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Zanthoxylum chiloperone leaves extract: First sustainable Chagas disease treatment

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ABSTRACT

Ethnopharmacological relevance: *Zanthoxylum chiloperone* var. *angustifolium* Engl. (Rutaceae) stem bark is used traditionally in Paraguay for its antiparasitic properties. Canthin-6-one is main compound isolated from *Zanthoxylum chiloperone* var *angustifolium* with broad spectrum antifungal, leishmanicidal and trypanocidal activities.

Aim of the study: The qualitative and quantitative characterization and the isolation of main alkaloidal components of different organs of *Zanthoxylum chiloperone* are investigated by HPLC–UV–MS. The *in vitro* biological activity of each extract against trypomastigote and amastigote forms of *Trypanosoma cruzi* parasites were evaluated, then comparison the *in vivo* efficacy of the ethanolic leaves extract of *Zanthoxylum chiloperone* with reference drug, benznidazole, in acute *Trypanosoma cruzi* infected mice when administered by oral route. We have also evaluated the mutagenic and cytotoxic activity of the main component of *Zanthoxylum chiloperone*, *i.e.* canthin-6-one, by mouse bone marrow micronucleus test.

Materials and methods: The compositions of the ethanol extracts obtained after the maceration process were studied by HPLC–UV–MS methods. The quantitation analysis was performed by external standard method, using a calibration curve constructed utilizing solutions containing different concentrations of the reference samples. The anti-trypomastigote activity was evaluated by the lysis effect on mouse blood trypomastigotes (Y strain *Trypanosoma cruzi*). The anti-amastigote *Trypanosoma cruzi* activity was evaluated by a modified colorimetric method with chlorophenol red-β-D-galactopyranoside (CPRG). The cytotoxicity of extracts and compounds was performed on NCTC 929 cells. The *in vivo* efficacy of the ethanolic leaves extract of *Zanthoxylum chiloperone* and benznidazole, in acute *Trypanosoma cruzi* (two different strains) was evaluated in *Trypanosoma cruzi* infected mice; the drugs were administered by oral route. The mortality rates were recorded and parasitaemias in control and treated mice were determined once weekly for 70 days. The mutagenic and cytotoxic activity of the main component of *Zanthoxylum chiloperone*, canthin-6-one, by mouse bone marrow micronucleus test.

Results: Canthin-6-one was the main compound of stem and root bark and 5-methoxy-canthin-6-one in leaves and fruits. The ethanolic leaves extract, canthin-6-one and benznidazole presented, approximately, the same level of *in vitro* activity against trypomastigote and amastigote forms of *Trypanosoma cruzi*. We have also evaluated the mutagenic and cytotoxic effects of canthin-6-one by micronucleus test in mice. This test showed any mutagenic and cytotoxic damages. The effects of oral or subcutaneous treatments at 10 mg/kg daily for 2 weeks with the ethanolic extract of leaves of *Zanthoxylum chiloperone* were examined in Balb/c mice infected acutely with *Trypanosoma cruzi* (CL or Y strain) and compared with benznidazole at 50 mg/kg for 2 weeks. In these experiments, 70 days after infection, parasitaemia and serological response were significantly reduced with the oral ethanolic extract treatment compared with reference drug.

Conclusions: This study have shown the efficacy of the leaves extract of *Zanthoxylum chiloperone* in reducing *Trypanosoma cruzi* parasitaemia *in vivo* assays and could be welcomed by scientific and rural communities of Paraguay because it could help them towards the use of local resources to treat an endemic infection, Chagas disease, affecting 20% of the population of this country.

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1. Introduction

Currently available treatment of Chagas disease on the market is limited to only two drugs: nifurtimox (Lampit®, Bayer) and benznidazole (Lafepe®, Brazil). These drugs present severe side effects and are most successfully used in the acute infection and early chronic diseases (de Andrade et al., 1996). Generally, the disease is diagnosed in the chronic phase, when the therapy becomes inefficient (Cerecetto and Gonzalez, 2002; Caldas et al., 2008). There is an urgent need for new drugs for the chemotherapy of Chagas disease (Rassi et al., 2010). Although new and efficient treatments against Chagas disease are urgently needed, only one class of drugs is in clinical development, i.e. triazoles, which have emerged as new potential treatments (Ribeiro et al., 2009).

Recently, we have reported the trypanocidal effect in experimental Chagas disease of canthin-6-one and some of its analogs isolated from a plant collected in Paraguay, *Zanthoxylum chiloperone* var. *angustifolium* Mart. Engl. (Rutaceae) (Ferreira et al., 2002b, 2007). In that study, the results indicated that canthin-6-one exhibited trypanocidal activity *in vivo* in the mouse model of acute or chronic infection. In addition, the total extract of alkaloids (totum) of *Zanthoxylum chiloperone* stem bark led to high levels of parasitological clearance. In this work, we had chosen an empirical approach associating the *in vitro* biological tests against parasites and activity-guided fractionation, mainly because no ethnopharmacological approach was possible to guide the selection of active plants against Chagas disease due to the absence of external symptoms, but this plant is used to treat parasitic diseases. After various chemical and biological studies with canthin-6-one type compounds (Thouvenel et al., 2003; Soriano-Agaton et al., 2005; Lagoutte et al., 2008) and the establishment of an agreement between IRD (Institut de Recherche pour le Développement) and the Drugs for Neglected Diseases initiative (DND*i*), a non-profit product development partnership, we have entered into two synergistic agreements to identify and develop new promising drug candidates against Chagas disease. This collaboration has allowed the optimization and development of canthin-6-one alkaloids. These molecules are the subject of patent owned by a joint cooperation between IRD and the National University of Asunción (UNA) in Paraguay (Ferreira et al., 2002a). In the present study, we wish to disclose a new approach to develop a treatment of Chagas disease from *Zanthoxylum chiloperone*, demonstrating that an extract prepared from *Zanthoxylum chiloperone* leaves holded the same activity as reference drug, benznidazole and canthin-6-one.

The aims of this study are (i) the qualitative and quantitative evaluation by HPLC–UV–MS methods of the content of active compounds, namely canthin-6-one and its close analogs in different organs of the plant (leaves, stem bark, roots bark, resin and fruits), (ii) the *in vitro* biological activity of each extract, against trypomastigote and amastigote forms of *Trypanosoma cruzi* parasites and (iii) finally comparison of the *in vivo* efficacy of the ethanolic leaves extract of *Zanthoxylum chiloperone* with reference drug, benznidazole, in acute *Trypanosoma cruzi* infected mice when administered by oral route. We have also evaluated the mutagenic and cytotoxic activity of the main component of *Zanthoxylum chiloperone*, canthin-6-one, by mouse bone marrow micronucleus test (Heddle, 1973).

2. Materials and methods

2.1. General experimental procedures

Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Extraction was monitored by TLC carried out on Merck Kieselgel silica gel plates (60F-254) using UV light as visualizing agent and sulfuric vanillin or Dragendorff reagent and heat as developing agent. Merck Kieselgel silica gel (60, particle size 40–63 μm) was used for flash chromatography. NMR spectra were recorded on an AM-400 Bruker spectrometer, calibrated using undeuterated solvent as an internal reference. IR spectra were recorded on Vector 22 Bruker spectrometer and values are reported in cm^{-1} units. Mass spectra were recorded on a Bruker Esquire-LC mass spectrometer. An ion trap mass spectrometer with Electrospray (ESI) and Atmospheric Pressure Chemical (APCI) Ionization source was employed at "*Service d'Analyse des Médicaments et Métabolites*", SAMM, Université Paris-Sud, Châtenay-Malabry, France.

2.2. Plant material

Leaves, stem bark, roots, fruits and resin of *Zanthoxylum chiloperone* var. *angustifolium* Engl. were collected by Maria Elena Ferreira, in Paraguay near Piribebuy, Department of Cordillera and identified by N. Soria (Department of Botany, National University of Asuncion, Paraguay) (Spichiger and Stutz de Ortega, 1987). A voucher specimen (MEF 55) has been deposited at the Herbarium of Chemical Sciences Faculty, San Lorenzo, Paraguay.

2.3. Extraction and isolation

Open air-sun drying was carried out with the different parts of the plant. Powdered dried stem bark of *Zanthoxylum chiloperone* var. *angustifolium* (1.3 kg) was basified with ammonia, extracted in a soxhlet apparatus with CH_2Cl_2 and MeOH separately and successively for 3 days to afford 23 g and 30 g of crude extracts respectively.

Canthin-6-one **1**, 5-methoxycanthin-6-one **2** and canthin-6-one *N*-oxide **3** were isolated as previously described (Thouvenel et al., 2003). Physical and spectral data were used to determine the chemical structure of the compounds and compared to reference samples and literature data. Benznidazole (*N*-benzyl-1,2-nitro-1-imidazole-acetamide) was purchased from Roche, Buenos Aires, Argentina, and used as a reference drug.

Powdered dried leaves of *Zanthoxylum chiloperone* var. *angustifolium* (500 g) were basified with ammonia, extracted in a Soxhlet apparatus separately and successively with CH_2Cl_2 and MeOH for 3 days to afford 10 g and 13 g respectively of crude extracts. The yields of the dichloromethane and methanolic extracts were approximately 2% and 2.6% respectively of the plant powder. The CH_2Cl_2 extract was subjected to silica gel flash column chromatography, eluted with cyclohexane/ethyl acetate (5:5), to obtain 42 fractions. Fractions 20–35 yielded **2** (105 mg) as a pure compound (1% and 0.02% respectively from the dichloromethane extract and plant powder).

To evaluate the chemical content of the different parts of the plant we have worked with powdered dried leaves, stem bark, fruits, roots and resin (2 g, 5 g, 24 g, 30 g and 400 mg). All specimens were basified with ammonia, extracted by ethanol maceration during 2 days, changing the solvent three times to afford 150 mg, 115 mg, 1 g, 6 g, 20 mg respectively of crude ethanol extracts. The yields of the alcohol extracts were approximately 7.5%, 2.3%, 4.1%, 20% and 5% respectively. The compositions of the ethanol extracts obtained after the maceration process were studied by HPLC–UV–MS methods.

2.4. Analysis by HPLC–UV–MS methods

2.4.1. HPLC–UV–MS qualitative study

HPLC analyses of natural products is usually performed using a large set of standards, and the measurements are typically achieve in the gradient elution mode, whereby the retention time depends