Trypanocidal Flavonoids from *Trixis vauthieri*

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The crude extract of *Trixis vauthieri* (Asteraceae) was active against the trypomastigote forms of *Trypanosoma cruzi*, the protozoan that causes Chagas' disease. Bioassay-guided fractionation of this extract afforded the trypanocidal flavonoids 5,4′-dihydroxy-7-methoxyflavanone (**1**) and 5,4′-dihydroxy-3,6,7-trimethoxyflavone (**2**) besides the inactive flavonoids 3,5,4′-trihydroxy-7-methoxyflavanone (**3**) and 5,4′-dihydroxy-3,6,7,8-tetramethoxy flavone (**4**). The trypanocidal activity of **1** and **2** and the presence of compounds **2** and **4** in *Trixis vauthieri* are reported here for the first time.

There is an urgent need for new drugs active against *Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease (American trypanosomiasis). This illness affects 18 million people in Latin America and is responsible for the death of 45 000 patients every year.[1] The treatment of Chagas' disease relies on two available drugs, nifurtimox and benznidazole, introduced in the 1970s. Although efficient in most cases of the acute phase of the disease, these drugs are almost ineffective in the chronic phase.[2]

Natural transmission of Chagas' disease occurs by contamination with the infective form of the parasite present in the Triatomine insect feces at the site of the insect bite or via neighboring intact mucosa. At present, the Triatomine vectors are under control in most affected areas, so that blood transfusion now causes the majority of new cases of Chagas' disease.[3] In highly endemic areas, it is strongly recommended to use chemoprophylatic measures such as the addition of gentian violet to clear trypomastigotes from blood banked for transfusion.[4] However, despite being effective, gentian violet is not completely accepted by clinicians or patients because of undesirable effects such as coloring the skin and possible mutagenic effects.[5] Thus, the development of new compounds to replace this dye remains a highly desirable goal. However, 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 for other parasitic diseases. During a screening of several natural products, Chiari and co-workers showed that some unusual isoflavonoids that carry a *para*-quinone ring present trypanocidal activity.[6] Previous studies indicate that naphthoquinones are the most promising class of trypanocidal natural products.[7,8] However, other structural groups may also be useful, as exemplified by the trypanocidal activity of the diterpene *ent*-kaur-16-en-19-oic acid.[9]

In an effort to discover new leads for the development of drugs to prevent or treat Chagas' disease, 54 Asteraceae species growing in the Brazilian savannas ("Cerrados") were screened in vitro in an assay with the trypomastigote form of *T. cruzi*.[10] This screening disclosed the trypanocidal activity of five species, including *Trixis vauthieri* L (Asteraceae). Previous work with this plant revealed the presence of several sesquiterpenes and flavonoids in its ligroin extract.[11] However, no information regarding the trypanocidal activity of these compounds could be found in the literature. In view of these observations we decided to investigate an extract of *Trixis vauthieri* with the aim to isolate and identify its trypanocidal component(s).

Table 1. *In Vitro* Activity of *Trixis vauthieri* Crude Extract, Isolated Flavonoids, and the Control Drug, Gentian Violet, on Trypomastigote Forms of *Trypanosoma cruzi* Present in Blood of Experimentally Infected Mice

compound (μg/mL)	trypanocidal activity (% ± S.D.)[a]
crude extract (1000)	100 ± 0
TVA-EP (1000)	10 ± 7
TVA-EE (500)	93 ± 7
TVA-MW (1000)	7 ± 5
1 (500)	100
2 (500)	86 ± 13
3 (1000)	0
4 (1000)	0
gentian violet (31)	50 ± 8

[a] The experiments were run in duplicate and repeated twice. The activity is expressed as percent reduction of the parasite number in infected murine blood ± standard deviation (S.D.).

A crude CH_2Cl_2−MeOH (1:1) extract from the leaves of *Trixis vauthieri* L. was tested in vitro against *T. cruzi* trypomastigotes present in murine blood (see Experimental Section). At 1000 μg/mL, this extract was able to kill 100% of the parasite. Gentian violet, the drug of choice to disinfect blood for transfusion, is used at 250 μg/mL. Using the bioassay with trypomastigotes to guide the isolation process, the active extract was subjected to a solvent partition protocol (see Experimental Section) to generate three fractions of different polarity: TVA-EP, TVA-EE, and TVA-MW. Only the ethyl ether fraction (TVA-EE) was active, causing the lysis of all parasites at 500 μg/mL. Successive chromatographic fractionation of TVA-EE using different stationary phases yielded four compounds (**1**−**4**) of high purity (>99%). The results of the in vitro trypomastigote bioassay with these compounds are summarized in Table 1. Compounds **1** and **2** were active at 500 μg/mL, while **3** and **4** proved to be inactive in the bioassay at the highest concentration used (1000 μg/mL).

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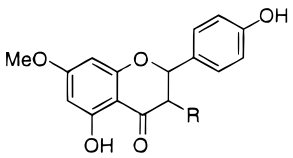
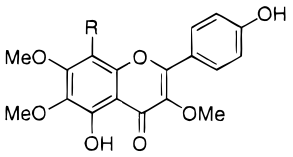


1: R = H (sakuranetin)

3: R = OH (7-Methoxy-aromadendrin)



2: R = H (Penduletin)

4: R = OMe (Calycopterin)

The flavonoid nature of compounds **1**−**4** was inferred from their behavior on TLC and their UV spectra with shift reagents.[12] Analysis of their spectral data, especially ^{1}H- and ^{13}C-NMR spectra, and DEPT, ^{1}H−^{1}H COSY and ^{1}H−^{13}C COSY NMR experiments, allowed us to elucidate their structures as 5,4′-dihydroxy-7-methoxyflavanone (sakuranetin, **1**) and 5,4′-dihydroxy-3,6,7-trimethoxy flavone (penduletin, **2**), 3,5,4′-trihydroxy-7-methoxyflavanone (7-methoxyaromadendrin, **3**) and 5,4′-dihydroxy-3,6,7,8-tetramethoxyflavone (calycopterin, **4**). The physical and spectral data of the isolated compounds are in complete agreement with those published in the literature.[13−16]

The flavonoids sakuranetin (**1**) and 7-methoxyaromadendrin (**3**) were reported to be present in this[11] and other plant species.[17] On the other hand, **2** and **4**, known to occur in other plants,[18] are reported here for the first time as constituents of the genus *Trixis*. Several biological activities are attributed to these compounds: sakuranetin (**1**) presents antifungal,[19] antifeeding,[20] and antioxidant effects[20] and inhibits the metabolism of the carcinogen benz(a)pyrene;[21] penduletin (**2**) is reported to present antifungal activity;[22] 7-methoxyaromadendrin (**3**) is described as a bactericide,[23] fungicide and larvicide[24] and to have hepatoprotective effects;[25] calycopterin (**4**) displays anthelmintic activity[26] and is toxic for fishes.[27] To the best of our knowledge, the trypanocidal activity of simple flavonoid compounds (**1** and **2**) is disclosed here for the first time.

The lack of defense mechanisms against oxidative stress in *T. cruzi* makes this parasite susceptible to drugs that are able to generate reactive oxygen species.[28] This is certainly the mode of action by which some quinones exhibit their trypanocidal activity.[8] Although flavonoids are well recognized for their antioxidant activity, they can also, under certain conditions, generate reactive oxygen species and suffer redox-cycling.[29] This behavior could explain the trypanocidal activity of flavonoids **1** and **2**, a hypothesis that deserves further evaluation.

In conclusion, the present investigation adds simple flavonoids to the list of potentially useful classes of very needed trypanocidal compounds. More extensive and detailed studies on the trypanocidal activity of the many known natural and synthetic flavonoids would provide an understanding of the structural requirements for trypanocidal activity and serve as a basis for the development of more efficient drugs for the treatment or prevention of Chagas' disease.

Experimental Section

General Experimental Procedures. Melting points were determined on a Mettler FP-5 and are uncorrected. IR spectra were obtained on a Mattson-Galaxy series FTIR 3000. UV spectra were recorded on a Beckman DU 640. ^{1}H- and ^{13}C-NMR spectra were measured on a Brucker AC-200 spectrometer, using TMS as internal standard. Mass spectra were obtained on a Hewlett-Packard 5989. TLC were run on precoated Si gel plates, using CH_2Cl_2 as a developing solvent system and vanillin−H_2SO_4 as spray reagent. Analytical and semi-preparative HPLC were run on a Shimadzu chromatograph equipped with a LC-6AD pump and a UV detector set at 254 nm. Analytical (4.6 × 250 mm) and semi-prep (20 × 250 mm) columns (Shimpak prep-ODS kit) were used throughout this work.

Plant Material. Leaves of *T. vauthieri* L. (Asteraceae) were collected in Belo Horizonte, Minas Gerais State, Brazil, in 1993. Plant identification was performed by Dr. T. M. S. Grandi from "Fundação Zoo-Botânica de Belo Horizonte", and a voucher specimen (BHCB 19072) is deposited at the Federal University of Minas Gerais Herbarium.

Bioactivity-guided Fractionation. Fresh leaves of *T. vauthieri* (3.5 kg) were crushed in the presence of CH_2Cl_2−MeOH (1:1) (5 L) and mechanically stirred for 24 h at room temperature (28−30 °C). The mixture was filtered and the residue extracted two more times with 10 L of the solvent. The filtrates were combined and the solvents removed under vacuum in a rotary evaporator to yield 176 g of crude extract. This extract was suspended in 2 L of MeOH−H_2O (9:1) and extracted three times with 1 L of petroleum ether (bp 45−60 °C) to afford the low polarity TVA-PE fraction (99 g). The aqueous alcoholic phase was then made 1:1 by the addition of H_2O (800 mL) and extracted with ethyl ether (3 × 1 L) to yield an organic fraction (TVA-EE, 65 g) and an aqueous alcoholic fraction (TVA-MW, 11 g). TVA-EE (18 g) was chromatographed on a Si gel column (200−400 mesh, 49 × 460 mm) using $CHCl_3$−MeOH mixtures of increasing polarity (5% steps) to elute 230 fractions of 30 mL each. According to their behavior on TLC, these fractions were pooled in 28 groups. Two groups showed activity and were fractionated using low-pressure liquid column chromatography on Lobar (Merck) size B columns filled with Si gel and eluted with $CHCl_3$−MeOH (95:5 and 90:10). Final purifications were performed by reversed-phase semi-preparative HPLC (MeOH−H_2O 7:3, UV 254 nm) followed by gel filtration on Sephadex LH-20 (20 × 500 mm, CH_2Cl_2−MeOH 1:1). In this way, the compounds **I** (125 mg), **II** (11 mg), **III** (26 mg), and **IV** (8 mg) were obtained.

Bioassay with Trypomastigote Form of *T. cruzi* in Infected Murine Blood. Blood infected with trypomastigotes of *T. cruzi* Y strain was obtained by retro-orbital bleeding of experimentally infected male Swiss albino mice and diluted with normal murine blood to 2 × 10^6 trypomastigotes/mL. Stock solutions of the extracts, fractions, and pure compounds were prepared by dissolving 2.0 or 1.0 mg in 100 μL of dimethylsulfoxide (DMSO). Of these solutions 5 μL were added to

195 μL of infected blood in a 96-well microtiter plate, attaining final concentrations of 1000 μg/mL or 500 μg/mL, respectively. Negative and positive controls containing either 2.5% DMSO or gentian violet at its IC_{50} (31 μg/mL) were run in parallel. After 24 h at 4 °C the number of parasites was determined by placing 5 μL of the tested blood on a glass plate, covering with a 22 × 22 mm coverslip, and counting the parasites in 50 fields at 400× magnification. Each experiment was performed in duplicate and repeated twice. The results were expressed as mean ± standard deviation (S.D.) of the percentage reduction of parasitemia compared to the control with DMSO. DMSO at 2.5% did not interfere with the parasite survival.

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