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Communication

# Effect of *Baccharis dracunculifolia* D.C. (Asteraceae) extracts and its isolated compounds on macrophage activation

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## Abstract

*Baccharis dracunculifolia* D.C. (Asteraceae), a shrub which grows wild in Brazil, is the main botanical source of Brazilian green propolis. Since Brazilian propolis shows an immunomodulatory activity, the goal of this work was to evaluate the action of *B. dracunculifolia* extracts and some of its isolated compounds on reactive oxygen intermediate ($H_2O_2$) production by macrophages obtained from male BALB/c mice. The results showed that the leaf (Bd-L) (25, 50, and 100 $\mu$g mL$^{-1}$), leaf rinse (Bd-LR) (25 $\mu$g mL$^{-1}$), and the root (Bd-R) (25 $\mu$g mL$^{-1}$) extracts enhanced $H_2O_2$ release by macrophages. A phytochemical study of the root and leaves of *B. dracunculifolia* was carried out. The chromatographic fractionation of Bd-R, using several techniques, afforded the isolation of baccharis oxide (**1**), friedelanol (**2**), viscidone (**11**), 11-hydroxy-10,11-dihydro-euparin (**12**), and 6-hydroxy-tremetona (**13**), while Bd-LR gave the following isolated compounds: baccharis oxide (**1**), friedelanol (**2**), isosakuranetin (**3**), aromadendrin-4′-methyl ether (**4**), dihydrocumaric acid (**5**), baccharin (**6**), hautriwaic acid lactone (**7**), hautriwaic acid acetate (**8**), drupanin (**9**), and cumaric acid (**10**). Among the isolated compounds, baccharis oxide (**1**) and friedelanol (**2**) increased $H_2O_2$ production at a concentration of 100 $\mu$M. This is the first time that the presence of compounds **7, 8, 12**, and **13** in *B. dracunculifolia* has been reported. Based on these results it is suggested that the crude extracts and some isolated compounds from *B. dracunculifolia* display an immunomodulatory action.

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## Introduction

*Baccharis dracunculifolia* D.C. (Asteraceae), a shrub which grows wild in Brazil, is used in folk medicine as an anti-inflammatory agent, mainly for the treatment of gastrointestinal diseases (Queiroga et al 1990). *B. dracunculifolia* has been described as the most important plant source of propolis in South Eastern Brazil, which due to its colour is called green propolis (Bankova et al 1999; Kumazawa et al 2003; Park et al 2004).

Propolis, a natural resinous substance collected by honey-bees (*Apis mellifera* L.) from buds and exudates of plants, is currently incorporated in food and beverages to improve health and to prevent several diseases (Park et al 2002). Besides its use in folk medicine, propolis possesses various biological activities, such as antibacterial (Sforcin et al 2000), antitumoral (Bazo et al 2002), antioxidant (Simões et al 2004), anti-inflammatory (Reis et al 2000) and immunomodulatory (Sforcin et al 2005).

With regards to the immune system, it was recently verified that Brazilian propolis increased natural killer activity against tumour cells (Sforcin et al 2002), and modulated in-vitro and in-vivo nitric oxide (NO) and hydrogen peroxide ($H_2O_2$) production by peritoneal macrophages (Orsi et al 2000). Additionally, it increased the fungicidal (Murad et al 2002) and bactericidal (Orsi et al 2005) activity of macrophages.

*B. dracunculifolia* and Brazilian green propolis have been reported to display anticariogenic (Leitão et al 2004) and trypanocidal (Da Silva Filho et al 2004) activities. However, it is still unknown whether *B. dracunculifolia* displays the same immunomodulatory effects

reported for Brazilian propolis. Moreover, as observed for propolis, a great variety of phenolic compounds, such as flavonoids, prenylated *p*-coumaric acids derivatives, as well as diterpenes have been identified from the aerial parts of *B. dracunculifolia* (Nagatami et al 2001; Kumazawa et al 2003). Also, this is the first time that the constituents of the roots of this species have been reported.

On the basis of the botanical origin of Brazilian green propolis and its immunomodulatory activity, the aim of this study was to evaluate the effect of *B. dracunculifolia* extracts and its pure isolated compounds on macrophage activation by in-vitro $H_2O_2$ determination.

## Materials and Methods

### Plant material

Leaves and roots of *B. dracunculifolia* D.C. (Asteraceae) were collected in Franca, São Paulo State, Brazil, in December 2001. The plant material was authenticated by J. N. Nakagima, and a voucher specimen (SPFR 06143) stored in the Herbarium of the Biology Department of the University of São Paulo, campus of Ribeirão Preto-SP, Brazil.

### Preparation of *B. dracunculifolia* extracts

Leaves (3 g) and roots (764 g) of *B. dracunculifolia* were air-dried and powdered, followed by exhaustive extraction with EtOH:$H_2O$ (9:1) at room temperature. The filtered extracts were concentrated under vacuum below 40 °C to give 400 mg total leaf crude extract (Bd-TL) and 31 g root crude extract (Bd-R), respectively. The leaf rinsed extract was obtained by immersing the air-dried leaves (658 g) into dichloromethane for 30 s at room temperature, affording 35 g leaf rinsed crude extract (Bd-LR) after removal of the solvent under vacuum below 40°C. After immersion into dichloromethane, the leaves (615 g) were air-dried, powdered, and submitted to exhaustive extraction with EtOH:$H_2O$ (9:1) at room temperature. The filtered extracts were combined, and the solvent was evaporated under vacuum below 40°C to give 37 g leaf (Bd-L) crude extract.

### General procedures

NMR spectra were recorded on a Brucker ARX 400 spectrometer. Vacuum-liquid chromatography (VLC) was carried out with silica gel 60 H 100–200 mesh ASTM (Merck Co, Darmstadt, Germany) in glass columns with 5–10 cm i.d. Flash chromatography was carried out with Silica gel 230–400 mesh (Merck) using a 450 × 25 mm glass column at 5 mL $min^{-1}$. Semi-preparative HPLC separations were carried out on a Shimadzu SCL-10 AVP liquid chromatography system equipped with a SPD-M10AVP Shimadzu UV-DAD detector (the channel was set at 281 nm), and Shimadzu column (ODS, 250 × 20 mm, 15 $\mu$m). Dichloromethane was acquired from Acros Co., New Jersey. Ethyl acetate, hexane and methanol were supplied by Mallinckrodt Co., Xalostoc, Mexico. Ethanol was bought from a local distillery and purified by distillation. Mercaptoethanol and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich Co. (St Louis, MO).

The crude Bd-LR extract (35 g) was dissolved in methanol:$H_2O$ (7:3), and submitted to sequential partition with hexane and dichloromethane, giving 2.6 g and 22.1 g of the crude fractions, respectively. The dichloromethane fraction was chromatographed over silica gel under a VLC system, using hexane–ethyl acetate mixtures in increasing proportions as eluent, resulting in five fractions. The fractions I (0.5 g) and II (0.7 g) were crystallized in methanol, affording compound **1** (25 mg) from fraction I and compound **2** (65 mg) from fraction II. The fractions III (hexane–ethyl acetate 1:1; 2.5 g) and IV (hexane–ethyl acetate 3:7; 6.5 g) were chromatographed over silica gel under a VLC system, using hexane–ethyl acetate mixtures in increasing proportions as eluent. The resulting sub-fractions III.2 and IV.2 were submitted to semi-preparative reverse-phase HPLC purification (column ODS 250 × 20 mm, 15 $\mu$m, UV-DAD detector at 281 nm) using methanol:$H_2O$ (75:25) as mobile phase. Fraction III.2 furnished compounds **3** (20 mg), **4** (15 mg), and **5** (13 mg), and fraction IV.2 furnished compounds **6** (27 mg), **7** (20 mg), **8** (18 mg), **9** (10 mg), and **10** (5 mg).

The crude Bd-R extract (31 g) was dissolved in methanol:$H_2O$ (7:3), and submitted to sequential partition with hexane and dichloromethane resulting in 3.1 g and 6.1 g of crude fractions, respectively. The hexane fraction was chromatographed over silica gel under a VLC system, using hexane–ethyl acetate mixtures in increasing proportions as eluent, giving four fractions. The fractions II (0.59 g) and III (0.52 g) were crystallized in methanol, affording compound **1** (200 mg) from fraction II and compound **2** (80 mg) from fraction III. The dichloromethane fraction was chromatographed over silica gel under a VLC system, using hexane–ethyl acetate mixtures in increasing proportions as eluent, giving four fractions. The fractions I (hexane–ethyl acetate 4:1; 0.93 g) and III (hexane–ethyl acetate 3:2; 0.6 g) were submitted to column chromatography over silica gel, using hexane–ethyl acetate mixtures in increasing proportions as eluent. The resulting sub-fractions I.3 and III.3 were submitted to semi-preparative reverse-phase HPLC purification (column ODS 250 × 20 mm, 15 $\mu$m, UV-DAD detector at 281 nm) using methanol:$H_2O$ (7:3) as mobile phase. Fraction III.3 furnished compounds **11** (10 mg) and **12** (5 mg). Fraction I.3 furnished compound **13** (3 mg).

### Animals and peritoneal macrophages

Male BALB/c mice (25–30 g, 6–8-weeks old) were used. Peritoneal macrophages were obtained by inoculation of 3–5 mL cold phosphate-buffered saline (PBS) into the abdominal cavity. After a soft abdominal massage for 30 s, the peritoneal liquid was collected and transferred to sterile plastic tubes (Falcon). This procedure was repeated three or four times for each animal and the tubes were centrifuged at 200 *g* for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37°C and counted in a haematocytometer to obtain a final concentration of $2 \times 10^6$ cells $mL^{-1}$. Cells were pooled from five animals and resuspended in cell

culture medium (RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES, $2.5 \times 10^{-5}$ M 2-mercaptoethanol), and cultured in a 96-welled flat-bottomed plate at a final concentration of $2 \times 10^5$ cells/well. Cells were incubated at 37°C and, after 2 h, non-adherent cells were removed and macrophages were re-incubated (37°C) for 24 h, with either crude extracts (25, 50 or 100 $\mu$g mL$^{-1}$) or pure compounds (1, 10 or 100 $\mu$M), with the exception of dihydroeuparin (**13**), which was not assayed. Crude extracts and pure compounds were dissolved in DMSO and water to achieve the desired concentrations.

Animals used in this study were housed and cared for in accordance with the protocols of the University of the State of São Paulo (UNESP). The experiments were undertaken following the Federal Government legislation on animal care.

### Determination of $H_2O_2$ production

Hydrogen peroxide ($H_2O_2$) production by peritoneal macrophages was determined according to Pick & Mizel (1981). Cells were incubated with the stimuli as described before over 24 h at 37°C. Afterwards, supernatants were removed and a 100 $\mu$L red phenol solution (containing in mM: 140 NaCl, 10 $K_2HPO_4$, 5.5 dextrose, and 5.5 horseradish peroxidase) was added to the adherent cells for $H_2O_2$ determination. After 1 h, 10 $\mu$L 1.0 M NaOH solution was added and the absorbance was measured at 620 nm.

### Statistical analysis

Analysis of variance was used for immunological assays, followed by multiple comparison tests by Tukey–Kramer methods (Zar 1996).

## Results and Discussion

### Phytochemical study

The phytochemical study led to the isolation of ten compounds (**1–10**) from Bd-LR, and five compounds (**1–2** and **11–13**) from Bd-R, respectively.

The chemical structures of all isolated compounds (Figure 1) were established by UV-vis, $^1$H and $^{13}$C NMR data analysis in comparison with the literature, as follows: baccharis oxide (**1**) (Bohlmann et al 1981a), friedelanol (**2**) (Chandler & Hooper 1979), isosakuranetin (**3**) (Bohlmann et al 1981a), aromadendrin-4′-methyl ether (**4**) (Banskota et al 1998), dihydrocumaric acid (**5**) (Mendez 2005; Loots et al 2006), baccharin (**6**) (Banskota et al 1998), hautriwaic lactone (**7**) (Bohlmann et al 1985), hautriwaic acetate (**8**) (Saad et al 1985), drupanin (**9**) (Banskota et al 1998), cumaric acid (**10**) (Langner & Schilcher 1999), viscidone (**11**) (Le-Van & Pham 1981), 11-hydroxy-10,11-dihydroeuparin (**12**) (Bohlmann et al 1981b), and 6-hydroxy-tremetona (**13**) (Bohlmann et al 1981a).

This is the first time that compounds **7, 8, 12**, and **13** from *B. dracunculifolia* have been reported.

### Immunological assays

Natural products with immunomodulatory action may be useful in the control of some infectious diseases.

Macrophage exposure to several stimuli, such as direct contact with antigen, complement system, cytokines, and others leads to metabolic changes. For that, production of oxygen metabolites such $O_2^-$, $H_2O_2$ and OH seems to be the main mechanism by which these cells become microbicidal (Moonis et al 1992). $H_2O_2$ comes from the respiratory burst of macrophages, and its production may be enhanced by different stimuli. Another indication of macrophage activation is NO generation, a microbicidal pathway of these cells, which is also related to the immune response (Grange & Davey 1990; Zhang & Morrison 1993).

The results indicated that Bd-LR (25 $\mu$g mL$^{-1}$), Bd-L (25, 50 and 100 $\mu$g mL$^{-1}$), and Bd-R (25 $\mu$g mL$^{-1}$) enhanced $H_2O_2$ release by macrophages (Table 1). Among all the tested pure compounds, only baccharis oxide (**1**) and friedelanol (**2**) increased $H_2O_2$ production, both at 100 $\mu$M (Table 1). Considering that *B. dracunculifolia* is the main botanical source for green propolis production, and that propolis has been used to treat mainly infectious diseases, it was important to investigate the activity of *B. dracunculifolia*. The aim was to corroborate the medicinal uses of propolis, and to investigate the potential of developing a new phytomedicine from the plant itself. It was observed that the lower concentrations of *B. dracunculifolia* extracts were more effective in enhancing $H_2O_2$ release than the higher concentrations. This might be due to the presence of $H_2O_2$ release-inhibitory compounds in the crude extracts, which might display activity at the higher tested concentrations, since for the pure compounds **1** and **2** a dose–response correlation was obtained. Moreover, there might be either a synergistic or an additive effect of the active compounds in the crude extracts at 25 $\mu$g mL$^{-1}$, as well as the presence of other active compounds, since the concentrations of compounds **1** and **2** in the crude extract were much lower than the ones displayed in Table 1.

We recently evaluated the action of three vegetal sources of Brazilian propolis (*Araucaria angustifolia, Baccharis dracunculifolia* and *Eucalyptus citriodora*) on macrophage activation, through oxygen ($H_2O_2$) and nitrogen (NO) metabolite determination. No effects were attributed to such extracts on production of these metabolites, while propolis, depending on the evaluated concentration, induced an elevation in $H_2O_2$ release and inhibited NO generation (Lopes et al 2003).

NO determination was also carried out in this study (data not shown). However, it was not possible to quantify accurately NO concentration because of the presence of chlorophyll and other colour compounds in the evaluated extracts.

The biological properties of propolis are a consequence of plant-derived products, since its composition was originated from plant exudates and substances secreted by bee metabolism (Marcucci 1995). The composition of propolis can be extraordinarily variable because of this, creating a problem for its medical use and quality control (Bankova et al 2000). Thus, the investigation of both the chemical and biological properties of propolis plant sources, such as *B. dracunculifolia*,

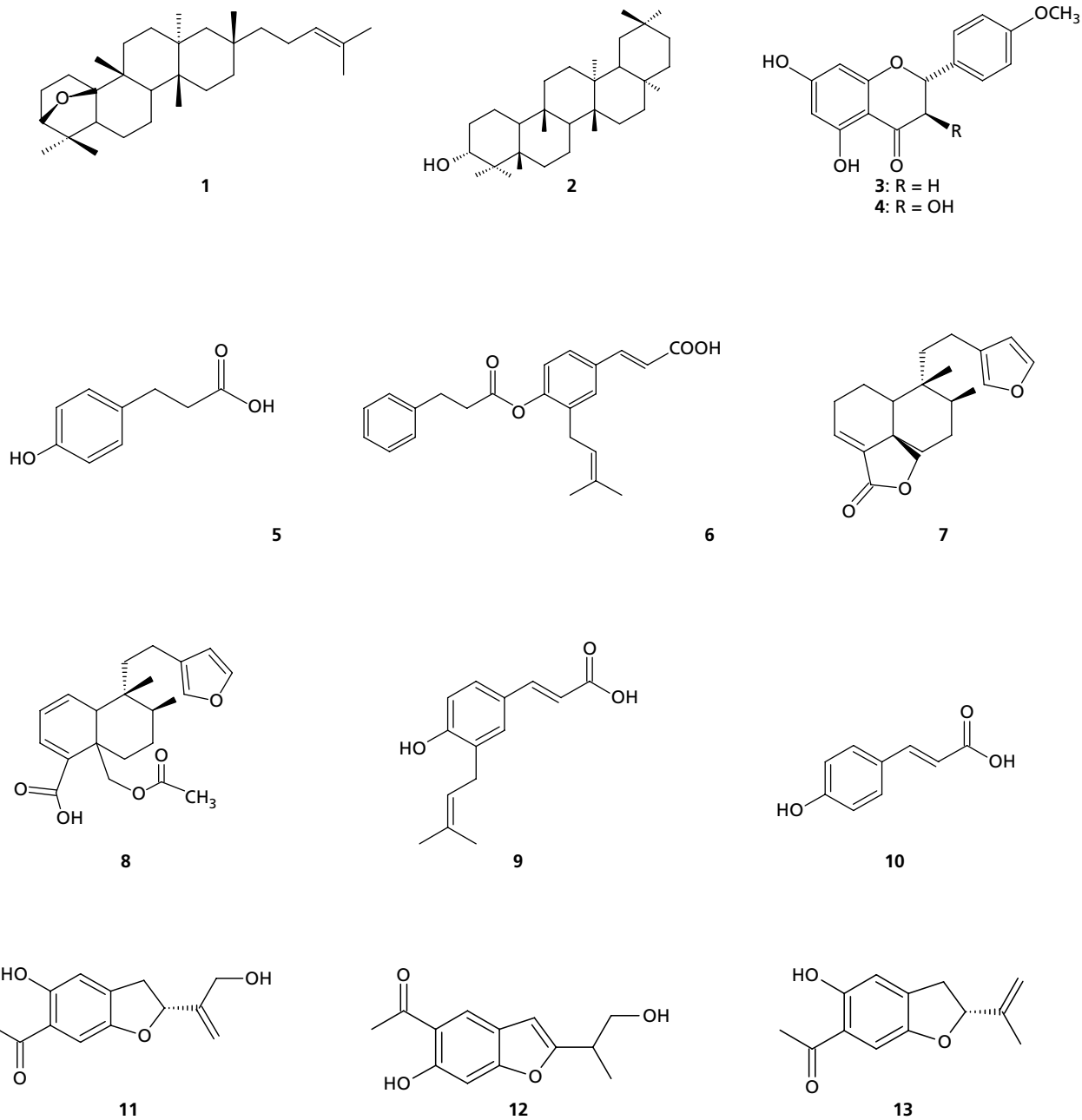


**Figure 1** Chemical structures of the isolated compounds from *B. dracunculifolia* extracts. **1,** Baccharis oxide; **2,** friedelanol; **3,** isosakuranetin; **4,** aromadendrin-4′-methyl ether; **5,** dihydrocumaric acid; **6,** baccharin; **7,** hautriwaic acid lactone; **8,** hautriwaic acid acetate; **9,** drupanin; **10,** cumaric acid; **11,** viscidone; **12,** 11-hydroxy-10,11-dihydro-euparin; **13,** 6-hydroxy-tremetona.

is important not only for its academic interest, but also for the chemical and biological standardization of propolis raw material (Bankova et al 2000).

## Conclusions

*B. dracunculifolia* extracts were able to activate macrophages in-vitro with a consequent $H_2O_2$ liberation, depending on its concentration. However, further biological and phytochemical investigations are necessary to investigate the immunomodulatory activity of *B. dracunculifolia* crude extracts, as well as its secondary metabolites. Furthermore, as suggested by some authors (Leitão et al 2004), if *B. dracunculifolia* and Brazilian green propolis display similar biological properties, *B. dracunculifolia* extracts could be used successfully in human and veterinary medicines.

**Table 1** Effect of the crude extracts and isolated compounds of *B. dracunculifolia* on the in-vitro $H_2O_2$ release by peritoneal macrophages

| **Crude extracts** | **Dose ($\mu$g mL$^{-1}$) × $H_2O_2$ concn[a] (± s.d.)** | | |
|---|---|---|---|
| | 25 | 50 | 100 |
| Bd-Tl | 0.42 ± 0.01 | 0.42 ± 0.02 | 0.37 ± 0.02 |
| Bd-LR | 0.51 ± 0.10 | 0.45 ± 0.06 | 0.41 ± 0.03 |
| Bd-L | 0.78 ± 0.10 | 0.47 ± 0.06 | 0.46 ± 0.04 |
| BD-R | 0.58 ± 0.01 | 0.45 ± 0.08 | 0.42 ± 0.05 |
| | Control: 0.30 ± 0.03 | | |
| **Compounds** | **Dose ($\mu$M) × $H_2O_2$ concn[a] (± s.d.)** | | |
| | 1 | 10 | 100 |
| **1** | 0.11 ± 0.05 | 0.11 ± 0.02 | 0.28 ± 0.02* |
| **2** | 0.07 ± 0.01 | 0.10 ± 0.01 | 0.44 ± 0.05* |
| **3** | 0.11 ± 0.02 | 0.08 ± 0.02 | 0.14 ± 0.01 |
| **4** | 0.10 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.02 |
| **5** | 0.08 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 |
| **6** | 0.10 ± 0.01 | 0.09 ± 0.02 | 0.10 ± 0.02 |
| **7** | 0.10 ± 0.02 | 0.10 ± 0.01 | 0.08 ± 0.03 |
| **8** | 0.10 ± 0.04 | 0.08 ± 0.02 | 0.10 ± 0.03 |
| **9** | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.10 ± 0.02 |
| **10** | 0.10 ± 0.02 | 0.10 ± 0.02 | 0.13 ± 0.05 |
| **11** | 0.09 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.01 |
| **12** | 0.08 ± 0.01 | 0.11 ± 0.01 | 0.09 ± 0.01 |
| | Control: 0.10 ± 0.01 | | |

[a]nmol $H_2O_2/2 \times 10^5$ cells. Results represent the mean ± s.d. of three assays. *$P < 0.05$ compared with control group (Tukey's test).

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