Trypanocidal Activity of Triterpenes from *Arrabidaea triplinervia* and Derivatives

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Ethanol extract from *Arrabidaea triplinervia* leaves showed *in vitro* activity (ED<sub>100</sub> 5.0 mg/ml) against trypomastigotes of *Trypanosoma cruzi*, the etiologic agent of Chagas’ disease. Bioactivity-directed fractionation of this extract led to the isolation of ursolic and oleanolic acids as trypanocidal compounds besides pomolic acid (not tested) and alpinetine (inactive). A series of natural and synthetic derivatives of ursolic and oleanolic acids was simultaneously assayed for structure activity relationships (SAR) studies. Ursolic acid (ED<sub>100</sub> 0.4 mg/ml) was four times more active than oleanolic acid (ED<sub>100</sub> 1.6 mg/ml). The presence of free hydroxy and/or carboxy groups is necessary for the trypanocidal activity as could be deduced from the effect of the acetates, methyl ester, and aldehyde derivatives.

**Key words** *Arrabidaea triplinervia*; Bignoniaceae; trypanocidal activity; ursolic acid; oleanolic acid; *Trypanosoma cruzi*

Chagas’ disease, caused by the protozoan *Trypanosoma cruzi*, is endemic in Latin America and affects 16—18 million people, 6 to 7 million of whom live in Brazil.1,2) Vector transmission is under control in Brazil,3) while infection via blood transfusion is becoming predominant and is estimated responsible for about 20,000 new cases annually. However, in some Latin American countries such as Mexico and Bolivia the incidence of infection, in some regions, is above 88%. Therefore the treatment of chronic cases and prophylaxis of disease transmission are still a challenge.4)

Gentian violet is the only chemoprophylactic agent used for blood sterilization but its use is limited.4,5) Benzidazole, a 2-nitroimidazole (Rochagan/Brazil; Radanil/Argentina, Roche), is the only available drug for human use but it has several limitations such as low efficacy for parasitologic cure, mainly in the chronic phase of the disease, besides being poorly tolerated and causing severe side effects.4,6)

Therapeutic agents of several pharmacological classes have been assayed aiming to identify more active and less toxic trypanocidal drugs but none could substitute gentian violet.4,7) Several natural products of different structural classes have proven active against *T. cruzi*8,9) and screening of plant extracts is a valid strategy being exploited to discover trypanocidal natural products.10—11) We have been carrying out screening *in vitro* against *T. cruzi* trypomastigotes of several species of Asteraceae10) and Bignoniaceae.12) Leaves ethanol extract from *Arrabidaea triplinervia* H. Bail. (Bignoniaceae) was among the active ones and its bioguided fractionation afforded ursolic and oleanolic acids. Natural and synthetic derivatives of these triterpenic acids were also assayed for structure activity relationship (SAR) studies.

**MATERIALS AND METHODS**

**Plant Material** Leaves of *A. triplinervia* were collected in the Pampulha Campus of the Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil, in March 1994, and a voucher specimen (BHCB–23863) is deposited in the herbarium at the Instituto de Ciências Biológicas (UFMG), Belo Horizonte, Brazil.

**Bioassays** Bioassays were performed according to previously described methodology that uses blood from Swiss albino mice infected with Y and CL strains of *T. cruzi*.13) The isolated compounds, and synthetic derivatives were dissolved in dimethyl sulfoxide (DMSO) (0.2 ml) and TCM199 (2.0 ml). Infected blood (0.2 ml) was added to aliquots of this solution (200, 100, 50, 25 µl) and a final volume of 0.4 ml was reached with TCM199. A parasite density of 2×10<sup>5</sup> trypomastigotes/0.1 ml was calculated for each test tube (4 ml, 56×13 mm). Controls containing only TCM199 and DMSO+TCM199, besides a control with gentian violet, were run in parallel. The solutions/suspensions were microscopically examined after incubation at 4°C for 24 h. Samples that caused 100% elimination of parasites were considered as active and those that caused a reduction of approximately 50% in the number of parasites were designated as partially active.

**Extraction and Bioguided Fractionation** The dried and powdered leaves of *A. triplinervia* (270 g) were submitted to exhaustive percolation with ethanol. The ethanol solution was concentrated until the solvent was completely removed affording the crude ethanol extract (49 g). Part of this extract (40 g) was submitted to exhaustive extraction with hexane under reflux in a water-bath for 2 h, affording a hexane-insoluble fraction (33 g) and a hexane-soluble fraction (5.8 g). The insoluble fraction caused complete elimination of the parasites from the blood at the concentration of 5.0 mg/ml while the soluble fraction was only partially active even at higher concentration (10.0 mg/ml). Chromatographic fractionation of the hexane-insoluble fraction (30 g) on a silica gel column (90 g) eluted with solvents of increasing polarity (hexane, dichloromethane, ethylacetate, and methanol) led to 25 groups, of which only three showed trypanocidal activity: G3 (0.859 g, eluted with dichloromethane-ethyl acetate 95 : 5), G4 (0.490 g, eluted with dichloromethane-ethyl acetate 90 : 10), and G5 (0.937 g, eluted with dichloromethane-ethyl acetate 80 : 20). G3 was rechromatographed on a silica gel column yielding ursolic acid (1) (80 mg) and oleanolic acid © 2006 Pharmaceutical Society of Japan

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Arrabidaea triplinervia

RESULTS AND DISCUSSION

1H-NMR, 13C-NMR, DEPT135, COSY45, HETCOR) and (Fig. 1. Chemical Structures of Compounds (1, 5, 10) Isolated from Arrabidaea triplinervia, Synthetic Triterpenoid Derivatives (2–4, 6) and 8 (5) (25 mg). G4 gave additional amount of 1 (20 mg) after recrystallization in ethanol. Rechromatography of G5 on silica gel column led to isolation of pomic acid (9) (6 mg) and alpinetine (10) (13 mg). Spectrometric analysis (IR, UV, MS, 1H-NMR, 13C-NMR, DEPT135, COSY45, HETCOR) and comparison with reported data were used to identify the compounds.

Synthetic (2, 3, 4, 6) and Natural Derivatives (7, 8)
The synthetic derivatives 2, 3, 4, and 6 were prepared by usual procedures of acetylation (Ac₂O–pyridine) and esterification (diazomethane). The natural derivatives 7 and 8 were previously isolated from Machaerium and Dalbergia species.14,15

RESULTS AND DISCUSSION

A. triplinervia belongs to Bignoniaceae, a family that afforded trypanocidal naphthoquinones.1,3 The in vitro assays against T. cruzi tryptomastigotes of the crude ethanol extract of leaves caused total elimination of blood parasites at the concentration of 5.0 mg/ml and a decrease of approximately 50% was observed at the concentration of 2.5 mg/ml. Partial lysis of erythrocytes was observed with both concentrations. Bioguided fractionation afforded three trypanocidal chromatic fractions that led to the isolation the triterpenic acids ursolic acid (1), oleanolic acid (5), and pomic acid (9) besides a flavanone, alpinetine (10).

Ursolic acid (1), oleanolic acid (5), and alpinetine (10) were assayed against T. cruzi tryptomastigotes; pomic acid (9) was not assayed due to insufficient available amount. Alpinetine (10) was completely inactive at the assayed concentrations. Ursolic and oleanonic acids (1, 5) disclosed trypanocidal activity (Table 1). A series of synthetic derivatives of these triterpenoids, including the acetates 2 and 6, the methyl ester 3, the acetate methyl ester 4, besides two available natural derivatives, oleanolic aldehyde acetate (7) and erythrodial (8), were simultaneously assayed aiming to evaluate SAR.

The results of the in vitro assays of this series of triterpenoids against tryptomastigote forms of Y and CL strains of T. cruzi are shown in Table 1. Ursolic acid (1) was the most active of the compounds tested. At the concentration of 0.4 mg/ml, it completely eliminated T. cruzi (Y strain) and at 0.2 mg/ml was partially active, causing 50% decrease in the number of parasites. CL strain showed a lower susceptibility, complete lysis occurring only at the higher concentrations tested (1.6 mg/ml and 0.8 mg/ml). Oleanolic acid (5) was less active, eliminating T. cruzi Y strain only at 1.6 mg/ml. No lysis of erythrocytes was observed for both of these acids while it occurred with the crude ethanol extract. Considering the higher activity of ursolic acid (1) than that of oleanolic acid (5), it would be expected that an urs-12-ene triterpene should have a higher trypanocidal activity than an olean-12-ene type. However, for both types, the presence of hydroxyl group and of carboxyl group is important for the trypanocidal effect since esterification of one of these functions led to significant decrease in activity of the corresponding derivatives (2, 3, 6), in comparison with the original acids (1, 5). The presence of a free hydroxyl group at C-3 and/or a carboxyl group at C-17 is undoubtedly necessary for the activity, as can also be inferred from the observations that methyl ursolate acetate (4) is completely inactive while the acetates 2 and 6 have shown lower activity. On the other hand, erythrodial (8) was shown inactive and thus the presence of a carboxyl group seems a structural requirement for the trypanocidal effect of these triterpenes.

Ursolic and oleanonic acids (1, 5) are ubiquitous triter-
penoids in the plant kingdom, medicinal herbs, and some vegetables used in the human diet. Several activities, such as anti-inflammatory, hepatic protection, antitumoral, antimicrobial, and hypoglycemic have been described for these triterpenic acids and motivated a great interest in their chemical modifications in the search for more effective derivatives.\(^{16—18}\) These triterpenic acids were shown responsible for the effect of a methanol extract of Rosmarinus officinalis L. leaves on the motility of \(T.\ cruzi\) epimastigotes. Ursolic acid (I) was active at the minimum concentration (MC\(_{100}\)) of 40 \(\mu\)g/ml (88 \(\mu\)M) after 48 h of incubation. Oleanolic acid (5) was less active (MC\(_{100}\) 250 \(\mu\)g/ml, 550 \(\mu\)M) and betulinic acid was practically inactive.\(^{19}\) It should be reminded that, due to differences in susceptibility, trypomastigotes, but not epimastigotes, were observed for these compounds while gypso- vic acid, a 6\(\alpha\)-hydroxy-derivative of oleanolic acid, was practically inactive.\(^{19}\) It should be reminded that, due to differences in susceptibility, trypomastigotes, but not epimastigotes, are recommended for screening of drugs.\(^{4}\) Recently, ursane and oleanane triterpenic acids, including ursolic and oleanolic acids, were shown responsible for the \textit{in vitro} trypanocidal effect of methylene chloride extracts of Brazilian \textit{Miconia} species (Melastomataceae) against \(T.\ cruzi\) trypomastigotes, causing lysis of approximately 99% at the highest concentration tested (500 \(\mu\)g/ml). No clearance of parasites was observed for these compounds while gypso- vic acid, a 6\(\alpha\)-hydroxy-derivative of oleanolic acid, was the most active (ED\(_{100}\) 500 \(\mu\)g/ml).\(^{20}\) The inconsistency with the presently reported results (I, ED\(_{100}\) 0.4 mg/ml Y strain, 0.8 mg/ml CL strain; 5, ED\(_{100}\) 1.6 mg/ml, Y strain) could be explained by differences in the susceptibility of parasite strains employed in the experiments and these were not specified by Cunha \textit{et al.}\(^{20}\) Several trypanocidal natural products have been described in the literature and some of them could be considered promising leads, although their potential utilization as chemoprophylactic agents is limited due to the low solubility of most of them in blood.\(^{13,15}\) As far as we are aware, the most promising candidate for Chagas’ disease prophylaxis is a hydrosoluble aminoquinoline [6-methoxy-8-(diethylamino)lepidine dihydrochloride, WR6026], which was effective for clearance of parasites from infected blood at doses comparable to gentian blue (65, 135 \(\mu\)g/ml).\(^{13,21}\)

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\textbf{REFERENCES}