-inflammatory effect on macrophages RAW 264.7 was securely evaluated. The selected concentration of each extract can be seen in Table 3.

Effect on NO production in RAW 264.7 macrophages

The anti-inflammatory potential of the extracts was established by determining their effect on the release of the NO inflammatory mediator in the RAW 264.7 macrophage cell line. When macrophages are stimulated, they produce NO, which is important in protecting the organism against viruses and pathogenic microorganisms (Bogdan, 2015; Wallace, 2005). The antiviral or antimicrobial effect of NO and other RNS such as peroxynitrite is related to their ability to react with structural elements, components of the replication machinery, metabolic enzymes, and molecules associated with the virulence of infectious pathogens (Bogdan, 2015). However, NO when it is in high concentrations or when it is produced continuously, as it happens during chronic inflammatory processes, can produce harmful results for the tissues, being able to react with the superoxide free radical (O^{2-})

Table 2. Phytochemical characterization of vegetable species in the study.

Scientific name	Part used	Metabolite							
		Alkaloids	Coumarins	Tannins	Glycosides	Flavonoids	Saponins	Terpenes/steroids	Quinones
A. cumanensis	Leaves	−	−	−	+	−	+	+	+
	Seeds	+	+	+	−	+	−	+	−
A. occidentale	Seeds	−	+	+	−	+	+	+	−
A. squamosa	Seeds	+	+	+	−	+	+	+	−
B. graveolens	Bark	−	+	−	+	+	−	−	+
B. simaruba	Bark	+	−	−	−	+	+	+	+
C. coriaria	Fruit	−	−	+	+	−	−	−	+
C. odoratissima	Leaves	+	+	−	−	−	−	+	−
C. ambrosioides	Leaves	−	−	−	−	+	−	−	−
C. peltata	Leaves	+	−	+	+	+	+	+	+
	Bark	+	+	−	+	+	+	+	−
C. tapia	Leaves	+	+	−	+	−	+	−	−
C. retusa	Seeds	+	−	−	−	+	+	−	−
D. inconstans	Bark	+	+	+	−	−	+	+	−
E. foetidum	Leaves	−	−	−	+	−	−	+	+
G. superba	Leaves	+	−	−	−	−	−	+	−
H. indicum	Seeds	−	−	−	+	+	−	−	+
H. mancinella	Leaves	+	+	+	+	+	−	+	−
	Fruit	−	+	−	−	−	+	+	−
	Seeds	+	+	+	+	+	−	−	+
H. crepitans	Bark	+	+	+	+	+	−	−	+
H. capitata	Leaves	+	+	+	−	+	+	−	−
	Seeds	+	+	−	+	+	−	−	+
I. vera	Seeds	−	+	−	−	+	−	−	−
M. americana	Leaves	−	+	+	+	+	+	+	−
M. charantia	Leaves	+	−	−	+	−	−	+	−
M. exotica	Leaves	+	+	−	−	+	+	+	−
P. tithymaloides	Leaves	+	−	+	+	+	−	−	+
P. peltatum	Leaves	+	−	+	+	+	+	+	+
R. tuberosa	Leaves	−	−	+	−	+	+	+	+
S. clausum	Leaves	−	−	−	+	+	−	−	+
S. apetala	Seeds	+	+	+	−	+	−	−	−
T. ochracea	Bark	−	−	+	−	+	−	−	+
T. cymosa	Seeds	+	−	−	+	+	+	+	+
	Bark	+	+	+	+	−	+	−	−
T. peruviana	Flowers	−	−	−	+	+	−	−	+
T. hirta	Seeds	+	+	−	+	−	+	+	−

+: presence and −: not detected.

to generate the peroxynitrite anion ($ONOO^-$), from which the formation of hydroxyl radicals (•OH), carbonate ($CO_3•^-$), and nitrogen dioxide ($•NO_2$) can be derived. These free radicals can interact with proteins, lipids, and nucleic acids, promoting various molecular modifications responsible for altering the cells' biological functions, leading to the appearance of various chronic diseases. Therefore, the iNOS inhibitors could have a therapeutic application in conditions that occur with chronic inflammatory processes such as arthritis, inflammatory bowel disease, and cancer (Bogdan, 2015; Lugrin *et al*., 2014).

The effect of the extracts under study on the inhibition of NO production in macrophages RAW 264.7 is presented in Table 3. The extracts were classified according to their activity as active (% inhibition ≥ 60), moderate (60 > % inhibition > 40), mild (40 > % inhibition > 20), and inactive (% inhibition < 20). Fifteen extracts corresponding to 40.5% of all the extracts evaluated were classified as actives, being the extracts of *A. cumanensis* (seeds), *T. hirta* (seeds), *Hyptis capitata* (seeds and leaves), *Mammea americana* (leaves), and *Crateva tapia* (leaves) the most active, with inhibition values greater than 95%, presenting itself as powerful inhibitors

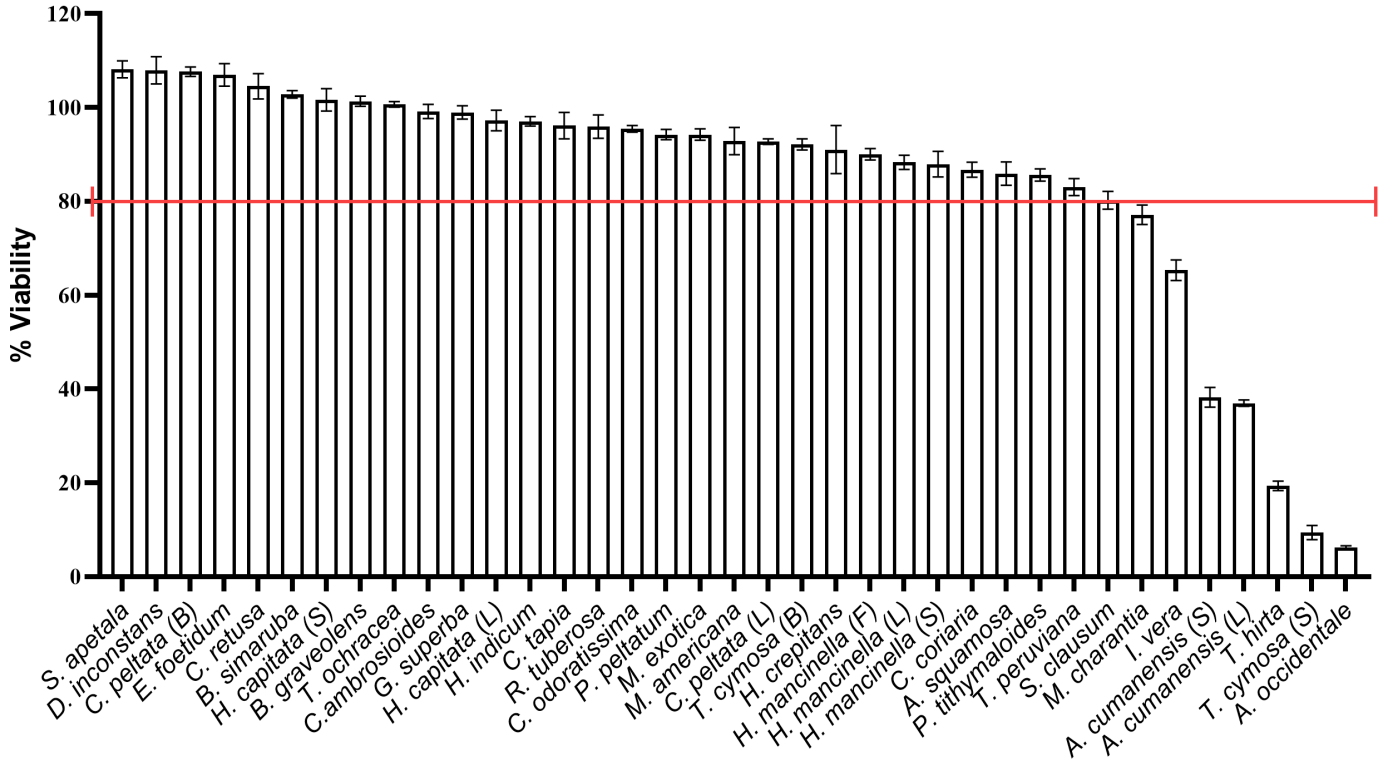

Figure 1. Effect of extracts on cell viability of RAW 264.7 macrophages. (S): seeds; (L): leaves; (B) bark; (F) fruit. Cytotoxicity was measured with MTT assay. Each value represents mean ± SEM (n = 9)

of the production of NO *in vitro*, and therefore, they could be the basis for developing new therapies to prevent the oxidation of macromolecules mediated by the NO during the chronic inflammatory processes; characteristic condition of pathologies such as cancer, diabetes, and inflammatory bowel disease. Plant species to which the most active extracts belong are widely used in traditional medicine to treat various health conditions (Table 4), including some related to chronic inflammatory processes. It is important to highlight the potent activity shown by the extracts of *A. cumanensis* (seeds) and *T. hirta* (seeds), which exerted their inhibitory effect at lower concentrations (25 and 12 µg/ml) than the required for the other extracts (100 µg/ml). The rest of evaluated extracts showed moderate activity (4 extracts, 10.8%) or mild activity (8 extracts, 21.6%) or were inactive (10 extracts, 27.0%).

Quantification of phenolic compounds and determination of free radical scavenging capacity

Phenolic compounds are widely known for their antioxidant effects, mainly associated with their free radical scavenging capacity and their impact on the decrease of various inflammatory mediators, including NO (Conforti and Menichini, 2011; Fernandez-Panchon *et al*., 2008). The content of total phenolic compounds, as well as their scavenger activity on DPPH and ABTS free radicals, was quantified in the extracts considered as active, observing a strong correlation between the presence of the phenolic compounds and the scavenger effect of free radicals DPPH (r = 0.86) and ABTS (r = 0.91); in fact, extracts of *A. occidentale* (seeds), *Bursera simaruba* (bark), *H. capitata* (seeds), and *M. americana* (leaves) showed the highest content of phenolic components as well as the best DPPH• and $ABTS^{\bullet+}$ radical scavenging activity (Table 5). With respect to the NO scavenging activity, a low correlation (r = 0.27) between the phenolic compounds content and this scavenging activity was observed (Table 6). Therefore, the effect on the inhibition of NO• production in LPS-induced RAW 264.7 macrophages is related to the impact on cells and not with the scavenging effects of NO by the active extracts.

In addition, phenolic compounds content showed a low correlation (r = 0.10) with the inhibition of the NO production by the macrophages RAW 264.7, in such a way that there is no direct relationship between the activity shown by extracts and the total phenolic content present in the extracts; for instance, extracts such as *A. cumanensis* (seeds and leaves), *T. hirta* (seeds), *H. capitata* (leaves), *C. tapia* (leaves), *Murraya exotica* (leaves), and *Eryngium foetidum* (leaves) that showed potent inhibitory activity of NO production in the cell line (% inhibition ≥ 90%) had a low content of phenolic compounds in their composition (< 30 mg of gallic acid/g of extract), while the extract of *A. occidentale* (seeds) that showed the highest content of phenolic compounds only inhibited in 60.28% of the NO in the cells. This low correlation is not sufficient reason to rule out that this group of metabolites can be associated with this biological activity since this not only depends on the concentration at

Table 3. Effect of evaluated extracts on the production intracellular of NO in LPS-stimulated RAW 264.7 macrophages.

Scientific name	Part used	Concentration (µg/ml)	Cell viability (%)	Nitrite inhibition (%)	Classification
A. cumanensis	Seeds	25	95.8 ± 3.1	113.6 ± 0.8	Active
T. hirta	Seeds	12	91.0 ± 3.3	108.2 ± 1.4	
H. capitata	Leaves	100	97.2 ± 2.2	101.6 ± 0.7	
H. capitata	Seeds	100	101.6 ± 2.4	100.3 ± 0.7	
M. americana	Leaves	100	92.8 ± 2.9	97.8 ± 2.4	
C. tapia	Leaves	100	96.1 ± 2.8	95.0 ± 5.8	
A. cumanensis	Leaves	25	94.0 ± 0.7	93.0 ± 0.8	
B. simaruba	Bark	100	102.8 ± 0.8	92.8 ± 1.1	
M. exotica	Leaves	100	94.2 ± 1.2	91.6 ± 5.3	
E. foetidum	Leaves	100	106.9 ± 2.4	90.4 ± 1.8	
A. squamosa	Seeds	100	85.9 ± 2.5	74.2 ± 3.7	
H. indicum	Seeds	100	97.0 ± 1.0	66.6 ± 3.8	
C. peltata	Leaves	100	92.7 ± 0.6	66.2 ± 1.9	
A. occidentale	Seeds	50	106.3 ± 5.7	60.3 ± 1.8	
C. ambrosioides	Leaves	100	99.1 ± 1.5	60.0 ± 2.0	
H. mancinella	Leaves	100	88.3 ± 1.5	48.2 ± 2.8	Moderately active
C. peltata	Bark	100	107.6 ± 1.0	44.8 ± 2.7	
T. cymosa	Bark	100	92.1 ± 1.2	41.0 ± 4.8	
D. inconstans	Bark	100	107.9 ± 2.9	40.7 ± 2.8	
R. tuberosa	Leaves	100	95.9 ± 2.5	38.1 ± 1.9	Mildly active
C. odoratissima	Leaves	100	95.4 ± 0.7	34.2 ± 3.6	
M. charantia	Leaves	50	97.4 ± 3.2	30.3 ± 2.3	
H. crepitans	Bark	100	91.0 ± 5.1	27.1 ± 2.3	
P. peltatum	Leaves	100	94.2 ± 1.1	22.6 ± 3.6	
C. coriaria	Fruit	100	86.7 ± 1.6	21.2 ± 3.4	
G. superba	Leaves	100	98.9 ± 1.4	20.8 ± 1.9	
C. retusa	Seeds	100	104.5 ± 2.7	20.2 ± 2.9	
H. mancinella	Seeds	100	87.9 ± 2.7	17.8 ± 1.9	Inactive
S. apetala	Seeds	100	108.1 ± 1.8	15.1 ± 3.7	
I. vera	Seeds	25	84.8 ± 2.8	13.5 ± 2.0	
T. cymosa	Seeds	50	84.8 ± 0.7	11.3 ± 1.9	
B. graveolens	Bark	100	101.3 ± 1.1	8.1 ± 1.0	
S. clausum	Leaves	100	80.2 ± 1.9	7.0 ± 3.1	
H. mancinella	Fruit	100	90.0 ± 1.2	5.2 ± 1.6	
T. peruviana	Flowers	100	83.0 ± 1.8	0.2 ± 5.6	
P. tithymaloides	Leaves	100	85.6 ± 1.3	0.0	
T. ochracea	Bark	100	100.6 ± 0.6	0.0	

The results represent the mean ± SEM (n = 12) from three independent experiments. Active (% inhibition ≥ 60), moderate (60 > % inhibition > 40), mild (40 > % inhibition > 20), and inactive (% inhibition < 20).

which the phenolic compounds are found but also on their structure, of which there are more than 8,000 known structures, in a range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (Conforti and Menichini, 2011; Sofi and Nabi, 2018). In fact, several phenolic compounds, including resveratrol, isovitexin, isoliquiritigenin, baicalin, baicalein, wogonin, apigenin, and luteolin, have shown the ability to inhibit the iNOS expression and NO production in LPS-induced RAW 264.7 macrophages (Conforti and Menichini, 2011).

Determination of inflammatory mediators

The most active extracts (nitrite inhibition > 95%) with anti-inflammatory potential were evaluated to identify their effects on the inflammatory mediators IL-6, TNF-α, and IL-1β, which is the same as NO, increase their production by activating the transcription factor NF-κB in LPS-induced macrophages (Girón *et al*., 2010). Results show that all evaluated extracts significantly decreased the production of IL-6, being the extracts of *A. cumanensis* (seeds) and *T. hirta* (seeds) the most potent, with inhibition percentages greater than 90%, with activity higher than

Table 4. Summary of ethnopharmacological antecedents of the most active plant species.

Scientific name	Part used	Mode of preparation	Principal medicinal indication	Way of administration	References
A. cumanensis	Leaves (fresh)	Decoction	Common cold	Bath	(Gómez-Estrada *et al*., 2011)
			Intestinal parasites	Orally	
		Poultice	Headache	Applied locally	
C. tapia	Bark	Paste of bark powder	Arthritis	Applied locally	(Sharma *et al*., 2013)
		Decoction	Abscess	Orally	
			Hypoglycemic		
	Leaves and stem	Decoction	Dysentery/diarrhea	Orally	
H. capitata	Whole plant	Juice	Malaria	Orally	(Animesh *et al*., 2010)
	Leaves, flowers, and roots	Decoction	Renal stones, liver disease, wounds, cough, shortness of breath, and diabetes	Orally	(Gailea *et al*., 2016)
M. americana	Seeds	Macerated	Lice control	Applied locally	(Germosén *et al*., 2017)
	Leaves	Poultice	Rheumatism		
T. hirta	Leaves and branch	Decoction	Flu	Orally	(Beyra *et al*., 2004)

Table 5. Quantification of phenolic compounds and scavenging effect on DPPH and ABTS radicals of the active extracts.

Scientific name	Part used	Phenolic compounds (mg of gallic acid/g of extract)	DPPH (µmoles Trolox/g of extract)	ABTS (µmoles Trolox/g of extract)
A. occidentale	Seeds	142.9 ± 2.4	1,652.2 ± 28.5	1,028.1 ± 19.4
B. simaruba	Bark	106.3 ± 2.4	831.6 ± 14.6	1,551.3 ± 22.2
M. americana	Leaves	97.3 ± 2.6	323.5 ± 6.7	1,155.0 ± 14.9
H. capitata	Seeds	96.8 ± 0.9	279.2 ± 3.1	769.1 ± 10.3
C. peltata	Leaves	53.0 ± 1.3	176.6 ± 4.1	207.3 ± 4.4
H. capitata	Leaves	30.0 ± 0.7	161.8 ± 1.4	158.8 ± 2.6
A. cumanensis	Leaves	26.4 ± 0.5	75.6 ± 1.8	51.8 ± 2.1
A. cumanensis	Seeds	23.3 ± 0.5	43.9 ± 0.7	127.9 ± 3.4
C. ambrosioides	Leaves	19.8 ± 0.3	43.5 ± 0.4	77.2 ± 0.9
E. foetidum	Leaves	19.7 ± 6.2	96.3 ± 1.0	220.6 ± 3.3
T. hirta	Seeds	15.7 ± 0.9	11.5 ± 0.2	23.0 ± 0.4
M. exotica	Leaves	14.8 ± 0.6	14.0 ± 0.2	64.7 ± 0.8
C. tapia	Leaves	7.5 ± 0.1	15.7 ± 0.2	45.5 ± 0.5
H. indicum	Seeds	3.3 ± 0.1	nd	34.8 ± 0.2
A. squamosa	Seeds	1.2 ± 0.0	nd	nd

The results represent the mean ± SEM (n = 9) from three independent experiments. nd = not determined.

Table 6. Scavenging effect of the active extracts on nitric oxide.

Scientific name	Part used	Phenolic compounds (mg of gallic acid/g of extract)	Concentration(µg/ml)	% scavenging NO
A. occidentale	Seeds	142.9 ± 2.4	50	0.0
B. simaruba	Bark	106.3 ± 2.4	100	12.0 ± 2.0
M. americana	Leaves	97.3 ± 2.6	100	24.9 ± 1.0
H. capitata	Seeds	96.8 ± 0.9	100	14.0 ± 1.1
C. peltata	Leaves	53.0 ± 1.3	100	22.1 ± 1.2
H. capitata	Leaves	30.0 ± 0.7	100	10.0 ± 0.5
A. cumanensis	Leaves	26.4 ± 0.5	25	4.9 ± 0.7
A. cumanensis	Seeds	23.3 ± 0.5	25	10.8 ± 1.8
C. ambrosioides	Leaves	19.8 ± 0.3	100	10.5 ± 0.7
E. foetidum	Leaves	19.7 ± 6.2	100	15.3 ± 1.4
T. hirta	Seeds	15.7 ± 0.9	12	4.3 ± 0.4
M. exotica	Leaves	14.8 ± 0.6	100	3.0 ± 0.3
C. tapia	Leaves	7.5 ± 0.1	100	10.3±0.7
H. indicum	Seeds	3.3 ± 0.1	100	2.3 ± 0.2
A. squamosa	Seeds	1.2 ± 0.0	100	0.0

The results represent the mean ± SEM (n = 9) from three independent experiments.

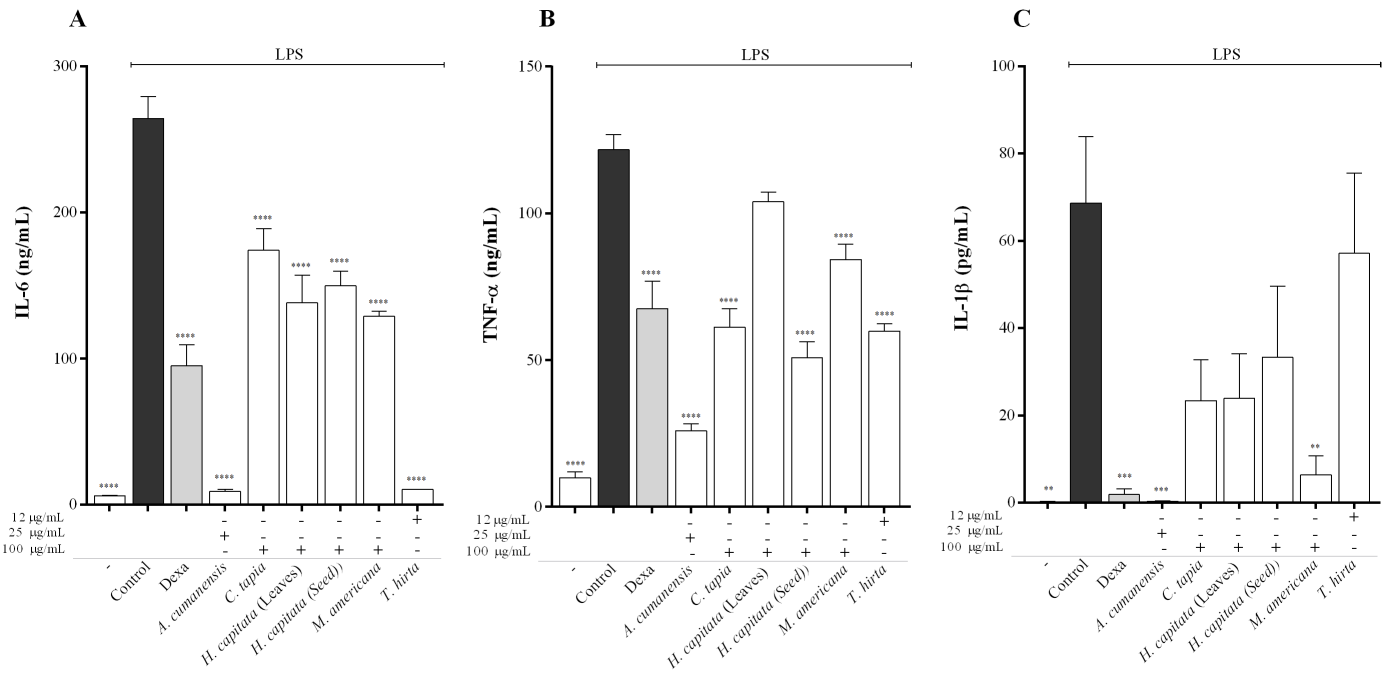

Figure 2. Effect of extracts on intracellular production of IL-6 (A), TNF-α (B), and IL-1β (C) in LPS-stimulated RAW 264.7 macrophages (1 µg/ml) for 24 hours. −: unstimulated cells. Dexamethasone (20 µM) was used as a positive control. Results represent the mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ ANOVA statistically significant compared with LPS-treated group).

shown by dexamethasone (68%) used as a reference drug (Fig. 2A). Regarding the effect on TNF-α, except for the extract of *H. capitata* (leaves), all others significantly inhibited the production of this mediator with similar percentages to that shown by the reference drug (Fig. 2B). Finally, the results presented in Figure 2C show that only *A. cumanensis* (seeds) and *M. americana* (seeds) extracts inhibited the production of IL-1β significantly. Inflammatory mediators IL-6, IL-1β, and TNF-α are produced by many cell types, mainly macrophages and mast cells. They have several roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute phase response (Medzhitov, 2008).

The simultaneous inhibition of the production of these proinflammatory cytokines, as in the case of the extracts of *A. cumanensis* (seeds) and *M. americana* (leaves) (Fig. 2), is a key point as a pharmacological target in the treatment of inflammation. Therefore, we evaluated the concentration-dependent effect of these two extracts on these proinflammatory cytokines. Figure 3 shows that the *M. americana* extract showed a significant concentration-dependent effect on all mediators with particular emphasis on the NO and IL-1β, even to the lower concentration tested of 50 µg/ml. *M. americana* is a species recognized for its high content of coumarins (Crombie *et al*., 1987; Yang *et al*., 2005). Studies of this species in terms of its health benefits are limited. Some studies have been conducted on its properties related to the use of leaves, seeds, and bark as antibacterial, anthelmintic, antiviral, and antimalarial (Gómez-Calderón *et al*., 2017; González-Stuart, 2011; Toma *et al*., 2005). Until now, there are no studies that validate the anti-inflammatory activity of Mamey (*M. americana*); therefore, the present work is an important contribution to the knowledge of the anti-inflammatory properties of this species.

On the other hand, *A. cumanensis* (seeds) extract exerts its effects even at concentrations as low as 6 and 12 µg/ml in all inflammatory mediators evaluated (Fig. 3). *Ambrosia cumanensis* is recognized for its high content of sesquiterpene lactones, compounds widely recognized for their anti-inflammatory properties (Jimenez-Usuga *et al*., 2016). The sesquiterpene lactones coronopilin and damsin isolated from *Ambrosia arborescens* modulate the activation of the transcription factor NF-κB, decreasing the production of IL-6, while ambrosanolide, a type of sesquiterpene lactone present in *Ambrosia psilostachya*, inhibits the production of NO in mouse peritoneal macrophages (Lastra *et al*., 2004; Svensson *et al*., 2018).

Considering the powerful activity shown by the *A. cumanensis* (seeds) extract on the evaluated cytokines, we decided to determine this extract's effect on the proinflammatory mediator PGE2 in macrophages RAW 264.7. Similar to the effect observed on cytokines, this extract inhibited LPS-induced PGE2 production in a concentration-dependent manner even at concentrations as low as 6 and 12 µg/ml, with activity at 12 µg/ml similar to that exerted by rofecoxib used as a control (Fig. 4). PGE2 is an essential homeostatic factor that plays an important role in the modulation of the inflammatory and immune response through the regulation of cytokine production, leukocyte migration, proliferation, and differentiation (Díaz-Muñoz *et al*., 2012; Kalinski, 2012).

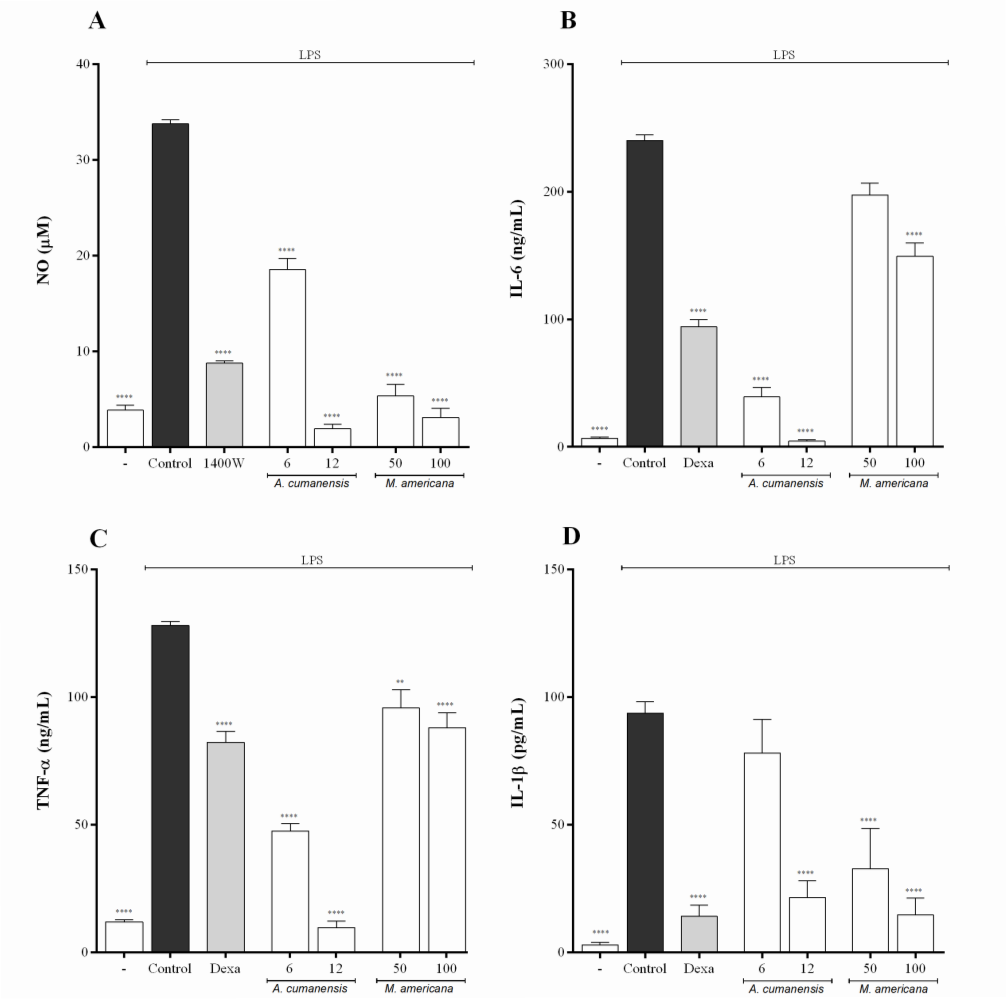

Figure 3. Effect of promissory extracts of *A. cumanensis* (seeds) and *M. americana* (leaves) on the production of NO (A), IL-6 (B), TNF-α (C), and IL-1β (D) in LPS-stimulated RAW 264.7 macrophages (1 μg/ml) for 24 hours. −: unstimulated cells. 1400W (10 μM) and dexamethasone (20 μM) were used as a positive control. Results represent the mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ ANOVA statistically significant compared with LPS-treated group).

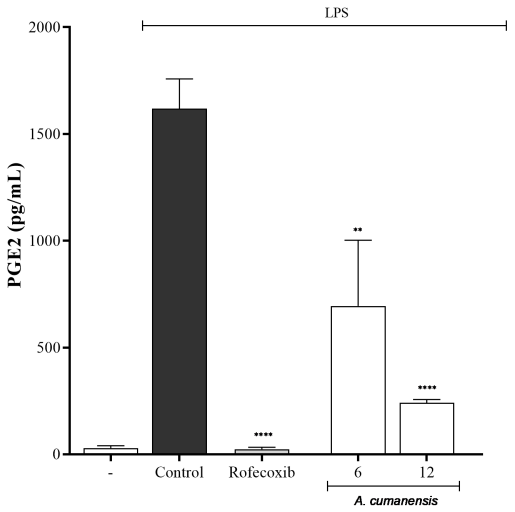

Figure 4. Effect of promissory extracts of *A. cumanensis* (seeds) on the production of PGE2 in LPS-stimulated RAW 264.7 macrophages (1 μg/ml) for 24 hours. −: unstimulated cells. Rofecoxib (10 μM) was used as positive control. Results represent the mean ± SEM (**$p < 0.01$; ****$p < 0.0001$ ANOVA statistically significant compared with LPS-treated group).

CONCLUSION

Our results show the anti-inflammatory potential of 15 extracts of 13 plant species of the Colombian Caribbean coast, which significantly inhibit the production of the NO inflammatory mediator. Extracts of the species *A. cumanensis* and *M. americana* are the most promising, inhibiting the production of all evaluated inflammatory mediators significantly, constituting a valuable biological resource of the Colombian diversity on which bioprospecting must be applied to develop new therapeutic alternatives for the treatment of diseases that occur with inflammatory processes.

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AUTHOR CONTRIBUTIONS

FD and LF conceived the study; FD, LF, and JC supervised the study, designed experiments, and carried out the experiments; JC, LF, and FD wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

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