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Immunomodulatory Effects of Three Species of *Baccharis* on Human Peripheral Blood Mononuclear Cells

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ABSTRACT

Species of the genus *Baccharis* are medicinal plants are popularly used to treat gastrointestinal and inflammatory diseases and have demonstrated biological activities. However, studies on human cells are limited. The effects of the extracts from aerial part of *B. trimera*, *B. punctulata*, *B. notosergila* on human peripheral blood mononuclear cells (PBMCs) were assessed. *B. trimera* and *B. punctulata* decreased phytohemagglutinin (PHA)-induced lymphocyte proliferation. Also, *B. punctulata* downregulated the production of IFN-γ cytokine. Additionally, different compounds were identified in the extracts (from *B. trimera* apigenin and genkwanin, from *B. punctulata* trans-phytol and a di-*O*-caffeoylquinic acid and from *B. notosergila* chrysoeriol) by using liquid chromatography coupled to mass spectrometry (LCMS). Our results show that *B. trimera* and *B. punctulata* have potential anti-inflammatory activity, indicating promising use as immunomodulators.

Keywords: *Baccharis*, Medicinal plant, Anti-inflammatory, Peripheral Blood Mononuclear Cells

Introduction

Baccharis species of the *Asteraceae* family are widely distributed in South America and are popularly known as "carqueja" or "jaguarete ka'a". The aerial parts of these plants are used in folk medicine not only to treat gastrointestinal and liver diseases, but also inflammatory processes.[1,2]

Extracts and compounds with activity on the immune system, or immunomodulators, are of great interest due to the therapeutic potential, mainly in pathologies that involve inflammation. The genus *Baccharis* has demonstrated diverse biological activities, including immunomodulatory activity.[3] Among the species with reported anti-inflammatory activity, *B. dracunculifolia* and *B. trimera* are the most studied.[3–5] However, most of these studies were performed in murine models.[6–8] For this reason, we evaluated the effects of three different species of the *Baccharis* genus (*B. trimera*, *B. punctulata*, *B. notosergila*) on human peripheral blood mononuclear cells (PBMCs). Cells were evaluated for phytohemagglutinin (PHA)-induced proliferation and the production of a relevant cytokine, interferon gamma (IFN-γ), by these cells. This study reveals new properties of methanolic extracts of *B. trimera*, *B. punctulata* and *B. notosergila* in an *in vitro* model of human inflammatory response.

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Materials and Methods

Plant material

Plant samples (aerial part) were collected from different regions of Paraguay and identified by botanist Dr. Rosa Degen. *B. trimera*, *B. punctulata* and *B. notosergila* samples used in this study were collected from San Lorenzo-Paraguay on April 2016, Ñemby-Paraguay on May 2016 and Presidente Hayes-Paraguay on August 2016, respectively (Table 1). Voucher specimens were deposited in the Herbarium-FCQ of the Department of Botany, Facultad de Ciencias Químicas, Universidad Nacional de Asunción (UNA) for indexing purposes (Table 1). The voucher numbers for *B. trimera*, *B. punctulata* and *B. notosergila* plant sample are R. Degen 4088, R. Degen 4302, R. Degen 4362, respectively.

Plant materials were air-dried and grounded to fine powder in a knife mill prior to extraction. Extracts were obtained by ultrasound-assisted maceration, suspending the powder in HPLC-grade methanol (1.5 L per 200 g) and sonicating it for 30 min three times. The extraction cycles were repeated for 3 days. The extracts were filtered through qualitative filter paper and the solvent was eliminated using a rotary evaporator (RVO 400 SD Boeco, Germany). The extracts were weighed, and the yield was calculated considering the initial dry material (Table 1). All extracts were resuspended in DMSO (Sigma, USA).

Analysis of extracts by liquid chromatography coupled to mass spectrometry (LC-MS)

The samples were dissolved in LC-MS grade acetonitrile (Merck KGaA, Germany) at a concentration of 10 mg/mL and filtered with a nylon sample filter (0.22 µ) (Microclar, Argentina). They were subsequently injected into a liquid chromatograph coupled to a tandem mass spectrometer (Xevo-TQD, Waters Corporation, USA) with an electrospray ionization source (ESI) under the following conditions: column Kinetex EVO C18 (100 x 2.1 mm x 1.7 µm, Phenomenex, USA), flow 0.3 mL/min, gradient elution with a mixture of water (with 0.1% formic acid, 10 mM ammonium formate, eluent A) and acetonitrile (with 0.1% formic acid, 10 mM ammonium formate,

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eluent B). All reagents were LCMS grade (Merck KGaA, Germany). The gradient conditions were initially 100% A, maintaining these conditions for 1 min, then decreasing A to 0% at 7.75 min and maintaining those conditions until 10 min, then increasing A to 50% at 15 min, increasing again to 95% at 18 min and maintaining these conditions until the end (20 min). The conditions of the mass spectrometer were as follows: ionization in negative mode, a cone ramp voltage of 18.80 to 30 V was used, capillary voltage -3.90 kV, source temperature 150ºC, desolvation temperature 500ºC and desolvation gas flow 1000 L/h.

Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral venous blood obtained from five adult volunteer donors. The donors did not report disease, fever, or infections in the seven days prior to taking the sample. Informed consent was obtained from all individual participants included in the study. This study was approved by the Ethics Committee of the Facultad de Ciencias Químicas-UNA (Approval Number: 437/18) and the Scientific and Ethics Committees of the Instituto de Investigaciones en Ciencias de la Salud-UNA (Approval Number: P42/2018).

Heparinized blood samples were subjected to density gradient centrifugation on Histopaque®-1077 (Sigma, USA) at 2000 rpm for 20 min. PBMCs were centrifuged and resuspended in RPMI medium (Sigma, USA), supplemented with 0.3 g/L of L-glutamine (Sigma, USA), 2.4 g/L of N-2-hydroxyethylpiperazine-N'-2'-acid ethanesulfonic acid (HEPES) (Gibco, USA), 2 g/L $NaHCO_3$ (Merck, Germany), 10% fetal bovine serum (PAA Laboratories GmbH, Austria), penicillin (100 UI/mL), and streptomycin (100 mg/mL) (Gibco, USA). Cell viability was determined by trypan blue dye exclusion and was greater than or equal to 95%. Cells were counted and adjusted to the desired concentration.

Cytotoxicity

PBMCs (1.5×10^6/mL) were seeded into 96-well flat-bottom plates, culture in the presence of each extract was dissolved in DMSO, and serially diluted (5, 10, 25 µg/mL). The final concentration of the vehicle (DMSO) was lower than 1%. After incubation, cytotoxic activity was determined by colorimetric 3-(4,5-dimethyl -2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Cell proliferation assay

Phytohemagglutinin (PHA) (Sigma, USA) was used for T-lymphocyte proliferation. PBMCs ($1.5x10^6$/mL) were seeded into a 96-well flat-bottom plate, culture in RPMI medium (Sigma, USA) in the presence of each extract, at 37°C and 5% CO_2. After 30 min, the cells were treated with PHA (6.5 µg/mL) (Sigma, USA). PHA alone was used as positive control and cells treated with the vehicle (DMSO) as negative control. After 72 h, the cells were incubated with 10 µL of MTT (5 mg/mL) (Sigma, USA) for three hours at 37°C. Subsequently, 100 µL of 0.04 N of hydrochloric acid (Biopack, Argentina)/isopropyl alcohol (Merck, Germany) was added to dissolve the formazan crystals. The optical density (OD) was measured in an ELISA reader (Awareness Technology Inc, USA) at 570 nm.

Real-Time Quantitative PCR

PBMCs ($1.5x10^6$/mL) were seeded into 6 wells dishes and treated with 10 µg/mL of each extract. After 30 min, 5 µg/mL PHA (Sigma, USA) was added and incubated for 18 h. As negative control cells treated with vehicle (DMSO). Total RNA was isolated using TRIzol® (Invitrogen, USA) following the manufacturer's instructions. The RNA was treated with DNase I (Promega®, USA) at 37°C for 20 min and converted into cDNA by reverse-transcription using: 3 µg of total RNA, 200 U of Moloney Murine Leukemia Virus (MMuLV) Reverse Transcriptase (Promega®, USA), 1 µL Oligo dT (100 µM) and 1 µL dNTPs (10 µM). The target genes were amplified using specific oligonucleotide primers, 5 uL SsoAdvanced™ Universal SYBR Green Supermix (BioRad, USA), and 1 µL of cDNA. The amplification step was carried out in a LightCycler Nano (Roche, USA). The used primers were as follows: *GAPDH* 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-CATGGGTGGAATCATATTGGAA-3', *INF-γ* 5'-AAAAATAATGCAGAGCCAAATTG-3' and 5'-TAGCTGCTGGCGACAGTTCA-3'.[9] The mRNA levels were analyzed through the $2^{-\Delta\Delta Ct}$ method, using GAPDH as a normalizing gene.

ELISA

For INF-γ quantitation, PBMCs ($1.5x10^6$/mL) were treated with 10 µg/mL of each extract. After 30 min, 5 µg/mL PHA (Sigma, USA) was added and incubated for 18 h. As negative control cells were treated with vehicle (DMSO). The cell supernatants were then collected and assayed according to the manufacturer's instructions (Duo-Set ELISA Kit, R&D Systems, USA).

Statistical analysis

The values are shown as arithmetic mean ± standard deviation. ANOVA or a t-test was used to compare means. The analyses were conducted using GraphPad Prism 7.0 (GraphPad Software Inc, USA). The level of statistical significance was defined as *$p<0.05$; ** $p< 0.01$; *** $p< 0.001$.

Results and Discussion

Extracts were examined by liquid chromatography-mass spectrometry (LCMS) (Figure 1). From the extract of *B. trimera*, the flavonoids apigenin and genkwanin were identified, both previously reported in this species. [4] From *B. punctulata*, trans-phytol and a di-*O*-caffeoylquinic acid were detected. The two compounds were described in the genus *Baccharis*.[10,11] From *B. notosergila*, the flavonoid chrysoeriol was identified; previously identified in other *Baccharis* species.[12] The methanolic extract of *B. trimera*, *B. notosergila* and *B. punctulata* showed no cytotoxic effects in concentrations up to 25 µg/mL on human PBMCs using the MTT assay (Supplemental Material, Figure 1S).

To test the effect of the extracts on cell proliferation, PBMCs were pretreated with 5 or 10 µg/mL of the extracts for 30 min and then exposed to T cell mitogen PHA. Both the extracts of *B. trimera* and *B. punctulata* decreased lymphocyte proliferation (Figure 2). This result is similar to a previous report for *B. trimera*.[13] It is noteworthy that this behavior was not observed with *B. notosergila.* Modulation of lymphocytes plays a crucial role in the treatment of many pathologies, such as inflammatory and autoimmune diseases. These results suggest that the extracts of these two species have a regulatory effect on lymphocyte proliferation.

The above results have demonstrated the effect of the extracts on mitogen-induced lymphocyte proliferation. Therefore, the activity of *Baccharis* extracts on IFN-γ production, a relevant pro-inflammatory cytokine, was assessed. A trend towards a reduction in mRNA level in cells treated with the extracts of *B. trimera* and *B. punctulata* (10 µg/mL) was observed. However, this trend did not reach statistical significance (Figure 3A). For that reason, protein levels of IFN-γ were analyzed. As is shown in Figure 3B, the extract of *B. punctulata* (10 µg/mL) decreases the production of the cytokine ($p<0.05$). Different compounds were detected in these extracts. Di-*O*-caffeoylquinic acid, identified in *B. punctulata* has been reported to have anti-inflammatory activity and inhibit nuclear factor kappa B (NF-κB) nucleus translocation in macrophages.[14] This compound could be responsible for the effect observed on IFN-γ production in cells treated with *B. punctulata* extract.

Apigenin is a natural flavonoid identified in the extract of *B. trimera*. It has been reported that this flavonoid decreases IL-6 and IFN-γ in human cells.[15] Also, genkwanin, present in the extract of *B. trimera*, has anti-inflammatory activity, decreasing IL-6 levels.[16] Additionally a trend towards a reduction in IFN-γ production by human PBMCs treated with *B. trimera* (10 µg/mL) was observed. Apigenin and genkwanin could be responsible for this effect. Chrysoeriol, identified in the extract of *B. notosergila* is a flavonoid with low anti-inflammatory activity compared to other flavonoids. Chrysoeriol does not inhibit the production of IL-6 induced by LPS in murine macrophages and shows low inhibition of the transcription factor NFκB.[17] This could explain the lower anti-inflammatory activity observed with *B. notosergila* on human PBMCs.

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It is noteworthy that in folk medicine these species are used interchangeably. Our results suggest that *B. trimera* and *B. punctulata* have inhibitory activity of inflammatory response. These results show the importance of the individual identification of the species since they can have different immunomodulatory activities, at least under the concentrations and conditions tested. Further *in vivo* studies in inflammation models are necessary to corroborate these results.

Table 1: Plant material used in the study

Species	Family	Place and month, year of collection	Collector/ Voucher N^{o}	Extract Yield (%)*
Baccharis trimera (Less.) DC.	Asteraceae	San Lorenzo, Paraguay, April 2016	R. Degen 4088	17.1 g (5.04)
Baccharis punctulata DC.	Asteraceae	Ñemby Paraguay, May 2016	R. Degen 4302	24.12 g (6.96)
Baccharis notosergila Griseb.	Asteraceae	Presidente Hayes, Paraguay, August 2016	R. Degen 4362	47.7 g (10.9)

* based on the dry weight

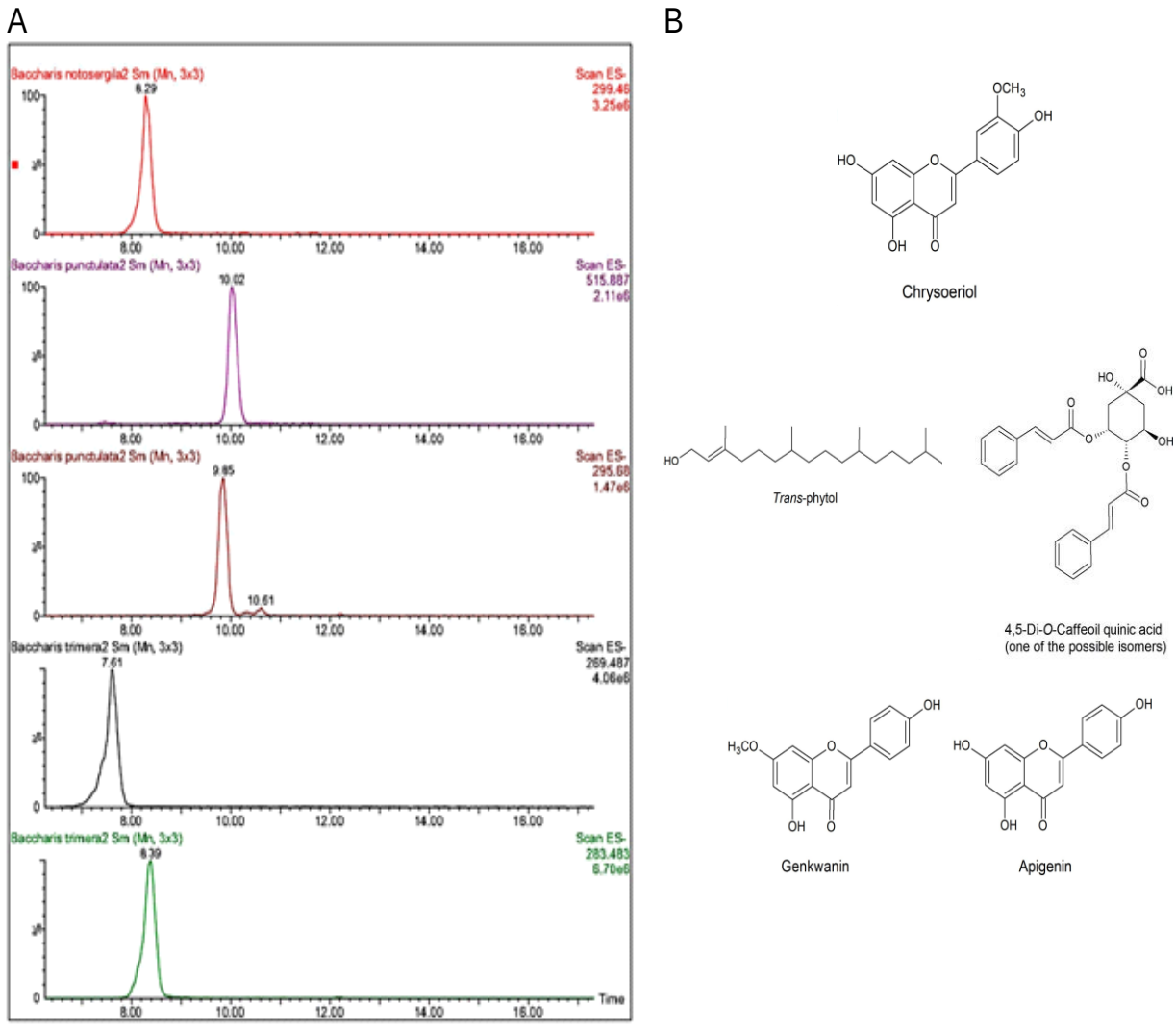

Figure 1: A. LCMS profile of *Baccharis* extracts. From up to down: *B. notosergila* (chrysoeriol, peak at 8.29 min and a m/z 299.46 molecular ion [M-H]$^{-}$), *B. punctulata* (trans-phytol and a di-O-caffeoylquinic acid, two peaks with retention time (RT) of 9.85 and 10.02 min, respectively and m/z 295.68 and 515.89 molecular ions [M-H]$^{-}$), *B. trimera* (apigenin and genkwanin, two peaks with RT of 7.61 and 8.39 min, respectively and their molecular ions were m/z 269.49 and 283.48 [M-H]$^{-}$). The di-caffeoyl acid could be the 3,4, the 3,5 or the 4,5 considering they were isomers and share the same molecular ion. **B.** The chemical structure of the identified compounds.

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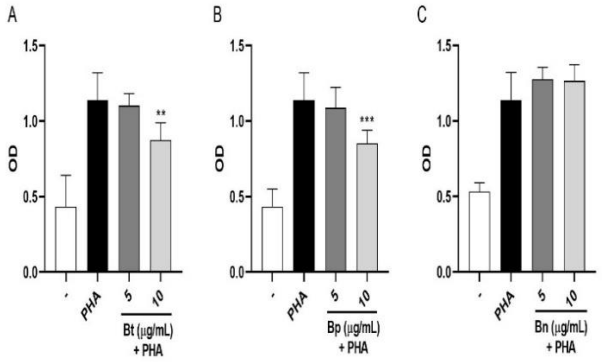

Figure 2: PBMCs ($1.5x10^6$/mL) were stimulated with PHA (6.5 µg/mL) for 72 h, after 30 min incubation with *B. trimera* (Bt), *B. punctulata* (Bp), *B. notosergila* (Bn) extract (5 - 10 µg/mL). PHA-treated cells were used as a positive control. The negative control corresponds to the cells treated with the vehicle (DMSO). Cell proliferation was determined by the colorimetric MTT assay. ** different from control (PHA), $p < 0.01$; *** different from control (PHA), $p < 0.001$.

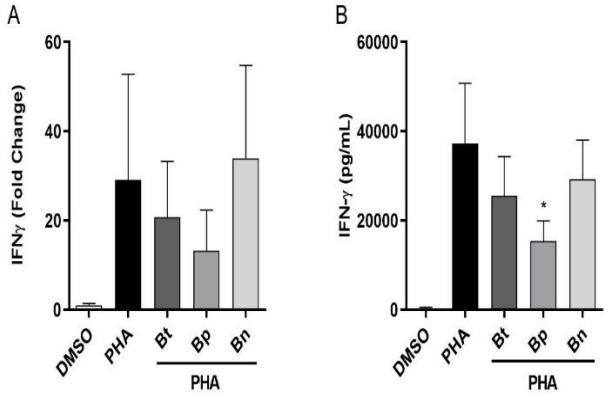

Figure 3: IFN-γ mRNA (**A**) and protein (**B**) production by PBMCs ($1.5x10^6$ cells/mL) stimulated with PHA (5 µg/mL) after 30 min incubation with *B. trimera* (Bt), *B. punctulata* (Bp), *B. notosergila* (Bn) extracts (10 µg/mL). *different from control (PHA), $p < 0.05$.

Conclusion

In conclusion, our results show immunomodulatory activity of the extracts studied. Essentially, *B. trimera* and *B. punctulata* show anti-inflammatory activities on human PBMCs, decreasing PHA-induced proliferation. Also, *B. punctulata* decreased the production of the pro-inflammatory cytokine IFN-γ. These results show that the studied species constitute a source for the isolation and development of new molecules with therapeutic effects on excessive inflammation.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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