

Medicinal Plants of Chile: Evaluation of their Anti-*Trypanosoma cruzi* Activity

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The extracts of several plants of Central Chile exhibited anti-*Trypanosoma cruzi* trypomastigotes activity. Most active extracts were those obtained from *Podanthus ovatifolius*, *Berberis microphylla*, *Kageneckia oblonga*, and *Drimys winteri*. The active extract of *Drimys winteri* (IC₅₀ 51.2 µg/mL) was purified and three drimane sesquiterpenes were obtained: polygodial, drimenol, and isodrimenin. Isodrimenin and drimenol were found to be active against the trypomastigote form of *T. cruzi* with IC₅₀ values of 27.9 and 25.1 µM, respectively.

Key words: Anti-*Trypanosoma cruzi*, Drimenol, Isodrimenin

Introduction

Chagas' disease, caused by *Trypanosoma cruzi*, is among the most important endemic parasitic diseases. Approximately 16 to 18 million people are infected in large areas of Latin America (Fournet and Munoz, 2002). Over 1 million of them will die of the disease unless considerable advances are made (Maguire, 2006). Furthermore, there is increasing evidence that it may be an emerging problem in the developed world, with more than 100,000 infected persons living in the United States of America alone (Maguire, 2006).

The drugs used for the treatment of this disease are nifurtimox, a nitrofurane derivative, and benznidazole, a nitroimidazole derivative. Both drugs generate severe side effects, and nifurtimox is no longer used in several countries because of its toxicity (Castro *et al.*, 2006; Croft *et al.*, 2005). Consequently, the need for effective anti-*Trypanosoma* compounds with less toxicity stimulates the search for natural products as novel drug candidates with a potential clinical use (Hoffmann *et al.*, 1992; González *et al.*, 1990).

The aim of the present work was to assess the *in vitro* activity of some Chilean plant extracts

against the trypomastigote forms of *Trypanosoma cruzi*. Thirty-one species of plants belonging to 28 genera in 21 families were investigated. The most active extracts were those obtained from *Podanthus ovatifolius*, *Berberis microphylla*, *Kageneckia oblonga*, and *Drimys winteri*.

Drimys winteri (Winteraceae) is a tree of economic and social importance with medicinal properties in Chile. This plant contains several sesquiterpenes of the drimane type. Leaves of *Drimys winteri* are used in Chilean folk medicine as analgesic and in anti-inflammatory medications (Houghton and Manby, 1998; San Martín, 1983; Cotoras *et al.*, 2001; Muñoz and Fajardo, 2005; Muñoz *et al.*, 2001; Ruiz *et al.*, 2010).

Experimental

General

Column chromatography (CC) was carried out using silica gel 60G (Merck Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on silica gel GF254 (Merck) with (i) *n*-hexane/ethyl acetate (8:2, v/v) and (ii) *n*-hexane/acetone (8:2) as eluents. Spots were detected under UV light or by spraying with Liebermann-Burchard reagent and heating to 110 °C for 2 min.

Preparative TLC was performed on 2 mm thick silica gel F254 plates (Merck) and on a chromatotron (Harrison-Research Model 7924 T; Palo Alto, CA, USA) with 1-mm and 2-mm discs, using silica gel 60 PF 254 (Merck). Flash chromatography was performed on silica gel 60 H (Merck) with an *n*-hexane/ethyl acetate gradient (0, 1, 5, 10, 50, 100% ethyl acetate). Melting points are uncorrected. Optical rotations were measured with a Perkin Elmer 241 MC polarimeter (Waltham, MA, USA).

^1H and ^{13}C NMR spectra were recorded in CDCl_3 , at 400 and 500 MHz for ^1H NMR and 100 and 125 MHz for ^{13}C NMR, on a Bruker Avance AM-400 spectrometer (Karlsruhe, Germany).

The ^1H NMR spectra used for the measurement of the coupling constants and the HMBC spectra used for the determination of the connectivity of substituents were recorded in CDCl_3 on a Bruker-DRX 500 MHz instrument. The ^1H Larmor frequency was determined using a 5-mm QPN direct detection probe. Chemical shifts (δ in ppm) are relative to the internal standard tetramethylsilane (TMS). 1D (^1H , ^{13}C) and 2D (COSY, HMQC, HMBC) experiments were performed using standard Bruker microprograms.

Plant materials

Ethnopharmacological and ethnobotanical literature was obtained from Instituto de Biología Vegetal y Biotecnología, Universidad de Talca, Talca, Chile, and from the Departamento de Botánica, Facultad de Ciencias, Universidad de Chile, Santiago, Chile. Thirty-one plant species were included in the study and collected in Central Chile (Coastal Range, Maule Region) during the flowering season in January 2009 and 2010, and identified by one of us (J. S. M.). Voucher specimens are deposited and kept in the Instituto de Biología Vegetal y Biotecnología, Universidad de Talca. Plants used in this study are listed in Table I.

Extraction

The collected plants were washed with distilled water and dried on absorbing paper at an ambient temperature of 25–30 °C in open air in the shade for 5–10 d. The dried plant samples were powdered and stored at ambient temperature in amber glass bottles until use. The powdered plant materials (10.0–80.0 g) were extracted with dichloromethane (4.0 mL/g plant) or methanol/water (4:1, v/v; 4.0 mL/g plant) for 4 h at room temperature. The extracts were filtered and evaporated to dryness under vacuum. The residues

Table I. Chilean plant species used in this study.

| No. Species, family | No. Species, family |
|--|--|
| 1 <i>Podanthus ovatifolius</i> Lag., Asteraceae | 18 <i>Margyricarpus pinnatus</i> (Lam.) Kuntze, Rosaceae |
| 2 <i>Cryptocarya alba</i> (Mol.) Looser, Lauraceae | 19 <i>Pseudognaphalium vira vira</i> (Mol.) A. Anderb., Asteraceae |
| 3 <i>Escallonia illinita</i> K. Presl., Saxifragaceae | 20 <i>Eupatorium salvia</i> Colla, Asteraceae |
| 4 <i>Blepharocalyx cruckshanksii</i> (H. et A.) Nied., Myrtaceae | 21 <i>Baccharis concava</i> (Ruiz et Pav.) Pers., Asteraceae |
| 5 <i>Satureja gilliesii</i> (Graham) Briq., Lamiaceae | 22 <i>Viviania crenata</i> (Hook.) G. Don ex H. et A., Rubiaceae |
| 6 <i>Drimys winteri</i> J. R. Forst. et G. Forst., Winteraceae | 23 <i>Fabiana imbricata</i> Ruiz et Pav., Solanaceae |
| 7 <i>Luma chequen</i> (Mol.) A. Gray, Myrtaceae | 24 <i>Myoschilos oblonga</i> Ruiz et Pav., Santalaceae |
| 8 <i>Luma apiculata</i> (DC.) Burret, Myrtaceae | 25 <i>Berberis darwinii</i> Hook., Berberidaceae |
| 9 <i>Fuchsia magellanica</i> Lam., Onagraceae | 26 <i>Maytenus chubutensis</i> (Speg.) Lourt., O'Donnell et Sleum., Celastraceae |
| 10 <i>Colliguaja odorifera</i> Mol., Euphorbiaceae | 27 <i>Myrceugenia chrysocarpa</i> (O. Berg) Kausel, Myrtaceae |
| 11 <i>Ugni molinae</i> Turcz., Myrtaceae | 28 <i>Elytropus chilensis</i> (A. DC.) Muell. Arg., Apocynaceae |
| 12 <i>Alstroemeria revoluta</i> Ruiz et Pav., Amaryllidaceae | 29 <i>Berberis serrato-dentata</i> Lechler, Berberidaceae |
| 13 <i>Pitavia punctata</i> Mol., Rutaceae | 30 <i>Berberis microphylla</i> G. Forst., Berberidaceae |
| 14 <i>Podocarpus saligna</i> D. Don, Podocarpaceae | 31 <i>Pseudopanax laetevirens</i> (Gay) Franch., Araliaceae |
| 15 <i>Lapageria rosea</i> Ruiz et Pav., Philesiaceae | |
| 16 <i>Schinus latifolius</i> (Gill. ex Lindl.) Engler, Anacardiaceae | |
| 17 <i>Kageneckia oblonga</i> Ruiz et Pav., Rosaceae | |

were weighed and solubilized in dimethylsulfoxide (DMSO) for biological assays.

Culture of trypomastigotes

African green monkey Vero cells were infected by co-incubation of a culture of epimastigotes in the late stationary phase, which contains about 5% of the infective trypomastigote form (Contreras *et al.*, 1985). Subsequently, the trypomastigotes harvested from this culture were used to further reinfect cultures of Vero cells at a density of $1 \cdot 10^6$ cells/25 cm², in a proportion of parasites to cells of 2:1. Vero cell cultures infected with trypomastigotes were incubated at 37 °C in humidified air and 5% CO₂ for 5–7 d. After that time, the culture medium was collected and centrifuged at 3,000 x *g* for 5 min, and the resulting trypomastigote-containing pellet was resuspended at a density of $1 \cdot 10^7$ parasites/mL in RPMI 1640 culture medium (without phenol red).

Trypomastigote viability assay

Viability assays were performed using the formazan formation method, called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (Faundez *et al.*, 2005; Mosmann, 1986). Briefly, $1 \cdot 10^7$ trypomastigotes were incubated in RPMI 1640 culture medium at 37 °C for 24 h with and without addition of the extracts at final concentrations of 10–500 µg/mL. Formazan formation was measured at 570 nm in a multi-well reader (Lab systems Multiskan MS, Vantaa, Finland).

Characterization of compounds isolated from *Drimys winteri*

Barks of *D. winteri* were dried in an air-forced oven at 40 °C for 48 h. Four hundred fifty g of chopped and powdered bark material were extracted with *n*-hexane (2 x 2.0 L) for 24 h in a Soxhlet extractor. Filtration followed by evaporation of the solvent under reduced pressure (0.21 atm) at 25–30 °C gave a yellowish oily residue (30.0 g).

The crude *n*-hexane extract was first subjected to flash CC (silica gel, 230–400 mesh, 650 g), then fractionated by gradient elution (100% *n*-hexane to 100% ethyl acetate) to give individual fractions which were further purified by two silica gel columns. Elution with *n*-hexane/ethyl acetate

(98:2–90:10) yielded 2.84 g of a yellow mixture consisting of two compounds. Further separation on the chromatotron afforded 0.85 g of polygodial and 0.015 g of drimenol. The ethanol fraction (2.5 g) was subjected to CC on silica gel and separated by gradient elution (dichloromethane/methanol) to afford isodrimenin. The compounds were identified by spectral data (IR, ¹H NMR, and ¹³C NMR), which were in good agreement to those previously published (Ciccio, 1984; Jansen and Groot, 2004; McCallion, 1982; Aasen *et al.*, 1977; White and Burton, 1985), and by direct comparison with authentic samples.

Results and Discussion

Table I shows the 31 plants used in this study from which dichloromethane and methanol/water extracts were prepared and tested against trypomastigotes in concentrations of up to 500 µg/mL. Table II shows the activities of extracts of *Podanthus ovatifolius*, *Berberis microphylla*, *Kageneckia oblonga*, and *Drimys winteri*, the four plants that produced significant inhibition in the MTT test. These plant extracts exhibited activities between 7 and 5% of those of the standard compounds benznidazole or nifurtimox, respectively. The extracts from all other plants in Table I showed an IC₅₀ value higher than 500 µg/mL. Table III shows the activities of isodrimenin, drimenol, and polygodial, the three

Table II. Activity of Chilean plant extracts on the *Trypanosoma cruzi* trypomastigotes.

| Plant | Extract | MTT viability test IC ₅₀ [µg/mL] |
|--|-----------------|---|
| Mitique (<i>Podanthus ovatifolius</i>) | Methanol/water | 40.1 ± 3.0 |
| Michay (<i>Berberis microphylla</i>) | Methanol/water | 38.4 ± 5.0 |
| Bollén (<i>Kageneckia oblonga</i>) | Methanol/water | 35.7 ± 4.0 |
| Canelo (<i>Drimys winteri</i>) | Dichloromethane | 51.2 ± 2.0 |
| Nifurtimox | - | 4.6 ± 0.1 |
| Benznidazole | - | 8.4 ± 0.1 |

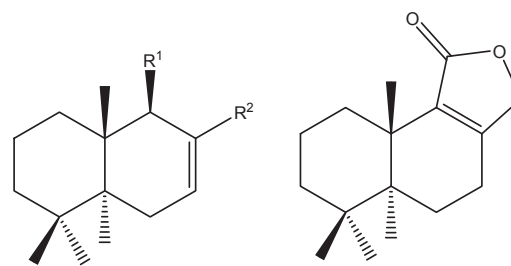
The MTT assay was carried out with the trypomastigote form of *T. cruzi*. Extracts were used at final concentrations of 10, 15, 20, 50, 100, and 500 µg/mL. IC₅₀ values were calculated from drug concentration-response curves (see Experimental). Results are shown as the average ± standard deviation of three independent experiments.

Table III. Activity of compounds isolated from *Drimys winteri* upon the *Trypanosoma cruzi* trypomastigote form.

| Compound | IC ₅₀ [μ M] |
|--------------|-----------------------------|
| Isodrimenin | 27.9 \pm 0.3 |
| Drimenol | 25.1 \pm 0.5 |
| Polygodial | 120.4 \pm 1.5 |
| Nifurtimox | 16.1 \pm 0.2 |
| Benznidazole | 32.2 \pm 0.3 |

The activity was measured on *T. cruzi* trypomastigotes using the MTT method at the concentrations of 10, 15, 20, 50, 100, and 200 μ M. IC₅₀ values were calculated from drug concentration-response curves (see Experimental). Results are shown as the average \pm standard deviation of three independent experiments.

sesquiterpenes isolated from the stem bark of *Drimys winteri* (Fig. 1). The results show that polygodial, the most active antifungal (Kubo *et al.*, 2001), antifeedant (Zapata *et al.*, 2009), and antibacterial (Kubo *et al.*, 2005) from *D. winteri*, has a weak anti-*Trypanosoma* activity with respect to the other sesquiterpenes. Isodrimenin and drimenol showed similar activities as nifurtimox or benznidazole (Table III). Some compounds of the other three active plant extracts are being isolated and their antiparasite activity will be tested later.



Polygodial R¹=R²=CHO
 Drimenol R¹=CH₂OH; R²=CH₃

Isodrimenin

Fig. 1. Chemical structure of compounds isolated from *Drimys winteri*.

Plant metabolites active against *T. cruzi* have been recently reviewed by our laboratories (Maya *et al.*, 2007; Salas *et al.*, 2011), and we concluded that the compounds isodrimenin and drimenol isolated from *Drimys winteri* represent new basic lead structures to obtain new selective antichagasic drugs.

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