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# Strategies for the isolation and identification of trypanocidal compounds from the Rutales*

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*Abstract*: Crude extracts of Rutales species were tested *in vitro* against the trypomastigote form of *Trypanosoma cruzi* at 4 mg/mL, and 20% of them showed significant activity (≥80%). Their inhibitory activity against the glycolytic enzyme GAPDH from *T. cruzi* has also been evaluated at the concentrations of 100 and 200 μg/mL. Additionally, the inhibitory activity of 13 purified coumarins were also assayed against *T. cruzi*-GAPDH. Chalepin was the most active substance with $IC_{50}$ = 64 μM. The 3D structure of the complex chalepin-enzyme was elucidated by X-ray crystallography, revealing the architecture of the interactions between the inhibitor and the enzyme active site.

## INTRODUCTION

Chagas' disease, caused by the flagellate protozoan *Trypanosoma cruzi*, affects about 16–18 million people in Latin America and is responsible for the death of 45 000 patients every year [1]. In Brazil, about 5–6 million people are infected, and 300 000 of them are situated in São Paulo State [2]. The only two drugs currently available are benznidazole and nifurtimox, but their use is restricted due to their toxicity, variable efficacy against early acute stage, and no effectiveness against the chronic phase of the disease [3]. At present, with the control of the insect vector population, infected blood transfusion is becoming the major mode of its transmission [4]. The only trypanocidal substance currently used to prevent infection by this route is gentian violet [5], whose use is limited due to undesirable effects in patients [4]. The discovery of drugs against Chagas' disease is still a challenge.

Estimates of the number of species of flowering plants in the world vary from 215 000 to 500 000. Only 15–17% of them have been systematically studied in the discovery of biologically active substances [6]. Yet, a large proportion of existing drugs are derived from natural compounds, including semisynthetic and synthetic derivatives based on natural products models.

Several species of the order Rutales (Rutaceae, Meliaceae, Simaroubaceae, Burseraceae, and Cneoraceae) have been used in traditional medicine for cancer treatment [7]. *Galipea longiflora* (Rutaceae) showed *in vitro* activity against *Leishmania ssp* and *T. cruzi*, and its bioactivity-guided fractionation provided 2-substituted quinoline alkaloids [8]. Additionally, lignans isolated from *Zanthoxylum naranjillo* showed high trypanocidal activity [9]. In fact, due to economical and technical reasons, the search for new leads of plant origin for the development of anti-chagasic drugs is much less intense than

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for the other parasitic diseases. Considering the extensive bioactive potentiality of the Rutales, some extracts of plants belonging to this order were tested *in vitro* against the trypomastigote form of *T. cruzi.*

The modern approach of target-directed drug development involves the identification of suitable enzymes taking part in essential metabolic pathways of *T. cruzi* life cycle, which are then targeted in the discovery of specific compounds with higher efficacy and lower toxicity. The bloodstream form of parasites of the family Trypanosomatidae possesses a microbody-like organelle where glycolysis takes place, the glycosome [10]. The bloodstream form of *T. cruzi* has no functional tricarboxylic acid cycle, and is highly dependent on glycolysis for ATP production [10]. This great dependence on glycolysis as a source of energy makes the glycolytic enzymes attractive targets for trypanocidal drug design. One of these proteins, glycossomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (BPG). The 3D structure of the glycolytic enzyme GAPDH from *T. cruzi* was determined by Souza *et. al* [11]. The binding site of the adenosine ring of the NAD+ cofactor and the catalytic site of GAPDH possess significant differences between the parasite and the homologous human enzyme [11]. Therefore, the *T. cruzi* enzyme gGAPDH is an attractive target for trypanocidal drug design.

The clear need for the development of new drugs against Chagas' disease has stimulated us to initiate a project aiming the discovery of lead compounds from the Rutales. For this purpose, extracts and pure compounds have been assayed against both trypomastigote form of *T. cruzi* and its glycolytic enzyme GAPDH.

## TRYPANOCIDAL ACTIVITY

The trypanocidal assays *in vitro* were carried out using blood collected by cardiac puncture of albino Swiss mice on the parasitemy peak (seventh day) after infection with Y strain of *T. cruzi* [12]. The infected blood was diluted to the concentration of $2 \times 106$ trypomastigotes per mL. The assays were performed on titration microplates (96 wells) with 387.5 mL of blood in triplicate. The extracts were solubilized in dimethyl sulfoxide (DMSO) and diluted in blood to give 4 mg/mL as final concentration. The plates were incubated at 4 °C, and the number of parasites counted after 24 h, according to the procedure described by Brenner [13]. Infected blood with the same volume of DMSO was used as control, and gentian violet was used as positive control.

## *T. CRUZI* GAPDH (TCGAPDH)-INHIBITORY ACTIVITY

The TcGAPDH used in the assays is a recombinant enzyme obtained in a *Escherichia coli* expression system. The preparation and purification of the TcGAPDH followed a procedure previously described [11]. TcGAPDH activity have been determined according to a modification of a previously reported procedure [14], by spectrophotometric measurements of the formation of NADH, at 340 nm after 30 s. The reaction mixture contains, in a final volume of 1.0 mL, 50 mM Tris-HCl pH 8.6 buffer with 1 mM EDTA and 1 mM β-mercapto-ethanol, 30 mM $Na_3AsO_3$, 2.5 mM $NAD^+$, 0.3 mM glyceraldehyde-3-phosphate, and 4-9 μg protein. The reaction initiates by the addition of enzyme. The specific activity (unit = U) of the enzyme is calculated according to the Eq. 1.

$$\text{U/mg} = \left( \frac{\left( \frac{\Delta \text{ Absorbance}}{\Delta t \text{ (min)}} \right) \text{ x volume of cell}}{6.22 \text{ x volume of enzyme x [enzyme]}} \right) \qquad \text{(Eq. 1)}$$

Where: $\Delta t$ = 0.5 min; volume of cell = 1.00 mL; $^{\varepsilon}$NADH = 6.22 $mM^{-1}cm^{-1}$; volume of enzyme = 0.005 mL; [enzyme] = concentration of enzyme in mg/mL.

The inhibitory activity has been recorded using the reaction medium as above, in a total volume of 1.0 mL. Plant extracts and pure compounds solutions have been prepared in DMSO and added to the

reaction mixture. The final DMSO concentration used in the assays is 10%, which do not affect significantly the specific activity of TcGAPDH. The extracts are tested in the following concentrations: 100 and 200 μg/mL. Pure compounds are assayed in a initial concentration of 100 μg/mL. If the inhibition observed is superior than 50%, the compound are assayed again in smaller concentrations until 50% of inhibition ($IC_{50}$). All the measurements are run in triplicate, and the averaged value is calculated. In each case, a blank experiment is performed with 10% DMSO alone in the reaction medium and it is used as a positive control. The inhibitory activity is calculated according to the Eq. 2.

$$\% \text{ inhibitory activity} = \frac{\text{U/mg control} - \text{U/mg compound}}{\text{U/mg control}} \times 100 \qquad \text{(Eq. 2)}$$

## RESULTS

Plant extracts from Rutales were *in vitro* tested against the trypomastigote form of *T. cruzi* and had their activity evaluated on the TcGAPDH. Table 1 summarizes the results obtained from both bioassays.

**Table 1** Extracts from the Rutales tested against trypomastigote form of *T. cruzi* (% of lysis) and glycolytic enzyme *T. cruzi*-GAPDH (% of inhibition).

| **Species** | **Part of plant** | **Solvent extraction** | **% of Lysis** (4 mg/mL) | **% of inhibition** (μg/mL) 100 | 200 |
|---|---|---|---|---|---|
| *Almeidea coerulea* | stems | hexane | 55.6 | 13.2 | 9.5 |
| *Almeidea coerulea* | leaves | dichloromethane | 70.0 | 7.9 | 38.6 |
| *Almeidea coerulea* | leaves | hexane | 80.5 | 0.0 | 30.1 |
| *Pilocarpus spicatus* | stems | dichloromethane | 97.3 | 2.3 | 25.6 |
| *Pilocarpus spicatus* | stems | hexane | 93.0 | 3.9 | 13.0 |
| *Rauia sp* | stems | methanol | 72.4 | 12.6 | 67.8 |
| *Rauia sp* | stems | methanol | 50.2 | 4.9 | 22.7 |
| *Rauia sp* | leaves | methanol | 49.8 | 5.1 | 26.9 |
| *Conchocarpus obovatus* | stems | methanol | 46.3 | 13.9 | 40.4 |
| *Conchocarpus obovatus* | stems | methanol | 85.6 | 11.8 | 39.2 |
| *Conchocarpus obovatus* | stems | dichloromethane | 60.3 | 7.9 | 36.7 |
| *Conchocarpus obovatus* | stems | hexane | 81.7 | 1.8 | 1.8 |
| *Conchocarpus obovatus* | leaves | hexane | 60.7 | 0.0 | 0.0 |
| *Conchocarpus obovatus* | leaves | methanol | 68.1 | 4.1 | 16.5 |
| *Azadirachta indica* | bark | methanol | - | 79.7 | - |
| *Azadirachta indica* | stems | methanol | - | - | 50.1 |
| *Azadirachta indica* | leaves | methanol | - | 87.5 | 95.3 |
| *Trichilia claussenii* | stems | methanol | 56.3 | 69.7 | 93.2 |
| *Trichilia claussenii* | leaves | dichloromethane | - | 0.0 | 29.6 |
| *Guarea macrophyla* | stems | petroleum ether | 0.0 | 12.5 | 45.9 |
| *Guarea macrophyla* | stems | dichloromethane | 0.0 | 3.4 | 59.5 |
| *Guarea macrophyla* | stems | methanol | 0.0 | 66.5 | 89.8 |
| *Guarea macrophyla* | leaves | petroleum ether | 0.0 | 11.7 | 37.8 |
| *Guarea guidonea* | stems | hexane | 0.0 | 7.2 | 3.6 |
| *Angostura trifoliata* | leaves | methanol | - | 0.0 | 63.3 |
| *Angostura trifoliata* | leaves | dichloromethane | - | 9.8 | 39.9 |
| *Angostura trifoliata* | leaves | petroleum ether | - | 16.6 | 26.8 |
| *Monnieira trifolia* | stems | methanol | 91.2 | 2.2 | 20.4 |
| *Monnieira trifolia* | leaves | methanol | 90.8 | 5.8 | 15.2 |
| *Ravenia infelix* | stems | methanol | 88.0 | 10.4 | 10.4 |
| *Ravenia infelix* | flowers | methanol | 87.0 | 0.0 | 25.6 |
| *Ravenia infelix* | leaves | methanol | 88.8 | 0.0 | 55.4 |

- not tested

Of the 25 crude extracts tested against the trypomastigote form of *T. cruzi*, 10 of them (40%) showed significant activity (>80%) and can be considered a promising source of active substances. In the gGAPDH bioassay, 32 crude extracts were tested. Only 4 extracts showed a good level of inhibition. Comparative analyses of the results showed no correlation between the trypomastigote form and the enzymatic bioassay. The different results suggest that the enzymatic assay is more selective than the trypomastigote form test. Thirteen coumarins, previously isolated from different Rutales species, have also been assayed in the inhibition of trypanosomal gGAPDH. Almost all tested coumarins **1–13** showed $IC_{50}$ at micromolar scale (Fig. 1).

Chalepin (**1**) was the most active substance with $IC_{50}$ = 64 μM. Molecular modeling studies using the program DOCK3.5 showed that coumarins could have favorable energetic and topologic complementarity to the cofactor ($NAD^+$) binding site of the enzyme gGAPDH [15]. Cocrystallization experiments of the enzyme with chalepin (**1**) were successful and allowed us to propose through X-ray crystallography how the coumarin is attached to the enzyme. Analysis of gGAPDH-chalepin complex displayed two important interactions. The oxygen atoms from lactonic ring of chalepin interact with Thr167 residue through water molecule W768 by hydrogen bonds. The prenyl group at position C-3 displayed hydrophobic interactions with Cys166 residue. Another important aspect is that chalepin approximates to Asp210 residue. In the homologous human enzyme this region is occupied by Leu194 residue that can guarantee a selectivity to gGAPDH of *T. cruzi*. Chalepin showed to be a promising lead compound for the process of structure-based design of new trypanocidal drugs. In order to improve its activity and selectivity, additional molecular modeling studies were conducted. Some coumarins analogs were selected by this approach and are being synthesized. The complete X-ray structural analysis of the complex of the enzyme with chalepin will be published elsewhere.

The coumarins escopoletin (**10**), osthol (**9**), phebalosin (**8**) had already been tested against trypomastigote form of *T. cruzi*, showing $IC_{50}$ values of 59, 268, and 1000 μg/ml, respectively.

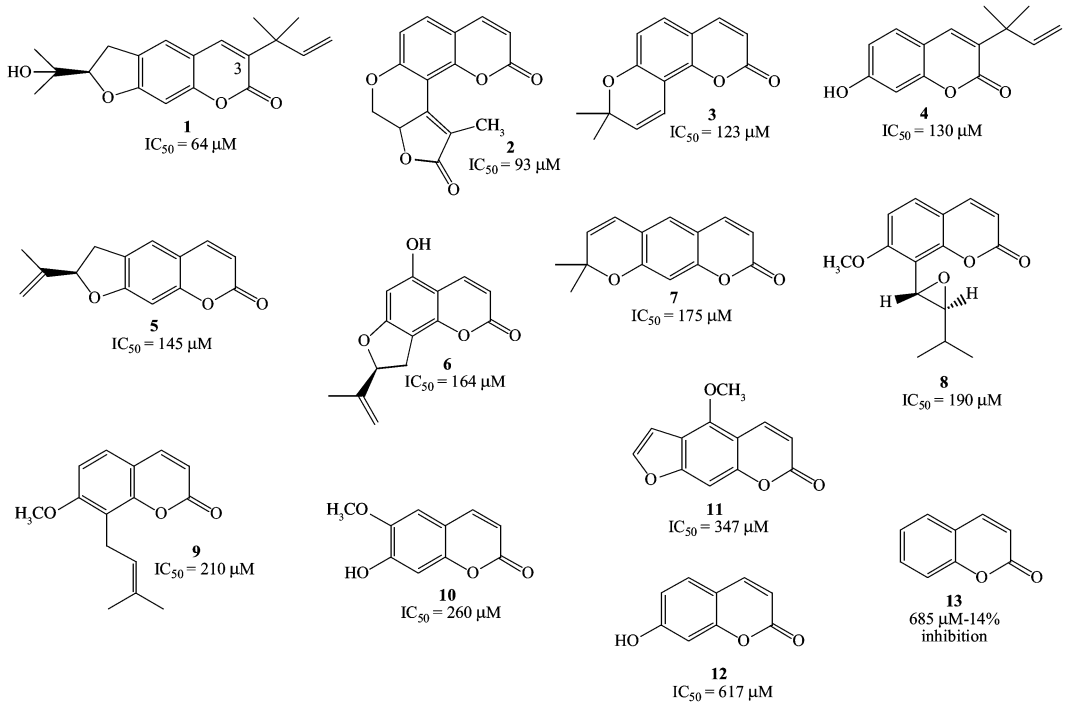


**Fig. 1** Coumarins assayed in the inhibition of glycolytic enzyme *T. cruzi*-GAPDH.

At this moment, coumarins have been the most promising class of substances in natural products with gGAPDH inhibitory activity. Recently, coumarins were isolated from *Vernonia brachycalyx* and showed *in vitro* activity against *Leishmania major* and *Plasmodium falciparum* [16] being another example of the potential antiparasitic activity from this class of substance. Furthermore, coumarins had already showed to be promising lead compounds for structure-based design of anti-HIV compounds. Several inhibitors of the enzyme HIV integrase were structurally based on coumarins [17]. In another approach, a coumarin was the lead compound for the designing of two potent inhibitors of the enzyme HIV protease, which are under clinical trials [18–20].

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