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ANTILEISHMANIAL ACTIVITY OF COMPOUNDS EXTRACTED AND CHARACTERIZED FROM *CENTROLOBIUM SCLEROPHYLLUM*

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Key Word Index—*Centrolobium sclerophyllum*; Leguminosae; diarylheptanoids; isoflavonoid; *Leishmania amazonensis*; promastigotes; antileishmanial activity.

Abstract—Drugs derived from plants, which had not been tested up to now in trypanosomatids, were studied. The compounds extracted from *Centrolobium sclerophyllum*, Leguminosae, were two diarylheptanoids, 2[β-(*p*-hydroxyphenyl)-ethyl]-6(*p*-hydroxyphenyl)-tetrahydropyran and 3-hydroxy-1,7-bis-(4′,4″-dihydroxyphenyl)-heptane, and one isoflavonoid, 7,3′-dihydroxy-4′-methoxy-isoflavone. Extracellular forms (promastigotes) of *Leishmania amazonensis* were assayed in presence of these compounds and the results showed that both diarylheptanoids and the isoflavonoid had a good antileishmanial activity with the LD50 = 77 nM, LD50 = 86nM and LD50 = 140nM, respectively.

INTRODUCTION

Leishmaniasis is a major public health problem and it can cause many different clinical manifestations in humans [1, 2]. *Leishmania amazonensis*, a species which is very common in Brazil, has been associated with different forms of the disease, including the cutaneous, the hyperergic mucocutaneous, the anergic diffuse cutaneous and visceral leishmaniasis [3–5]. Members of the genus *Leishmania* differentiate from proliferative promastigotes in the sandfly vector gut to infective metacyclic promastigotes in the insect foregut. Parasites are inoculated by the vector as the flagellate promastigotes enter the mammalian host, where they infect macrophages differentiating into nonmotile amastigotes and multiplying as such. Despite the enormous progress made in understanding the biology of *Leishmania* and clinical possibilities presented by some experimental chemotherapeutic agents, no new drugs have been developed for the treatment of leishmaniasis since the introduction of the pentavalent antimonials more than 80 years ago by Gaspar Viana [6]. The lack of an effective anti-leishmanial drug has led a renewed interest in the study of traditional remedies as sources for the development of new chemotherapeutic compounds with better activity and less toxic effects. Several medicinal plants have been used for the treatment of parasitic diseases. As a part of our research program, we have studied some compounds derived from plants widely spread in Brazil, which have not been tested previously in trypanosomatids. Guided fractionation of a chloroform extract of the wood of *Centrolobium sclerophyllum* led to the isolation of two diarylheptanoids and one isoflavonoid. Samples of those classes of compounds, extracted and characterized from other plant species, had been shown to have some anti-inflammatory and anti-bacterial effects, but had never been tested against parasitic diseases. Our results are promising, showing that these compounds are biologically active against *Leishmania amazonensis* promastigotes.

EXPERIMENTAL

Culture and maintenance of the parasite

Leishmania amazonensis promastigotes, strain MHOM/BR/77/LTB0016, were grown at 25°C in Schneider's *Drosophila* medium [7] supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). Cells were harvested in the late log phase, resuspended in fresh medium, counted in Neubauer's chamber and adjusted to a concentration of 4×10^6/mL. The characterization of this strain was performed by molecular techniques, such as isoenzyme electrophoresis and indirect radioimmune assay using specific monoclonal antibodies [8].

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In vitro *assay with* Leishmania amazonensis *promastigotes*

The compounds were added to the promastigotes culture, in the above concentration, for screening (from 160 μg/ml to 5 μg/ml), aseptically solubilized in DMSO (the highest concentration used was 1.6%, v/v) and incubated at 25°C. After 24 h of incubation, the surviving parasites were counted in a Neubauer chamber and compared with controls, which only had DMSO. All tests were done in triplicate and pentamidine isethionate (May & Baker Lab., U.K.) was used as a reference drug, in the same concentration range used for our compounds.

Plant material

The wood of *Centrolobium sclerophyllum* Lima sp. *nova* was collected in 1985 in the CURD Reserve, Linhares, Espirito Santo State, Brazil. A voucher specimen has been deposited at the herbarium of the Botanical Garden of Rio de Janeiro (No. 1535 RB).

General experimental methods

^{1}H NMR (100 MHz) and ^{13}C NMR (25.2 MHz) were recorded on an XL-100 Varian instrument whereas ^{1}H NMR (60 MHz) spectra were obtained on a T-60 Varian instrument. Optical rotations were measured in acetone solutions on a Perkin-Elmer 243 polarimeter. IR spectra were recorded on a Perkin-Elmer 257 spectrophotometer. Electron impact mass spectra were obtained with V6 Micromass NMRF at 70 ev. Column chromatography was performed with silica gel (Si 60 H, Merck Darmstadt, Germany).

Extraction and purification of the compounds

Air-dried wood (1 kg) was powdered and exhaustively percolated at room temperature sequentially with hexane, chloroform and methanol, to give yields of 14 g, 24 g and 37 g, respectively. The chloroform extract (15 g) was chromatographed on silica gel (300 g), using three stepwise gradients of increasing solvent polarity: chloroform (500 ml), chloroform–methanol (400ml:100 ml; 200 ml:300 ml), methanol (500 ml), to yield five fractions (A–E). Fractions A–C were eluted with pure chloroform, whereas D and E were obtained with chloroform–methanol mixtures (4:1 and 2:3, respectively).

Characterization of the isolates

Fractions A–C were repeatedly crystallized in acetone to give pure 7,3′-dihydroxy-4′-methoxy-isoflavone (15 mg). Prisms from acetone, m.p. 226–228°. IR (KBr) γ_{max} cm^{-1}: 3400, 3150 (OH), 1620 (C=O), 1595 (Ar). MS *m/z* (rel. int.): 283 (M^+, 100), 283 (18), 269 (27), 241 (21), 213 (22), 148 (10), 136 (27), 133 (16), 105 (17); ^{1}H NMR ($CDCl_3$+CD_3OD, 60 MHz) δ ppm: 8.05 (s, H-2), 8.2 (*d*, J = 8.5, H-5), 6.8–7.2 (*m*, H-6, H-8, H-2′, H-5′, H-6′). Fraction D was repeatedly crystallized in acetone+petroleum ether to give pure 2-[β-(*p*-hydroxyphenyl)-ethyl]-6-(*p*-hydroxyphenyl)-tetrahydropyran (30 mg). Prisms from acetone+petroleum ether, m.p. 158–160°, $[\alpha]_D^{22}$ = −95.9, in acetone. IR (KBr) γ_{max} cm^{-1}: 3380 (OH), 1615, 1605, 1540 (Ar). MS *m/z* (rel. int.): 298 (M^+, 21), 160 (23), 150 (21), 149 (20), 134 (15), 133 (37), 123 (5), 120 (24), 107 (100). ^{1}H NMR (CD_3COCD_3, 100 MHz) δ ppm: 7.3 (*d*, J = 8.5, H-2″, H-6′), 7.0 (*d*, J = 8.5, H-2′, H-6′), 6.8 (*d*, J = 8.5, H-3″, H-5″), 6.7 (*d*, J = 8.5, H-3′, H-5′), 2.5–2.8 (*m*, H-1′), 3.2–3.6 (*m*, H-3′), 4.25 (*d*, J = 10, H-7ax), 8.0 (*br s*, OH); ^{13}C NMR (CD_3COCD_3, 25.2 MHz) δ ppm: 34.5, 39.4, 77.7, 32.0, 24.7, 31.4, 79.9, 134.0, 127.8, 115.6, 157.1, 115.1, 130.0, 135.9, 127.8, 115.6, 156.1, 115.9, 130.0. Fraction E was also repeatedly crystallized in acetone+petroleum ether to give pure 3-hydroxy-1,7-bis (4′,4″-dihydroxyphenyl) heptane (1.3 g). Prisms from acetone+petroleum ether, m.p. 118–119°, $[\alpha]_D^{22}$ = −10.9, in acetone. IR (KBr) γ_{max} cm^{-1}: 3570, 3450 (OH), 1610, 1595 (Ar). MS *m/z* (rel. int.): 300 (M^+, 12), 282 (29), 134 (12), 133 (74), 120 (55), 108 (30), 107 (100); ^{1}H NMR (CD_3COCD_3, 100 MHz) δ ppm: 6.8–7.0 (*m*, H-2′, H-2″, H-3′, H-3″, H-5′, H-5″, H-6′, H-6″), 2.6–2.8 (*m*, H-1, H-7), 1.3–2.0 (*m*, H-2, H-6, H-4, H-5), 3.5–4.0 (*m*, H-3); ^{13}C NMR (CD_3COCD_3, 25.2 MHz) δ ppm: 31.5, 40.0, 71.1, 37.6, 25.7, 32.3, 35.3, 133.8, 129.6, 115.5, 155.4, 115.5, 129.6, 133.6, 129.6, 115.5, 155.4, 115.5, 129.6.

RESULTS AND DISCUSSION

The activity of antileishmanial plant extracts has been attributed thus far to compounds belonging to diverse chemical groups, such as isoquinolines, indole alkaloids, quinones and terpenes [9, 10]. However, there are no data regarding the use of diarylheptanoids and isoflavonoids to treat parasitic diseases.

In Brazil, diarylheptanoids were extracted and characterized from *Centrolobium tormentosum*, *C. robustum* and *C. sclerophillum* [11–13], Leguminosae. In India, they were isolated from *Garuga pinnata*, Burseraceae, where they are used in indigenous medicine to treat asthma, opacity of cornea and pulmonary infections [14]. Other diarylheptanoids from Zingiberaceae plants were studied: from the rhizomes of *Alpinia conchigera*, used in traditional Thai medicine to relieve gastro-intestinal disorders [15], from *Alpina officinarum*, for use as inhibitors of prostaglandin biosynthesis [16, 17]. Furthermore, two new phenolic and three non-phenolic diarylheptanoids were characterized from rhizomes of *Curcuma xanthorrhiza* which showed anti-inflammatory activity [18, 19].

Here, we analyzed the activity of one isoflavonoid [7,3′-dihydroxy-4′-methoxy-isoflavone (A)] and two diarylheptanoids 2-[β-(*p*-hydroxyphenyl)-ethyl]-6-(*p*-hydroxyphenyl)-tetrahydropyran (B) and 3-hydroxy-1,7-bis-(4′,4″-dihydroxyphenyl)-heptane (C)α isolated

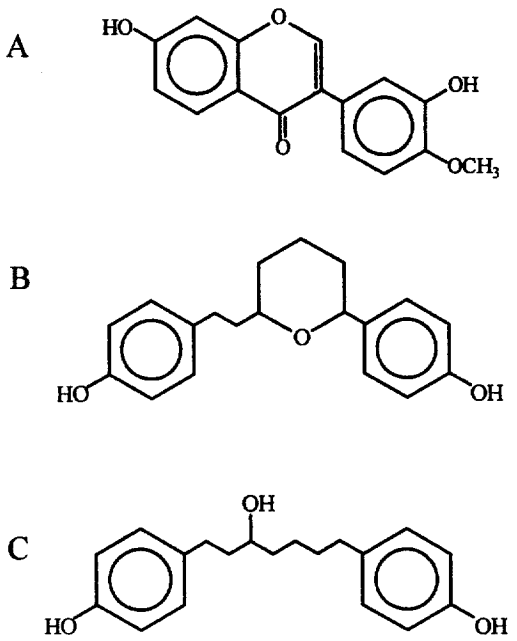

Fig. 1. Basic chemical structures of the compounds. (A) 7,3′-dihydroxy-4′-methoxy-isoflavone. (B) 2-[β-(*p*-hydroxyphenyl)-ethyl]-6-(*p*-hydroxyphenyl)-tetrahydropyran. C: 3-hydroxy-1,7-bis-(4′-4″-dihydroxyphenyl)-heptane.

from *Centrolobium sclerophyllum* (Fig. 1), against *L. amazonensis* promastigotes. This study is a new approach, since these specific compounds are never been used before on trypanosomatids. All three compounds showed a good antileishmanial activity (Fig. 2), with calculated LD50 = 140nM, LD50 = 77nM and LD50 = 86nM, respectively. These compounds showed a high activity when compared with glucantime which is a drug used in clinical practice, but which did not show any activity in our *in vitro* experiments, at the same concentrations as used with our compounds. *In vitro* studies of promastigotes' antimony susceptibility have revealed a wide variability of responses with reports of considerable insensitivity for promastigotes as compared with amastigotes within macrophages [20, 21]. In leishmaniasis, the therapeutic response to the antimonial compounds (glucantime and pentostam) varies with the parasite species involved and with the clinical form of the disease [22]. On the other hand, pentamidine, which is also a clinical drug, showed much better activity *in vitro* than our compounds. For example, when we used 10 μg/ml (16.8 μM) and 0.625μg/ml (1.05 μM), the inhibition of growth was 100% and 89.6%, respectively. However, clinical data demonstrated that both glucantime and pentamidine are associated with very serious side effects, including cardiac arrest problems [1, 23].

It is interesting to notice that, when the diarylheptanoids were tested in parasites with different degree of virulence, those with low virulence (several passages in axenic culture as promastigotes), showed higher sensitivity to these compounds. On the other hand, no difference was observed with the isofla-

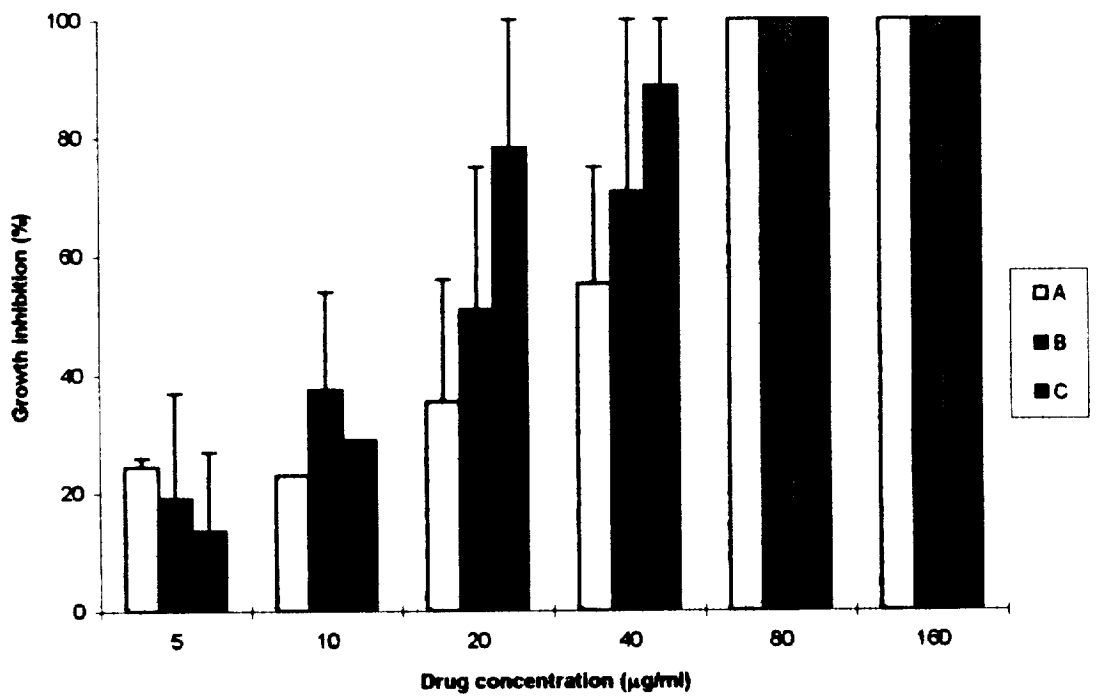

Fig. 2. Effect of different concentrations of one isoflavonoid (A) and two diarylheptanoids (B and C) on the growth of *Leishmania amazonensis* promastigotes.

vonoid. The surviving parasites from all experiments were subcultured, observed for viability, and were found to grow as well as the controls. Although we have some preliminary data concerning the lack of toxicity in mice treated with 2-[β-(*p*-hydroxyphenyl)-ethyl]-6-(*p*-hydroxyphenyl)-tetrahydropyran (one month follow up), we cannot assume that our compounds are ready to be used in clinical trials.

The continued search for new drugs with high activity and low side effects is an important approach, considering that in our country diseases associated to protozoan parasites, such as leishmaniasis, are important public health problem.

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