Suppression of inflammation by *Baccharis punctulata* and *Baccharis trimera* through modulation of innate and adaptive immune responses

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**ABSTRACT**

Inflammatory processes, involving both innate and adaptive responses, are essential for controlling pathogens and maintaining homeostasis. However, excessive inflammation can cause chronic conditions including autoimmune diseases, atherosclerosis, and cancer. Alternative treatment options for inflammation are crucial, and exploring plants with anti-inflammatory properties holds significance. The genus *Baccharis* (Asteraceae family) is widespread across the Americas and is traditionally used to treat various disorders, including inflammatory diseases. Several biological activities of *Baccharis* species have been described; however, their immunomodulatory effects have not been widely evaluated. In this study, we analyzed the immunomodulatory activity of two *Baccharis* species, *Baccharis punctulata* and *Baccharis trimera*, using an *in vitro* inflammation model involving monocytic cells and splenocytes to evaluate both innate and adaptive immune responses. Both *B. punctulata* and *B. trimera* reduced lipopolysaccharide (LPS)-induced inflammatory mediators, including soluble CD14 and pro-inflammatory cytokines, in THP-1 cells. Furthermore, they suppressed nitric oxide (NO) production in splenocytes, demonstrating a dampened innate immune response. In addition, both species attenuated concanavalin A (ConA)-induced splenocyte proliferation, suggesting an anti-inflammatory effect on the adaptive immune response. In summary, the extracts demonstrated significant anti-inflammatory activity, affecting both innate and adaptive immune responses, and underscoring their potential as treatments for inflammatory diseases and sources of anti-inflammatory molecules.

**INTRODUCTION**

A diverse array of plants has been employed in traditional medicine since ancient times, with a substantial proportion specifically harnessed for the alleviation of inflammatory conditions such as rheumatic diseases and diabetes [1]. For example, in South America, *Baccharis trimera*, *Annona neosalicifolia*, and *Artemisia absinthium* L. CTES0032064 are used frequently for the treatment of diabetes and *Dysphania ambrosioides*, *Sida cordifolia*, and *Maytenus ilicifolia* as anti-inflammatory agents [2].

Inflammation is a protective reaction that contributes to the return to a homeostatic state after injury. Innate and adaptive immune cells participate in this response [3]. In this context, monocytes/macrophages play a significant role in inflammation, defense mechanisms, and removal of damaged cells. Excessive responses of these cells, on the other hand, can lead to chronic inflammatory conditions including autoimmune diseases, atherosclerosis, and cancer, among others. Overproduction of inflammatory mediators by monocytes, such as pro-inflammatory cytokines, reactive nitrogen species, and reactive oxygen species, characterizes this response [4]. In addition, lymphocytes are highly activated under chronic inflammatory conditions [5] the histology of inflamed tissue begins to resemble that of peripheral lymphoid organs, which can be referred to as lymphoid neogenesis or formation of tertiary lymphoid tissues. Lymphocyte recruitment to inflamed tissues is also reminiscent of lymphocyte homing to peripheral
lymphoid organs. In the latter, under physiological conditions, homing receptors expressed on lymphocytes adhere to vascular addressin expressed on high endothelial venules (HEVs). Thus, overactivation of innate and adaptive responses can lead to chronic inflammation and autoimmune diseases.

Currently used anti-inflammatory drugs are effective; however, they have some disadvantages, such as undesirable adverse effects and unresponsiveness to treatment in certain patients [6]. Therefore, alternative treatment options are needed, and the study of plants with anti-inflammatory activity is relevant because of their potential pharmacological activity.

*Baccharis* (Asteraceae) is a widespread genus in the Americas and some species are commonly named “carqueja” [7]. *Baccharis* species are used in South American traditional medicine to treat gastrointestinal and liver disorders, infections, fever, and inflammation [8].

The *Baccharis* genus has been associated with numerous biological activities, including antioxidant, antibacterial, and antifungal properties [7,9–11]. Furthermore, previous studies have recognized potential anti-inflammatory effects [12–16]; however, its impact on the immune system has not been extensively analyzed.

*Baccharis trimera* is one of the most studied species within the genus *Baccharis*. The anti-inflammatory activity of *B. trimera* has been demonstrated in murine models of inflammation [12–14,17]. However, studies of its anti-inflammatory activity in human cells are limited.

*Baccharis punctulata* has rarely been studied. It has been reported that *B. punctulata* exhibits anti-inflammatory properties in a murine model of 12-O-tetradecanoyl-phorbol-13-acetate-induced inflammation [18]. In addition, *B. punctulata* has been shown to decrease lymphocyte proliferation [19].

Therefore, in the present study, we analyzed the immunomodulatory activity of two species of the *Baccharis* genus, *B. punctulata* and *B. trimera*, in *in vitro* models of inflammation using monocytes and splenocytes, encompassing the assessment of both innate and adaptive immune responses.

**MATERIALS AND METHODS**

**Plant material and sample preparation**

*Baccharis punctulata* DC and *B. trimera* (Less.) DC were collected from Ñemby, Paraguay, and San Lorenzo, Paraguay, respectively. Taxonomical evaluations were performed by botanists and voucher specimens (*B. trimera*: Voucher N° 4088 and *B. punctulata*: Voucher N° 4302) were deposited in the Herbarium-FCQ of the Department of Botany, Facultad de Ciencias Químicas, Universidad Nacional de Asunción (UNA). The aerial parts of the collected plants were used for extract preparation. The plant material was air-dried and ground into a fine powder using a cutting mill. To obtain the extracts, 200 g of the powder was suspended in HPLC-grade methanol (1.5 l) and sonicated three times for 30 minutes (*in vitro method*). The extraction procedure was repeated thrice, after which the extracts were filtered. A rotary evaporator (RVO 400 SD; Boeco, Germany) was used to remove the solvent. The yield was calculated considering the initial powdered dry material as the starting point (*B. trimera*: 17.1 g, 5.04%, and *B. punctulata*: 24.12 g, 6.96%). Plant methanol extracts were chemically characterized as previously described by our group [19]. The extracts were resuspended in dimethyl sulfoxide (DMSO; Sigma, USA) at a concentration of 10 or 100 mg/ml for further experiments.

**Cells and cell culture**

In this study, THP-1 (human acute monocytic leukemia) cells and splenocytes isolated from murine spleens were used. These cellular models were used to evaluate the immunomodulatory activity of the extracts on monocytes and lymphocytes, which are the primary cells involved in the innate and adaptive immune responses, respectively. To investigate the effect of *B. punctulata* and *B. trimera* extracts on innate immunity, their effects on human monocytic cells were determined using an *in vitro* model of lipopolysaccharide (LPS)-induced inflammation.

THP-1 cells were cultured at 37°C in 5% CO₂ in Roswell Park Memorial Institute (RPMI) medium (Gibco, USA), 10% fetal bovine serum (Gibco, Brazil), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, USA). Splenocyte isolation was performed as described in Coligan et al. [20] with minor modifications. Briefly, the spleen of 8–10-week-old healthy female Swiss albino mice were surgically removed in a sterile manner (under a laminar flow cabinet). The spleens were then gently pressed and subsequently subjected to a 70 µm cell strainer. After that, the cells were incubated in red blood cell lysis using an ammonium chloride-potassium solution, washed with phosphate buffer saline, and cultured in RPMI medium (Gibco, USA), 10% fetal bovine serum (Gibco, Brazil), 100 µl penicillin, and 0.1 mg/ml streptomycin (Gibco, USA). Cell viability was assessed by trypan blue staining. The study was approved by the Ethics Research Committee of Facultad de Ciencias Químicas, UNA (N° 798/21).

**Measurement of cell viability**

THP-1 cells were seeded in 96-well plates (Corning Costar®, USA) at a density of 2 × 10⁴ cells/well. The cells were treated with 10, 25, 50, 100, or 200 µg/ml of the extract for 24 hours. Cell viability was measured using an MTT colorimetric assay. Splenocytes, isolated as previously described, were cultured at a density of 5 × 10⁶ cells/ml and 200 µl/well in a 96-well plate in RPMI medium in the presence of increasing extract concentrations. Resazurin was added 48 hours later and incubated for 24 hours at 37°C and 5% CO₂. After this time, cell viability was determined by absorbance measurements at 570 and 600 nm on a MultiskanTM GO (Thermo Scientific, Waltham, USA). Cells cultured in medium alone or 0.1% DMSO (vehicle control) were used as controls.

**Determination of soluble CD14 (sCD14) levels**

LPS induces monocyte activation and subsequent production of pro-inflammatory mediators such as cytokines and sCD14 [21–23] Cells (2 × 10⁴ cells/well) were seeded in 96-well plates and pretreated for 15 minutes with the methanol extracts, followed by 1 µg/ml LPS (*E. coli* 0111:B4, Sigma, USA) for 24 hours. Cells treated with DMSO and cells pretreated with DMSO and stimulated with LPS were used as
the negative and positive controls, respectively. sCD14 levels in the culture medium were assessed using an enzyme-linked immunosorbent assay (ELISA) assay (Quantikine ELISA, R&D Systems, USA) following the manufacturer’s instructions. The optical density of each well was determined at 450 nm using a microplate reader (Multiscan GO; Thermo Fisher Scientific Waltham, USA).

Reverse transcription-polymerase chain reaction

THP-1 cells at a density of 1 × 10⁶ cells/ml were treated with the methanolic extracts (15 minutes), followed by stimulation with LPS (1 µg/ml) for 4 hours as reported previously [24]. RNA extraction was performed using TRIzol reagent (Thermo Fisher Scientific, USA) and subsequently processed with DNase RQ1 (Promega, USA) at 37°C for 30 minutes. cDNA synthesis was performed with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega®, USA), 100 µM Oligo dT, and dNTPs. cDNA was amplified (StepOnePlus real-time PCR system, Thermo Fisher Scientific, USA), using a SsoAdvanced Universal SYBR Green Supermix (BioRad, USA), and primers (Table 1).

A negative PCR control (with no template) was included. Relative mRNA expression was determined by the 2^-ΔΔCT method [29], using RPL13A as a normalizing gene.

Proliferation assay

Splenocytes (5 × 10⁶ cells/ml) were seeded in RPMI medium and stimulated with 20 µg/ml concanavalin A (ConA) in the presence of extracts. After 48 hours of treatment, resazurin (AlamarBlue®) was added, and the absorbance at 570 and 600 nm was measured 24 hours later on MultiskanTM GO (Thermo Scientific, Waltham, USA). The proliferation index was calculated using the following equation:

\[ IR = \frac{(E_{ox})A_2 - (E_{ox})A_1}{(E_{ox})A_2} \]

\[ E_{ox} = \text{molar extinction coefficient of oxidized resazurin}; \lambda_1 = 570 \text{ nm}; \lambda_2 = 600 \text{ nm}. \]

Measurement of NO production

Griess assay [30] was performed using the culture supernatant of splenocytes treated with or without the extract for 15 minutes, followed by stimulation with LPS (10 µg/ml) for 18 hours. The cell culture medium and Griess solution (0.1% N-1-naphthyl ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) were mixed in a 1:1 ratio and incubated for 15 minutes. The absorbance was measured at 545 nm (Multiscan GO, Thermo Fisher Scientific, USA). A standard curve was prepared by dissolving increasing concentrations of sodium nitrite (Cicarelli, Argentina) in the RPMI culture medium. The NO concentration was determined by extrapolation to the standard curve.

Statistical analyses

The results are expressed as mean ± standard deviation and statistical significance was determined by one-way analysis of variance (ANOVA) and Dunnett’s post-test (p < 0.05) using GraphPad Prism 8.01 (GraphPad Software Inc., USA).

RESULTS AND DISCUSSION

Cytotoxicity on THP-1 monocytic cells by B. punctulata and B. trimera extracts

First, the effect of the extracts on cell viability was determined by treating THP-1 cells with different concentrations of B. punctulata or B. trimera extracts. The species had no cytotoxic effects at 10, 25, or 50 µg/ml (Fig. 1A and B) compared with cells treated only with the vehicle (DMSO) used to dissolve the plant extract. It should be noted that unstimulated cells (with culture medium only) showed no difference in viability from cells treated with the vehicle (DMSO). Based on these results, concentrations of up to 50 µg/ml were selected for subsequent assays.

Effects of B. punctulata and B. trimera extracts on LPS-induced soluble CD14 and cytokines production in monocytic cells

THP-1 cells treated with the inflammatory stimulus LPS showed a significant increase in sCD14 production compared to unstimulated cells (Fig. 2). This indicated that the inflammation model functioned properly.

In addition, THP-1 cells were pretreated with vegetal extracts before stimulation with LPS. The levels of sCD14 in

Table 1. List of primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer direction</th>
<th>Primer sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL13A</td>
<td>Forward</td>
<td>CATCGTGCTAAACAGGATCTG</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGACACCTTGAGGCAGC</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>TCTCCACAAGCCCTTCG</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGAGGCTCATAGGTCG</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward</td>
<td>GATCTCGAGTGAGGCTCG</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGCTTTCCAGGGTGTCAT</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>TGATGGCTTATTACAGTGCCAAATG</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTAGTGGTGCCGAGATTCG</td>
<td></td>
</tr>
</tbody>
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RPL13A: ribosomal protein L13a; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein-1; IL-1β: interleukin 1 beta.

Figure 1. Effects of B. punctulata and B. trimera on cell viability. THP-1 cells were treated with B. punctulata (A) or B. trimera (B) extract for 24 hours. Cell viability was determined using an MTT assay. ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle (DMSO) control.
The flavonoid apigenin has been shown to decrease IL-6 and contained apigenin and genkwanin extract of B. trimera in previous study conducted by our group. This phenomenon is that different compounds were identified those in cells stimulated with LPS alone. One explanation for reduced IL-1β and IL-6 mRNA levels, compared to B. punctulata reduced IL-6 and MCP-1 mRNA levels, and in the effects of the species on cytokine expression:

macrophages[15]. which decreased IL-1β, IL-6, and IL-10 levels in murine, B. dracunculifolia the results observed in another species, in innate immune response. These findings are in line with the results observed in another species, B. dracunculifolia, which decreased IL-1β, IL-6, and IL-10 levels in murine macrophages[15].

LPS acts as a stimulus that triggers the production of inflammatory cytokines and chemokines in monocytes/macrophages[22]. THP-1 cells were pretreated with B. punctulata or B. trimera extracts and subsequently stimulated with LPS. The cells were then collected, and RNA extraction and RT-qPCR were performed to evaluate IL-1, IL-6, and MCP-1 mRNA levels. Baccharipunctulata significantly reduced LPS-induced sCD14 production (Fig. 2A and B), suggesting an immunomodulatory effect of these extracts on human monocytic cells. These results indicate a decrease in monocyte activation in the presence of the extracts compared to cells stimulated with LPS alone and without treatment with plant extracts. This suggests that the species modulates innate immunity.

It should be noted that there were some differences in the effects of the species on cytokine expression: B. punctulata reduced IL-6 and MCP-1 mRNA levels, and B. trimera reduced IL-1β and IL-6 mRNA levels, compared to those in cells stimulated with LPS alone. One explanation for this phenomenon is that different compounds were identified in these extracts in a previous study conducted by our group[19]. In our previous study, we showed that the methanol extract of B. trimera contained apigenin and genkwanin[19]. The flavonoid apigenin has been shown to decrease IL-6 and IL-1β[31]. In addition, genkwanin has anti-inflammatory activity and decreases IL-6 levels[32]. These compounds may be responsible for the action of the extract on the production of pro-inflammatory cytokines. In the B. punctulata methanol extract, trans-phytol and di-O-cafeoylquinic acid were previously identified by our group[19]. Di-O-cafeoylquinic acid and phytol have been shown to inhibit the production of the pro-inflammatory cytokines IL-6 and TNF-α[33,34]. Furthermore, di-O-cafeoylquinic acid has been shown to suppress the expression of inflammatory mediators such as nitric oxide(NO), prostaglandin E2, NO synthase, and cyclooxygenase-2 and to inhibit carrageenan-induced edema in vivo[33]. Therefore, these compounds could be responsible for the effects of the B. punctulata extract. These compounds have been documented to decrease the activation of the transcription factor NF-κB, which stimulates the expression of pro-inflammatory cytokines and other inflammatory mediators[33,35]. Consequently, it is hypothesized that the mechanism of action of B. punctulata extract involves inhibition of NF-κB activation.

Cytotoxicity on splenocytes and effects of B. punctulata and B. trimera extracts on cell proliferation

To gain a more comprehensive understanding of the effects of the extracts on immune cells, we evaluated the
NO, produced by immune cells, is an important mediator of the immune response. However, uncontrolled NO release can damage or destroy the host tissue. In the present study, *B. punctulata* and *B. trimera* reduced the LPS-induced NO production. Similarly, other *Baccharis* species (*B. obtusifolia*, *B. latifolia*, *B. pentlandii*, and *B. subulate*) have been shown to inhibit NO production [38]. Inhibition of iNOS activity, responsible for NO production, has been suggested to be beneficial for the treatment of pathologies involving inflammation [39]. Thus, *B. punctulata* and *B. trimera* present this potential action as immunomodulators of inflammatory response.

**CONCLUSION**

Evaluation of the activity of plant extracts on immune cells is a necessary step to recognize their potential in the management of inflammatory disorders such as autoimmunity, chronic infections, and allergies. In this study, we found that *B. punctulata* and *B. trimera* have anti-inflammatory activities, acting on both innate and adaptive immune responses. Our findings are significant because they show how these species globally modulate the inflammatory response.

In conclusion, the extracts tested in this study exhibited significant anti-inflammatory activity, acting on the innate and adaptive immune responses. Therefore, these species have important potential for the anti-inflammatory modulation of the inflammatory response.

**AUTHOR CONTRIBUTIONS**

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit
to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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DATA AVAILABILITY
All data generated and analyzed are included in this research article.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY
The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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