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In-vitro trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D. C. (Asteraceae)

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Abstract

We have performed a trypanocidal bioactivity-guided study of *Baccharis dracunculifolia* (Asteraceae), the main botanical origin of Brazilian green propolis. The leaf rinse extract of *B. dracunculifolia*, at a concentration of 3.0 mg mL^{-1}, displayed 100% lysis of trypomastigote forms of the Y strain of *Trypanosoma cruzi* (2×10^6 parasites mL^{-1}). The chromatographic fractionation of the leaf rinse, using several techniques, afforded the isolation of the compounds isosakuranetin (**1**), aromadendrin-4′-methylether (**2**), baccharis oxide (**3**), ferulic acid (**4**), dihydrocinnamic acid (**5**), 3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid (**6**), and friedelanol (**7**). The chemical structures of all compounds were established by UV-vis, ^{1}H and ^{13}C NMR data analysis in comparison with the literature. Compounds **1** and **3** were the most active in the trypanocidal assay, showing IC50 values (inhibitory concentration required for 50% inhibition) of 247.6 and 249.8 μM, respectively. Compounds **2**, **4**, and **6** displayed moderate activity, whilst compounds **5** and **7** were inactive.

Introduction

Chagas' disease affects more than 18 million people in Latin America, leading to approximately 400 000 deaths per year. In Brazil, it is estimated that five to six million people are infected and that 300 000 of them are living in São Paulo State (Takeara et al 2003). *Trypanosoma cruzi*, the aetiologic agent of the disease, causes a pathology of which blood transfusion is the most important mechanism of transmission. Crystal violet (gentian violet) is the only effective compound available to eliminate the parasite in the blood before its transfusion. Despite its good activity, crystal violet causes alteration of blood colour and is rejected by the patients (Neves-Pinto et al 2002). Thus, the search for new chemo-prophylactic trypanocidal agents has been the main goal in the prevention of Chagas' disease (Bastos et al 1999). In the last decade the search for trypanocidal compounds from natural sources has been intensified, leading to the identification of several classes of active plant metabolites (Da Silva Filho et al 2004, Zuleta et al 2003).

Baccharis dracunculifolia D. C. (Asteraceae), a shrub which grows wildly in the Brazilian 'cerrado', is used in folk medicine as an anti-inflammatory and for the treatment of gastrointestinal diseases (Queiroga et al 1990). This plant was selected for study based on the fact that it is the most important botanical origin of a Brazilian propolis, called green propolis because of its colour. Propolis is the generic name for the resinous substance collected by honey bees (*Apis mellifera*) from buds and resins of different plant species. Currently, it is incorporated in food and beverages to improve health and to prevent several diseases (Park et al 2004). Besides its use in folk medicine, propolis possesses antibacterial, fungicidal and antitumoral activity (Park et al 2004).

In recent years, there has been growing interest in the study of the chemical profile of Brazilian green propolis, as well as in its biological activity. Higashi & De Castro (1994) reported trypanocidal activity of the Brazilian propolis, however, so far, any report on the biological activity of *B. dracunculifolia* has not been found.

Therefore, on the basis of the botanical origin and the biological activity described for Brazilian green propolis, the aim of this work was to evaluate the trypanocidal activity of different crude extracts and isolated compounds from *B. dracunculifolia*.

Materials and Methods

Plant material

Baccharis dracunculifolia De Candole (Asteraceae) was collected by A. A. Da Silva Filho in Cajuru (São Paulo State, Brazil), in November 2001. Jimi N. Nakagima kindly authenticated the plant material and a voucher specimen (SPFR 06143) was deposited in the Herbarium of the Biology Department of the University of São Paulo (FFCLRP-USP), Ribeirão Preto, SP, Brazil.

Extraction and isolation

Branches (601 g) and roots (764 g) of *B. dracunculifolia* were air-dried (35°C) and powdered, followed by exhaustive extraction with ethanol:H_2O (9:1) at room temperature. The filtered extracts were concentrated under vacuum below 40°C to furnish 18 and 31 g, respectively, of the dried crude extracts of branches and roots. The leaf rinsed extract was obtained by immersing the air-dried leaves (615 g) in dichloromethane for 30 s at room temperature, and the solvent was removed under vacuum below 40°C, affording 35 g of the leaf rinse extract.

All the obtained extracts were screened against the trypomastigote forms of *T. cruzi* using in-vitro bioassay, leading to the selection of the leaf rinse extract. Afterwards, the crude leaf rinse extract was dissolved in methanol:H_2O (7:3), and submitted to sequential partition with hexane and dichloromethane. After in-vitro assay of both fractions at 3.0 mg mL^{-1}, the dichloromethane fraction (22.1 g) was selected to continue the work, since it displayed total lysis of *T. cruzi*. Then, this fraction was chromatographed over silica gel under a VLC (vacuum liquid chromatography) system, using hexane–ethyl acetate mixtures in increasing proportions as eluent, furnishing five fractions. The fractions I (0.5 g) and II (0.7 g) were crystallized in methanol, affording compound **3** (250 mg) from fraction I and compound **7** (65 mg) from fraction II. The fractions III (2.5 g) and IV (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 μm, UV-diode array detector at 281 nm) using methanol:H_2O (75:25) as mobile phase. Fraction III.2 furnished compounds **1** (20 mg), **2** (15 mg), and **4** (7 mg). Fraction IV.2 furnished compounds **5** (13 mg) and **6** (27 mg).

Bioassay against *T. cruzi*

The bioassays were carried out using trypomastigote forms of the Y strain of *T. cruzi*, obtained from peritoneal mouse macrophage culture cells. Approximately 2×10^6 parasites mL^{-1} were incubated in RPMI-1640 medium (Sigma) in 96-well microtitre plates containing the tested samples. The isolated compounds were dissolved in dimethylsulfoxide (DMSO) and diluted into the medium to give 100, 250 and 500 μg mL^{-1}, as their final concentrations, as well as 1% DMSO, while the crude extracts and its fractions were evaluated at 3.0 mg mL^{-1}. The plates were incubated at 4°C and the bioassays were performed in triplicate. The lysis percentage was determined by a MTT colorimetric method, which was described by Muelas-Serrano et al (2000). The infected blood, added with the same volume of pure DMSO, was used as negative control, and crystal violet was used as positive control (250 μg mL^{-1}). All the experiments were authorized by the Ethical Committee for Animal Care of the University of Franca (Process number 002/04), in accordance with the Federal Government legislation on animal care.

Statistical analysis

Statistical analysis was performed using non-linear regression to obtain the IC50 values (inhibitory concentration required for 50% inhibition) followed by one-way analysis of variance. The trypanocidal activity was expressed as lysis percentage of trypomastigote forms of *T. cruzi* (± s.d.).

Results

Leaf rinse extract and branch extract displayed total lysis of trypomastigote forms of *T. cruzi* at a concentration of 3.0 mg mL^{-1} (Table 1). Nevertheless, the branch extract presented a haemolytic effect. The root extract was barely active, showing only 37.3% of lysis of trypomastigote forms.

The bioactivity-guided phytochemical study of the leaf rinse extract of *B. dracunculifolia* led to the isolation of seven compounds (Figure 1), from which five displayed significant trypanocidal activity. The chemical structures of all compounds were established by UV-vis, ^{1}H and ^{13}C NMR data analysis in comparison with the literature, as follows: isosakuranetin (**1**) by Arakawa & Masui (1969); aromadendrin-4′-methyl ether (**2**) by Agrawal (1989); baccharis oxide (**3**) by Numberg et al (1998); ferulic acid (**4**) by Schmitt & Schneider (1999); dihydrocinnamic acid (**5**) by Schmitt & Schneider (1999); 3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid (**6**) by Zdero et al (1986); and friedelanol (**7**) by Chandler & Hooper (1979). Among all isolated compounds from *B. dracunculifolia*, the flavonoid isosakuranetin (**1**) and the triterpene baccharis oxide (**3**)

Table 1 In-vitro trypanocidal activity of the crude extracts and isolated compounds from *B. dracunculifolia* against the Y strain of *T. cruzi*

Compounds	**% of lysis ± s.d.**			**IC50 (μM) ± s.d.**
	100 μg mL^{-1}	250 μg mL^{-1}	500 μg mL^{-1}	
1	66.3 ± 9.1	92.6 ± 3.9	97.5 ± 3.1	247.6 ± 1.13
2	6.8 ± 5.9	45.5 ± 4.5	74.3 ± 3.7	947.7 ± 1.05
3	43.3 ± 2.6	97.5 ± 2.5	97.5 ± 2.5	249.8 ± 1.02
4	7.8 ± 3.0	60.4 ± 6.9	88.1 ± 3.0	1135.9 ± 1.07
5	4.8 ± 4.2	32.2 ± 3.1	87.6 ± 7.6	2013.9 ± 1.03
6	16.8 ± 1.5	66.8 ± 9.0	88.6 ± 5.2	523.8 ± 1.05
7	3.3 ± 1.3	41.7 ± 2.9	47.5 ± 9.8	1403.5 ± 1.43
Extracts and fractions (3.0 mg mL^{-1})				**% of lysis ± s.d.**
Root extract				37.3 ± 3.1
Branch extract				100 ± 0.0
Leaf rinse extract				100 ± 0.0
Leaf rinse extract – hexanic fraction				77.4 ± 1.9
Leaf rinse extract – dichloromethane fraction				100 ± 0.0

[a]Positive control, crystal violet at 250 μg mL^{-1} (IC50 = 76 μM) displayed 100% lysis. [b]Negative control, RPMI-1640 medium plus 1% DMSO displayed 0% lysis. [c]The activity was expressed as lysis percentages of trypomastigote forms of *T. cruzi*.

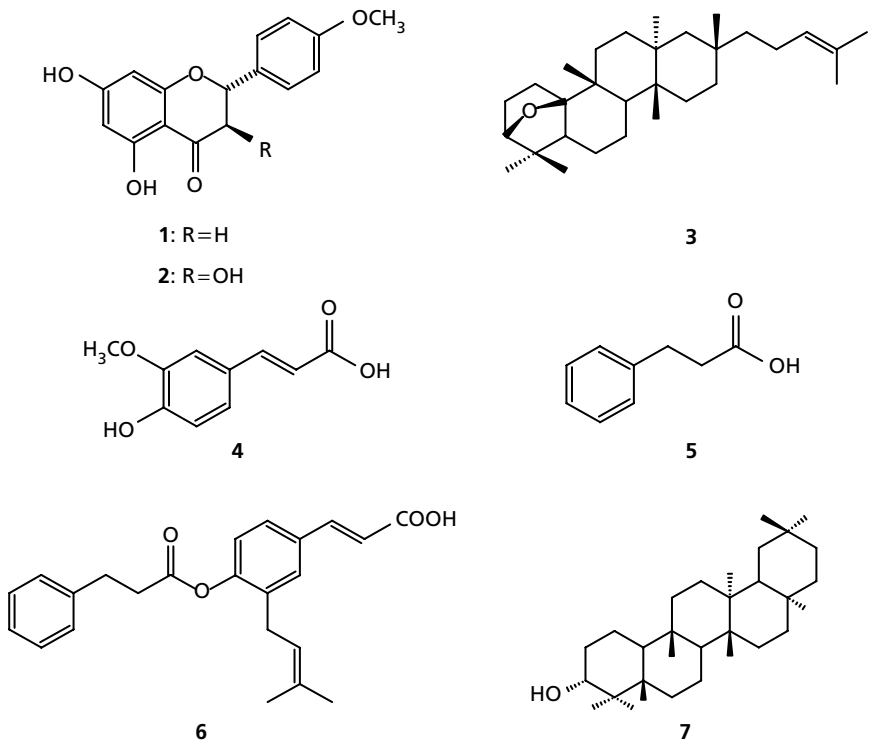

Figure 1 Chemical structures of compounds from *B. dracunculifolia*.

showed the highest trypanocidal activity, displaying IC50 values of 247.6 and 249.8 μM, respectively (Table 1). Compounds **2**, **4**, and **6** displayed moderate activity, while compounds **5** and **7** were inactive. Crystal violet, used as positive control, showed an IC50 value of 76 μM and displayed 100% lysis of *T. cruzi* trypomastigote forms, while the negative control (RPMI-1640 medium plus 1% DMSO) did not show any reduction in parasite numbers.

Discussion

The bioactivity-guided fractionation of the crude extracts of *B. dracunculifolia* successfully allowed the isolation of five active compounds. Similar studies against *T. cruzi*, involving plant extracts (Takeara et al 2003), synthetic and semi-synthetic compounds (Chiari et al 1996; Letelier et al 1990), and propolis (Prytzyk et al 2003) have been reported in the literature, from which several isolated flavonoids and other antioxidant compounds were tested.

There are many biological activities described for flavonoids, including antioxidant and anti-inflammatory (Harborne & Williams 2000). It has been reported that some highly oxygenated flavonoids possess the structural requirements for inhibiting the trypanosomal GAPDH, the enzyme that catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate (Tomazela et al 2000). Considering that the chemical structure of isosakuranetin (**1**) is quite similar to aromadendrin-4′-methylether (**2**), the oxygenation pattern of the C-Ring may interfere with the trypanocidal activity of flavonoids, since **1** showed an IC50 value of 247.6 μM but **2** showed an IC50 value of 947.7 μM. These results were in accordance with the findings of Takeara et al (2003), for related flavonoids. Nevertheless, according to Prytzyk et al (2003), different flavonoids may have similar activity toward *T. cruzi*. Furthermore, it is difficult to compare the activity among the compounds, due to the diversity of parasite strains, stages of its life cycle and experimental conditions applied (Abe et al 2002a). Therefore, more extensive and detailed studies on the trypanocidal activity of flavonoids are necessary to better understand the mechanism of action.

The tetracyclic triterpene baccharis oxide (**3**), a compound frequently found in the *Baccharis* species, was highly active against *T. cruzi*, showing an IC50 value of 249.8 μM and lysis of 97% of the parasites at 500 μM. It is the first report of any biological activity of this compound. Recently, Cunha et al (2003) reported the fully trypanocidal activity of some pentacyclic triterpene acids. On the other hand, the pentacyclic triterpene friedelanol (**7**) was inactive, which was in accordance with the results obtained for Biavatti et al (2001). As reported previously (Cunha et al 2003), the presence of polar groups in the triterpenes may be important for their trypanocidal activity. Hence, as suggested by Abe et al (2002b), this class of compounds should also be considered for further trypanocidal studies.

In conclusion, *B. dracunculifolia*, like Brazilian green propolis, could be a promising source of natural compounds for the development of new chemo-prophylactic trypanocidal agents to replace crystal violet. It is important to emphasize that, considering the results, more biological, toxicological and chemical investigations of other active metabolites from *B. dracunculifolia* against trypomastigote forms of *T. cruzi*, especially from the leaf rinse extract, should be undertaken, as well as detailing their mechanisms of action.

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