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Antiprotozoal and Cytotoxicity Assays of the Isolates of *Tephrosia tinctoria*

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The *in vitro* antiprotozoal assays of the flavonoids isolated from the roots of *Tephrosia tinctoria*, were studied for their potential to inhibit parasitic protozoa *Trypanosoma*, *Leishmania* and *Plasmodium*. The cytotoxicity of the flavonoid derivatives was performed using Cell line L-6 (rat skeletal muscle myoblasts). Of the three flavonoids studied, 2-hydroxy tephrosin and tephrinone exhibited moderate activity against both *Trypanosoma brucei rhodensiense* and *T. cruzi*, mild activity against *Leishmania donovani* and no activity against *Plasmodium falciparum*.

Key Words: *Tephrosia tinctoria*, Prenylated flavonoids, Cytotoxicity assay, Antiprotozoal activity.

INTRODUCTION

Tephrosia tinctoria is a tropical herb or an undershrub growing up to a height of 1 metre recorded in the hill areas of Chittoor, Vijayanagaram and Visakhapatnam districts[1]. Flavonoids isolated from varied plant sources have shown to exhibit antileishmanial activity against the promastigote forms of *L. major, L. donovani*, *L. infantum* and *L. enrietti*[2]. Prenylated flavonoids like 5,7,4'-trihydroxyflavan exhibited toxic activity on amastigotes of *L. amazonensis*[3]. 7-Hydroxy-3'-4'- methylene-dioxyflavan isolated from the fruits of *Terminalia bellerica* showed significant activity against *Plasmodium falciparum*[4].

In view of the above, the prenylated flavonoids 2-hydroxy tephrosin, tephrinone and rotenone isolated from the species *T. tinctoria*, were studied for their potential to inhibit parasitic protozoa belonging to the genera *Trypanosoma*, *Leishmania* and *Plasmodium* using *in vitro* antiprotozoal assays.

EXPERIMENTAL

The roots (2 kg) of *T. tinctoria* were collected, air-dried and coarsely powdered in a Willey mill. The root material was extracted with chloroform and methanol successively and subsequently concentrated under reduced pressure. The root extracts were later subjected to column chromatography which resulted in three prenylated flavonoids namely 2-hydroxy tephrosin, tephrinone and rotenone apart from sterols.

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The *in vitro* antiprotozoal assays of the three prenylated flavonoids for *T. brucei rhodensiense, T. cruzi* and *Leishmania donovani* were performed according to the established procedures[5,6] while the antiplasmodium activity was determined as described by Matile and Pink[7].

***In vitro* assay for *Traypanosoma brucei rhodesiense*:** *T. brucei rhodesiense* STIB 900 strain and the standard drug melarsoprol (Arsobal) were used for the assay. Minimum essential medium (50 µL) supplemented with 2-mercaptoethanol and 15 % heat-activated horse serum was added to each well of a 96 well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 µg/mL and then added to the wells. Then 10^4 bloodstream forms of *Trypanosoma b. rhodesiense* STIB 900 in 50 µL were added to each well and the plate incubated at 37 ºC under a 5 % CO_2 atmosphere for 72 h. 10 µL of resazurin solution[8,9] (12.5 mg resazurin dissolved in 100 mL distilled water) was then added to each well and incubation continued for further 2-4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programm Softmax Pro (Molecular Devices) which calculated IC_{50} values.

***In vitro* assay for *Trypanosoma cruzi*:** Rat skeletal myoblasts (L-6 cells) were seeded in 96 well microtiter plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10 % FBS and 2 mM L-gultamine. After 24 h the medium was removed and replaced by 100 µL per well containing 5000 trypomostigote forms of *T. cruzi* (Tulhahuen strain C-2, C-4 containing the β-galactosidase (Lac Z) gene). After 48 h, the medium was removed from the wells and replaced by 100 µL fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90 to 0.123 µg/mL. Each drug was tested in duplicate. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 µL) was added to all wells. A colour reaction developed within 2-6 h and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Benznidazole was the standard drug used.

***In vitro* assay for *Leishmania donovani* (Axenic amastigote assay):** 50 µL of culture medium, a 1:1 mixture of SM medium[10] and SDM-79 medium[11] at pH 5.4 supplemented with 10 % heat-inactivated FBS, was added to each well of a 96-well microtiter plated (Costar, USA). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041 µg/mL. Then 10^5 axenically grown *Leishmania donavani* amastigotes (strain MHOM-ET/67/L82) in 50 µL medium were added to each well and the plate incubated at 37 ºC under a 5 % CO_2 atmosphere for 72 h. 10 µL of resazurin solution[9] (12.5 mg resazurin dissolved in 100 mL distilled water) were added to each well and incubation continued for a further 2-4 h. The plate was

then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Miltefosin (Zentaris GmbH, Germany) was used as a positive reference.

***In vitro* assay for *Plasmodium falciparum*:** Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [3*H*]-hypoxanthine incorporation assay was used[7]. Briefly, infected human red blood cells in RPMI 1640 medium with 5 % Albumax II were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 ºC in a reduced oxygen atmosphere, 0.5 µCi 3*H*-hypoxanthine was added to each well. Cultures were incubated at 37 ºC in a reduced oxygen atmosphere, 0.5 µCi 3*H*-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate TM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated. The IC_{50} values are the means of four values of two independent assays carried out in duplicate.

Cytotoxicity assay: The cytotoxicity assay of the tested flavonoid derivatives was done following the method of Page *et al.*[12] with the modification of Ahmed *et al.*[13]. Cell line L-6 (rat skeletal muscle myoblasts) were seeded in 96-well Costar microtiter plates at 2 × 103 cells/100 mL, 50 mL per well in MEM supplemented with 10 % heat inactivated FBS. A three-fold serial dilution ranging from 90 to 0.13 mg/mL of compounds in test medium was added. Plates with a final volume of 100 mL per well were incubated at 37 ºC for 72 h in a humidified incubator containing 5 % CO_2. Resazurin was added as viability indicator according to Ahmed *et al.*[13]. After an additional 2 h incubation, the plate was measured with a fluorescence scanner using as excitation wavelength of 536 nm and an emission wavelength of 588 nm (Spectra-Max GeminiXS, Molecular Devices). Podophyllotoxin (Polysciences INC., USA) was used as a positive reference.

RESULTS AND DISCUSSION

Flavonoid 2-hydroxy tephrosin exhibited good activity against *T. brucei, T. cruzi* and *L. donovani* with IC_{50} of 3.7, 3.35 and 17.12 µg/mL and displayed high toxicity towards L-6 cells (IC_{50} of 17.2 µg/mL) (Table-1). It showed considerable activity against *Trypanosoma brucei rhodensiense* (strain STIB 900, stage trypomastigotes) with IC_{50} 3.7 µg/mL and with *T. cruzi* (strain Tulahuen C4, stage trypomastigotes) 3.35 µg/mL.

TABLE-1
ANTIPROTOZOAL ACTIVITY OF THE COMPOUNDS ISOLATED *T. tinctoria*

Compounds (µg/mL)	IC_{50} values (µg/mL)				
	T. b. rhod	*T. cruzi*	*L. don*. Axen	*P. falc. K1*	L-6 Cells
2-Hydroxy tephrosin	3.7	3.35	17.2	>5	17.126
Tephrinone	33	5.2	16.6	>5	33.478
Rotenone	78.5	>30	>30	>5	>90
Standards	0.0010	0.1830	0.2300	0.0916	0.0060

Standards used: For *T.b. rhodesiense*: melarsoprol, *T. cruzi*: benznidazole, *L. donovani: miltefosine, P. falciparum*: chloroquine and L-6 cells: podophyllotoxin.

Tephrinone exhibited moderate activity against both *Trypanosoma brucei rhodensiense* and *T. cruzi* of IC_{50} 33 and 5.2 µg/mL. All three flavonoids showed low activity against *Leishmania donovani* (strain MHOM-ET-67, stage amastigotes) with IC_{50} of 17.2, 16.6 and > 30 µg/mL, respectively (Table-1).

Rotenone exhibited no activity against any of the parasitic protozoa used in this study. All compounds exhibited dismal activity against *Plasmodium falciparum* (strain K1 and NF54, stages IEF). Cytotoxicity studies towards L-6 cells was showed by 2-hydroxy tephrosin, tephrinone and rotenone were 17.126, 33.478 and > 90, respectively. The *in vitro* parasitic assays of the above compounds were much lesser than observed for the standard drugs melarsoprol, benznidazole, miltefosine, chloroquine/artemisinin and podophyllotoxin.

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