

## Drimanes from *Drimys brasiliensis* with leishmanicidal and antimalarial activity

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This paper evaluates  $CHCl_3$  and  $CH_3OH$  extracts of the stem bark, branches and leaves of *Drimys brasiliensis* and drimane sesquiterpenes isolated from the stem bark against strains of *Leishmania amazonensis* and *Leishmania braziliensis promastigotes* and *Plasmodium falciparum* trophozoites. All of the extracts and compounds were tested in cell lines in comparison with reference standards and cell viability was determined by the XTT method. The  $CHCl_3$  and  $CH_3OH$  extracts from the stem bark and branches yielded promising results against two strains of *Leishmania*, with 50% inhibitory concentrations ( $IC_{50}$ ) values ranging from 39-100  $\mu g/mL$ . The  $CHCl_3$  extract of the stem bark returned  $IC_{50}$  values of 39 and 40.6  $\mu g/mL$  for *L. amazonensis* and *L. braziliensis*, respectively. The drimanes were relatively effective: 1- $\beta$ -(*p*-coumaroyloxy)-polygodial produced  $IC_{50}$  values of 5.55 and 2.52  $\mu M$  for *L. amazonensis* and *L. braziliensis*, respectively, compared with 1- $\beta$ -(*p*-methoxycinnamoyl)-polygodial, which produced respective  $IC_{50}$  values of 15.85 and 17.80  $\mu M$ . The  $CHCl_3$  extract demonstrated activity ( $IC_{50}$  of 3.0  $\mu g/mL$ ) against *P. falciparum*. The  $IC_{50}$  values of 1- $\beta$ -(*p*-cumaroyloxy)-polygodial and 1- $\beta$ -(*p*-methoxycinnamoyl)-polygodial were 1.01 and 4.87  $\mu M$ , respectively, for the trophozoite strain. Therefore, the results suggest that *D. brasiliensis* is a promising plant from which to obtain new and effective antiparasitic agents.

Key words: leishmaniasis - malaria - *Drimys* - drimane sesquiterpenes

Neglected tropical diseases, including leishmaniasis, are among the most common causes of illness among the poorest populations living in developing countries. Cutaneous leishmaniasis has a worldwide distribution and is a public health problem, with 1-1.5 million cases recorded annually worldwide (Hotez et al. 2008). The World Health Organization (WHO) considers leishmaniasis to be one of six major infectious diseases, with a high detection rate and the capacity to produce deformities. It is a disease with a variety of agents, hosts, vectors and transmission patterns, but limited knowledge about some aspects of the disease makes it difficult to control (WHO 2010).

Another serious public health problem is malaria, which is caused by protozoa of the genus *Plasmodium* and is transmitted to humans by female *Anopheles* mosquitoes, producing fever and other symptoms. In Brazil, the greatest number of cases is recorded in the Amazon Region, where the environmental and sociocultural conditions favour the expansion of plasmodial transmission (WHO 2009).

The intense search for new ways of treating these neglected diseases has motivated scientific research in this field. The importance of parasitic diseases as a public health issue, coupled not only with the limited number of drugs available for their treatment, but also their significant side effects, has led to the important strategy of searching for new antiparasitic agents among natural or synthetic compounds.

Our research group has taken part in an Ibero-American program (Ribiofar/CYTED) to search for natural bioactive products from plants, with the aim of evaluating the pharmacological potential of Brazilian plants. In this context, *Drimys* has been extensively investigated for its chemical composition and biological activity. In *Drimys angustifolia* Miers [synonym, *Drimys winteri* f. *angustifolia* (Miers) Eichl], sesquiterpene drimanes are predominant, including polygodial, 1- $\beta$ -(*p*-methoxycinnamoyl)-polygodial and drimanial. These compounds exhibit antifungal (Castelli et al. 2005, Malheiros et al. 2005), antinociceptive (El Sayah et al. 2000), anti-inflammatory (Cunha et al. 2001, Malheiros et al. 2001) and antihyperalgesic activities (Mendes et al. 1998, 2000).

It has recently been demonstrated that a hexane extract of the stem bark of *Drimys brasiliensis* and polygodial, a drimane, exhibit activity against *Leishmania* spp and *Trypanosoma cruzi* (Corrêa et al. 2011). This information contributes to the further study of this plant species, with the aim of obtaining new drugs to treat the neglected diseases.

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This paper analyses chloroform and methanol extracts of the stem bark, branches and leaves of *D. brasiliensis* and selected pure drimane sesquiterpenes for their inhibitory activity against strains of *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes and *Plasmodium falciparum* trophozoites.

## MATERIALS AND METHODS

**General experimental procedures** - NMR spectra for  $^1\text{H}$  (300 MHz) and  $^{13}\text{C}$  (75.5 MHz) were obtained on a Varian Gemini AC 300 spectrometer with  $\text{CDCl}_3$  as the solvent. The chemical shifts are given in  $\delta$  (ppm) with TMS as the internal standard. Silica gel 60 (230-400 mesh) was used for the column chromatography and silica gel 60  $F_{234}$  (230-400 mesh) was used for the thin layer chromatography.

**Plant material** - The stem bark, branches and leaves of *D. brasiliensis* were collected in Rancho Queimado, in the Brazilian state of Santa Catarina (SC), in December 1998. The plant was identified as *D. brasiliensis* Miers subsp. *sylvatica* (Saint Hilaire) Ehrendofer & Gottsb by Dr Ademir Reis (Department of Botany, Federal University of Santa Catarina) and a voucher specimen was compared with one deposited in the Barbosa Rodrigues Herbarium (Itajaí, SC), under the protocol VC Filho 010.

**Preparation of extracts** - The stem bark of *D. brasiliensis* (774 g) was dried at 40°C for two days, powdered and successively extracted with chloroform and  $\text{CH}_3\text{OH}$  at room temperature for seven days each. The extracts were concentrated under reduced pressure, yielding residues of 30.2 and 60.6 g, respectively. The branches (700 g) were processed in the same way as the bark to produce a yield of 17.6 and 14.8 g from the  $\text{CHCl}_3$  and MeOH extracts, respectively. From 250 g of leaves, the same method yielded 24.5 and 10.6 g of material from the  $\text{CHCl}_3$  and MeOH extracts, respectively.

**Isolation and identification of compounds from the  $\text{CHCl}_3$  bark extract** - An aliquot of the stem bark extract (25 g) was chromatographed ( $\theta$ i 5.4 cm) on a column that was packed with silica gel (238 g) and the extract

components were eluted with hexane gradually enriched with ethyl acetate and ethanol. Seventy fractions of 100 mL each were collected. Spathulenol (Figure, compound 1) was obtained from fractions 4-21 [elution solvent Hex-AcOEt (9:1)] with a yield of 603 mg (0.0645% of the dry plant material). Polygodial (Figure, compound 2) in the amount of 732.8 mg (0.078% of the dry plant material) was obtained from fractions 26-30 [elution solvent Hex-AcOEt (9:1)].

Fractions 48-55 (5.0 g) were chromatographed on a column ( $\theta$ i 3.5 cm) packed with silica gel (76 g) and the adsorbent was eluted with hexane gradually enriched with ethyl acetate and ethanol. Ninety-one subfractions of 25 mL each were collected. From subfractions 44-47 eluted with a 6:4 ratio of hexane:ethyl acetate, 1- $\beta$ -(p-methoxycinnamoyl)-polygodial (Figure, compound 3) was obtained (yield of 0.144 g, 0.172% of the dry plant material).

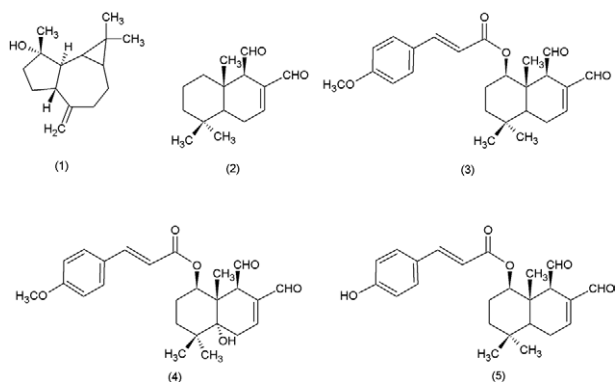
Fractions 56-59 (3.60 g) were chromatographed ( $\theta$ i 5.4 cm) on a column packed with silica gel (140 g) and the adsorbent was eluted with hexane gradually enriched with ethyl acetate and ethanol. Sixty subfractions of 25 mL each were collected. Drimanal (Figure, compound 4) was obtained from subfractions 25-46 [elution solvent Hex-AcOEt (6:4)] at a yield of 1.994 g (0.028% of the dry plant material). A yield of 0.175 mg of 1- $\beta$ -(p-coumaroyloxy)-polygodial (Figure, compound 5) (0.050% of the dry plant material) was obtained from subfractions 50-55 [elution solvent Hex-AcOEt (6:4)].

**Isolation and identification of compounds from the  $\text{CHCl}_3$  branch extract** - An aliquot of the branch extract (13.3 g) was chromatographed on a column ( $\theta$ i 3,5 cm) packed with silica gel (100 g) and the adsorbent was eluted with hexane gradually enriched with ethyl acetate and ethanol. Seventy-five fractions of 100 mL each were collected. Using this procedure, only mixtures of fatty acids and alcohols were obtained.

**Isolation and identification of compounds from the  $\text{CHCl}_3$  leaf extract** - Part of the leaf extract (17.1 g) was subjected to chromatography ( $\theta$ i 4.0 cm) on a column packed with silica gel (150 g) and the adsorbent was eluted with hexane gradually enriched with ethyl acetate and ethanol. Fifty-five fractions of 100 mL each were collected. Fraction 34 collected in hexane:ethyl acetate at a 1:1 ratio (1.134 g) was chromatographed ( $\theta$ i 2,0 cm) on a column packed with silica gel (27 g) and eluted with hexane gradually enriched with ethyl acetate and ethanol. Thirty-five subfractions of 35 mL each were collected. Fractions 12-16, which were eluted with hexane:ethyl acetate at an 8:2 ratio, yielded 157.4 mg of a mixture of  $\alpha$  and  $\beta$ -cubebin (0.0438% of the dry plant material).

All of the compounds were identified based on their spectral data through comparison with the literature (Malleiros et al. 2001) and directly with authentic samples.

**Biological activity assays - Leishmanicidal activity** - Promastigotes of *Leishmania* [*L. amazonensis* clone 1, AML (MHOM/BR/76/LTB-012) and *L. braziliensis* (M2904 C192 RJA)] obtained from in vitro cultures of IIFB (20  $\mu\text{L}$ ) were fixed with glutaraldehyde (5%, 180



Compounds isolated from *Drimys brasiliensis*. 1: spathulenol; 2: polygodial; 3: 1- $\beta$ -(p-methoxycinnamoyl)-polygodial; 4: drimanal; 5: 1- $\beta$ -(p-coumaroyloxy)-polygodial.

μL) and counted in a Neubauer chamber. The population was adapted to  $3 \times 10^6$  parasites/mL with Schneider medium (pH 6.8) and foetal bovine serum (10%) distributed (100 μL/well) in 96-microwell plates. Solutions of the samples to be assessed at final concentrations of 100, 50 and 25 μg/mL were added (100 μL) to the wells of the plates with the parasites. Dimethyl sulfoxide (DMSO) (1%) and amphotericin B (0.5 μg/mL) were used as parasite growth controls. Each test was performed in triplicate and the plates were incubated for 72 h at 26°C.

Then, 50 μL of a solution of XTT (1 mg/mL) in phosphate buffer (pH 7.0, 37°C) with phenazine methosulfate (Sigma-Aldrich, 0.06 mg/mL) was added to each well of the plates, which were incubated for another 4 h at 26°C. The plates were read on a Stat Fax 2100 microplate reader at 450 nm. The 50% inhibitory concentrations (IC<sub>50</sub>) values for the parasites were calculated using Microsoft Excel 2000 (Capusiri et al. 2008).

**Antiplasmodial activity** - The *P. falciparum* parasites belonged to the F32-Tanzania strain (chloroquine-sensitive, kindly provided by Dr T Fandeur, Pasteur Institute, Kayenne) and were cultivated under anaerobic conditions at 37°C in RPMI-1640 medium supplemented with 10% human serum containing erythrocytes at a haematocrit of 4% (blood group O, RH<sup>+</sup>) (Trager & Jensen 1976). The parasites were synchronised at 1% parasitaemia and 2% haematocrit before their distribution into 96-well plates at a volume of 100 μL in duplicate. A total of 100 μL of each concentration of each compound or extract (10, 1 or 0.1 μg/mL in DMSO - final concentrations did not exceed 0.1%) was added to each well and chloroquine

(100 nM) was used as a control. The parasites were then incubated at 37°C for 48 h. Next, a smear of the parasites was fixed with methanol and stained with Giemsa. The antiplasmodial activity was determined by examining the slides under a microscope and counting the non-infected red cells (GRL) and infected cells (GRI) to obtain the inhibition percentage (formula 1).

$$\% \text{ Inhibition} = \frac{(\text{GRL} - \text{GRI})}{\text{GRL}} \times 100 \text{ (1)}$$

The IC<sub>50</sub> for schizont ripening was determined by a graphic method using the program CRICKET GRAPH 1.3 and those treatments with IC<sub>50</sub> values < 10 μg/mL were considered to have inhibitory activity.

**Statistical analysis of data** - The samples were analysed in replicates with n = 4 using two methods to assess viability (MTT and neutral red). The data are reported as means ± standard errors and the significant differences among the treatments were determined using a one-way ANOVA followed by the Tukey-Kramer test (\*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001).

## RESULTS

In the search for new potential antiparasitic agents, the chloroform and methanol extracts of the stem bark, branches and leaves and the isolated compounds from the stem bark of *D. brasiliensis* Miers were assessed in vitro for their capacity to inhibit development of the promastigotes of *L. amazonensis* and *L. brasiliensis* and the trophozoites of *P. falciparum*. The resulting minimal IC are shown in Tables I, II.

TABLE I

Leishmanicidal activity of extracts and compounds isolated from stem bark of *Drimys brasiliensis* in vitro on promastigote forms of *Leishmania amazonensis* and *Leishmania brasiliensis*

Species	Samples	<i>L. amazonensis</i>		<i>L. brasiliensis</i>	
		IC <sub>50</sub> (μg/mL)	IC <sub>50</sub> (μg/mL)	IC <sub>50</sub> (μg/mL)	IC <sub>50</sub> (μg/mL)
Extracts	DBCH <sub>3</sub> OH stem bark	83.8 ± 4.03	> 100	> 100	> 100
	DBCHCl <sub>3</sub> stem bark	39.0 ± 0.28	40.6 ± 1.40	40.6 ± 1.40	40.6 ± 1.40
	DBCH <sub>3</sub> OH branches	85.0 ± 1.9	78.0 ± 10	78.0 ± 10	78.0 ± 10
	DBCHCl <sub>3</sub> branches	99.0 ± 6.9	81.9 ± 9.4	81.9 ± 9.4	81.9 ± 9.4
	DBCH <sub>3</sub> OH leaves	> 100	> 100	> 100	> 100
	DBCHCl <sub>3</sub> leaves	> 100	> 100	> 100	> 100
Compounds		IC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)
	Spathulenol	81.82 ± 0.63	89.10 ± 0.90	89.10 ± 0.90	89.10 ± 0.90
	Polygodial	41.45 ± 1.79	25.64 ± 1.12	25.64 ± 1.12	25.64 ± 1.12
	1β-(p-methoxycinnamoyl)-polygodial	15.85 ± 0.41	17.80 ± 0.68	17.80 ± 0.68	17.80 ± 0.68
	Drimanial	20.42 ± 1.31	12.67 ± 1.15	12.67 ± 1.15	12.67 ± 1.15
	1β-(p-cumaroyloxy)-polygodial	5.55 ± 0.98	2.52 ± 0.78	2.52 ± 0.78	2.52 ± 0.78
	Amphotericin B	0.21 ± 0.04	1.20 ± 0.04	1.20 ± 0.04	1.20 ± 0.04

data are expressed as mean standard deviation of three determinations. IC<sub>50</sub>: 50% inhibitory concentrations.

For the initial screening of substances inhibiting the *Leishmania* spp, those extracts with IC<sub>50</sub> values less than 50 µg/mL were considered active. Thus, the IC<sub>50</sub> values of 39.0 and 40.6 µg/mL for the stem bark chloroform extract tested against *L. amazonensis* and *L. brasiliensis*, respectively, indicated the leishmanicidal activity of the extract (Table I). The methanol and chloroform extracts of the branches presented IC<sub>50</sub> values between 78.0-99.0 µg/mL, against the promastigote forms of the test *Leishmania* and the leaf extracts were inactive.

It is possible to compare these results with those reported in the literature for various plant extracts. For example, extracts of *Bidens pilosa* L. and *Punica granatum* L. inhibited the growth of intracellular amastigotes, with IC<sub>50</sub> values of 42.6 and 69.6 µg/mL, respectively. Extracts of *Julocroton triqueter*, a *Dichorisandra* sp. and *Tephrosia cinerea* were shown to be more effective in inducing the death of promastigotes, with IC<sub>50</sub> values of 29.5, 32.9 and 43.6 µg/mL, respectively (Luize et al. 2005, Rocha et al. 2005, Bezerra et al. 2006, García et al. 2010).

### DISCUSSION

Based on the results presented above, *D. brasiliensis* can be considered a source of compounds with leishmanicidal activity. The chloroform extracts of the stem bark, branches and leaves were subjected to column chromatography. The fractionation of the chloroform extract of the stem bark led to the isolation of five compounds, which were identified as spathulenol (Figure, compound 1), which is an aromadendrane sesquiterpene and the drimanes polygodial (Figure, compound 2), 1-β-(p-methoxycinnamoyl)-polygodial (Figure, compound 3), drimanal (Figure, compound 4) and 1-β-(p-cumaroyloxy)-polygodial (Figure, compound 5). The column chromatography of the chloroform extracts

yielded the isolation of principally fatty alcohols and acids from the branches and fatty alcohols and acids and a mixture of the lignans α and β-cubebin from the leaves. The compounds were characterised by H<sup>1</sup> and C<sup>13</sup> NMR and comparison of the NMR spectra with previously reported data (Malheiros et al. 2001, Lago & Roque 2009).

Considering that the stem bark extract was rich in drimane sesquiterpenes and exhibited excellent inhibitory activity when evaluated in vitro against the promastigotes of *L. amazonensis* and *L. brasiliensis*, the drimanes were evaluated to identify the compound or compounds responsible for this inhibition. All of the drimanes exhibited activity against the promastigotes, with IC<sub>50</sub> values < 42 µM. The compound 1-β-(p-cumaroyloxy)-polygodial (Figure, compound 5) exhibited the best activity, with IC<sub>50</sub> values of 5.55 and 2.52 µM, respectively, against *L. amazonensis* and *L. brasiliensis*. Amphotericin B, which was used as a positive control, activity against *L. brasiliensis* and had an IC<sub>50</sub> of 1.1 µM.

Comparing the drimanes with each other, those with a cinnamoyl group at the C-1 position exhibited higher activity than did polygodial, which lacks this group. This increased activity can be related to the lipophilicity caused by the addition of the aromatic ring to these compounds. Moreover, the substitution of a hydroxycinnamoyl group (Figure, compound 5) for the methoxycinnamoyl group (Figure, compounds 3, 4) led to increased leishmanicidal activity against the promastigotes of both of the *Leishmania* spp analysed. These results highlight the importance of the p-hydroxycinnamoyl group for the leishmanial inhibition, which may be explained by the lipophilicity associated with the aromatic group and the possibility of hydrogen bond formation to maintain the biological activity. Another compound evaluated was the aromadendrane spathulenol (Figure, compound 1),

TABLE II  
Plasmodicidal activity of extracts and compounds isolated from stem bark of *Drimys brasiliensis* in vitro on trophozoite forms of *Plasmodium falciparum*

Species	Samples	Inhibition (10 µg/mL)	IC <sub>50</sub> (µg/mL)	<i>P. falciparum</i> (FcR3)
Extracts	DBCHCl <sub>3</sub> stem bark	97 ± 3.0	3 ± 0.4	Active
	DBCH <sub>3</sub> OH stem bark	0	> 10	Inactive
	DBCHCl <sub>3</sub> branches	0	> 10	Inactive
	DBCH <sub>3</sub> OH branches	0	> 10	Inactive
	DBCHCl <sub>3</sub> leaves	40 ± 6.0	> 10	Inactive
	DBCH <sub>3</sub> OH leaves	0	> 10	Inactive
				IC <sub>50</sub> (µM)
Compounds	1-β-(p-methoxycinnamoyl)-polygodial	100	4.87 ± 0.97	Active
	1-β-(p-cumaroyloxy)-polygodial	100	1.01 ± 0.10	Active
Positive control	Chloroquine	100	0.1 ± 0.01	Active

IC<sub>50</sub>: 50% inhibitory concentrations.



which was isolated from the chloroform extracts. As can be seen from the data in Table I, spathulenol did not contribute to the activity of this extract.

The extracts obtained from the different parts (the stem bark, branches and leaves) of *D. brasiliensis* were also evaluated for their antiplasmodial activity. The initial screening revealed plasmodicidal activity only for the chloroform extract of the stem bark, which produced an  $IC_{50}$  value of 3.0  $\mu\text{g/mL}$  and 97% inhibition (Table II).

Compounds 4, 5 (Figure) exhibited the greatest leishmanicidal activity and they were therefore investigated for their plasmodicidal activity. The more effective of the two compounds against the trophozoite form of *P. falciparum* was 1- $\beta$ -(p-cumaroyloxy)-polygodial (Figure, compound 5), with an  $IC_{50} = 1.01 \mu\text{M}$  (100% inhibition), the  $IC_{50}$  for 1- $\beta$ -(p-methoxycinnamoyl)-polygodial (Figure, compound 3) was 4.87  $\mu\text{M}$  (100% inhibition). In this experiment, it was again observed that the p-cinnamoyl group at the C-1 position in the drimane ring is important for maintaining the inhibitory activity.

Previous studies have indicated that both a crude hexane extract of *D. brasiliensis* and polygodial show activity against *Leishmania* spp when applied in the range of 22-62  $\mu\text{g/mL}$  and polygodial demonstrated high selectivity towards *T. cruzi* trypomastigotes (2  $\mu\text{g/mL}$ ) (Corrêa et al. 2011). The drimanes 9-epideoxymuzigadial, 9-deoxymuzigadial, muzigadial and 3- $\beta$ -acetoxypolygodial isolated from a hexane extract of the leaves of *Canella winterana* exhibited strong activity against the chloroquine-sensitive strain of *P. falciparum*, producing  $IC_{50}$  values of 1.01, 2.19, 0.31 and 2.77  $\mu\text{g/mL}$ , respectively (Grace et al. 2010).

Our results, together with those reported in the literature, strongly suggest that *D. brasiliensis* and the drimanes could be promising for the treatment of diseases such as leishmaniasis and malaria, two of the so-called neglected diseases caused by protozoans, which demand the search for new chemotherapeutic agents. However, further studies (in vitro and in vivo) are imperative for understanding the mechanisms of action of these bioactive compounds and for evaluating their toxicity to humans before these agents can be exploited clinically.

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