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Cytotoxicity and antiprotozoal activity of flavonoids from three *Tephrosia* species

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Abstract

In the present study we analyzed the cytotoxicity on RAW and HT-29 cell lines by MTT assay and antiprotozoal activity on *Trypanosoma, Leishmania* and *Plasmodium* parasites of different flavonoids isolated from three species of *Tephrosia, T. calophylla, T. pulcherrima* and *T. pumila*. Pulcherrimin exhibited toxicity towards RAW and HT-29 cell lines at low concentrations. Candidone, millettone and pongachin showed toxicity towards HT-29 cell lines at low concentrations. The isolated compounds were characterized by spectral data, including 2D NMR, ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY experiments.

Key words: cytotoxicity, antiprotozoal, flavonoids, *T. calophylla, T. pulcherrima,* and *T. pumila.*

1. Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in plant foods. Over 6000 flavonoids, have been identified in plant sources. Flavonoids possess a remarkable spectrum of biochemical and pharmacological activities [1] and affect cell functions such as growth and apoptosis. Flavonoids inhibit and protect against various stages of the cancer process and are associated with a reduced incidence of coronary heart disease [2]. In addition, the flavonoids exhibit a wide range of biological activities, including anticarcinogenic [3-5], antiallergic [6,7], anti-inflammatory, antioxidant [8] and antiviral activities [9,10].

Flavonoids isolated from varied plant sources have shown to exhibit antileishmanial activity against the promastigote forms of *Leishmania major, Leishmania donovani, Leishmania infantum* and *Leishmania enrietti* [11]. Prenylated flavonoids like 5, 7, 4'-trihydroxyflavan exhibited toxic activity on

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amastigotes of *L. amazonensis* [12]. 7-hydroxy-3' 4'-methylenedioxyflavan isolated from the fruits of *Terminalia bellerica* showed significant activity against *Plasmodium falciparum* [13]. In addition, six isoflavones, formononetin, prunetin, biochanin A, calycosin, genistein, pratensein isolated from the stems and leaves of *Andira inermis* showed considerable activity on malarial parasite *P. falciparum* with IC_{50} of 2.0, 4.2 µg/ml for genistein and calycosin [14].

2. Materials and Methods

2.1 Materials

RAW (mouse macrophage) and HT-29 (Colon cancer) cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. DMEM (Dulbeccos Modified Eagles Medium), MTT [3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Trypsin and EDTA (Ethylene Diamine Tetra Aceticacid)were purchased from Sigma Chemicals Co (st.Louis, MO), Fetal bovine serum (FBS) was procured from Arrow labs, 96 well flat bottom tissue culture plates were purchased from Tarson. Dimethyl sulfoxide (DMSO) is used as control. The isolates of *T. calophylla* spinoflavanone B, 7-methyl glabranin, tephcalostan, millettone, tephcalostan C, the compounds of *T. pulcherrima* pulcherrimin, pongachin, candidone and the isolates of *T. pumila* 2-methoxymacckianin and dehydrovillosin were tested for cytotoxicity by MTT assay using RAW and HT-29 cell lines and antiprotozoal activity was studied for their potential to inhibit parasitic protozoa belonging to the genera *Trypanosoma, Leishmania* and *Plasmodium* using *in vitro* antiprotozoal assays.

2.2 Plant Material

The root parts of *T. calophylla* (Bedd), *T. pulcherrima* (Drumm) *and T. pumila* (Pers) were collected from Khailasa hills, Visakhapatnam, India, during February 2006. The sample was authenticated by Dr. M. Venkayya, Taxonomist, Department of Botany, College of Science and Technology, Andhra University. Voucher specimens (SG/TCR/06/113), (SG/TPR/06/114) and (SG/SGPR/06/115) respectively have been deposited at the Herbarium, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India.

2.3 Extraction and Isolation

The plant materials were air dried and finely powered in willy mill and exhaustively extracted with chloroform. The extracts so obtained, were then concentrated and *vacuum* to get the corresponding residues. The residues on column chromatography over silica gel (Acme 100-200 mesh) and eluted with solvents of increasing polarity of petroleum ether, chloroform and methanol.

The root parts of *T. calophylla* afforded spinoflavanone B, 7-methyl glabranin, tephcalostan, tephcalostan C and millettone, the root parts of *T. pulcherrima* afforded 7-methyl glabranin, pulcherrimin, pongachin, and candidone, the root parts of *T. pumila* afforded 2-methoxy maackianin, pumilanol and dehydrovillosin.

2.4 Methods

2.4.1 Cytotoxicity assay

Maintenance of cell lines: RAW, HT-29 cell line was grown as adherent in DMEM media supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine, and culture was maintained in a humidified atmosphere with 5% carbon dioxide.

Preparation of samples for cytotoxicity: Stock solution of 10mg/ml was prepared in DMSO. From the above stock solutions various dilutions were made with sterile water to get required concentration.

Table 1: Cytotoxicity of the isolates of *T. calophylla* and *T. pulcherrima* on RAW and HT-29 cell lines

Compound	concen (µg/ml)	RAW		HT-29	
		inhibition	IC_{50}	inhibition	IC_{50}
Candidone	10.000	16.4649376	30.1 µg/ml	48.63045	10.96 µg/ml
	25.000	17.0172911		66.8205922	
	50.000	84.8530259		76.7485096	
	100.000	97.3371758		95.4393337	
Millettone	10.000	21.1565802	36.03 µg/ml	40.6322034	16 µg/ml
	25.000	38.8126801		59.6064874	
	50.000	66.9663785		70.0643094	
	100.000	65.2257445		84.0103448	
7-Methyl- glabranin	10.000	0.80979827	177 µg/ml	15.4568673	35.48 µg/ml
	25.000	9.4543708		39.033723	
	50.000	27.0720461		60.7013637	
	100.000	41.0835735		76.9082603	
Pulcherrimin	10.000	-7.5389049	19.95 µg/ml	48.6401909	10.71 µg/ml
	25.000	98.6897214		63.1112605	
	50.000	97.6195965		74.4087473	
	100.000	98.8607109		85.2795831	
Spinoflavanone B	10.000	14.9779059	37.15 µg/ml	24.8879992	58.8 µg/ml
	25.000	25.5292988		26.1718488	
	50.000	67.0566763		45.7403273	
	100.000	77.4121037		63.3177674	
Pongachin	10.000	-16.90682	117 µg/ml	48.63045	12.02 µg/ml
	25.000	1.30259366		61.0150205	
	50.000	19.2122959		70.5338204	
	100.000	50.3227666		90.1792519	
Tephcalostan	10.000	8.14226925	64.5 µg/ml	27.0368401	24.54 µg/ml
	25.000	28.6831276		49.8519579	
	50.000	50.8759553		74.2938048	
	100.000	54.3601803		77.3358854	
Control (DMSO)		1.738		1.710	

Table 2. Antiprotozoal activity of the isolates of *T. calophylla*, *T. pulcherrima*

Compound	*T.b.rhod.*	*T.cruzi*	*L.don. axen.*	*P. falc. K1*	*Cytotox. L6*
Melarsoprol	0.004				
Benznidazole		0.405			
Miltefosine			0.173		
Chloroquine				0.079	
Podophyllotoxin					0.009
Spinoflavanone B	12.1	16.49	3.09	0.285	24.3
7-Methyl glabranin	29.8	8.83	2.51	3.73	19.9
Tephcalostan	63.4	>30	>90	>5	>90
Millettone	4.84	22.8	9.81	3.72	20.1
Tephcalostan C	67.4	>30	59.1	>5	>90
Pulcherrimin	1.29	4.1	3.13	1.49	6.1
Pongachin	6.3	12.41	7.05	1.32	23.5
Candidone	6.16	6.71	0.697	2.11	7.5
2-Methoxymaackianin	39.1	>30	>90	>5	>90
Pumilanol	3.7	3.35	17.2	>5	17.126
Dehydro villosin	27.5	>30	>90	0.638	28.5

MTT assay

1. RAW, HT-29 cell lines were seeded at a density of $1x10^4$ cells (cell number was determined by Trypan blue exclusion dye method) per each well in 100µl of DMEM supplemented with 10% FBS
2. 12 hrs after seeding, above media was replaced with fresh DMEM supplemented with 10% FBS then 20 µl sample from above stock solutions were added to each well in triplicates which gives final concentration of 100, 50, 25, 10 µg/well.
3. The above cells were incubated for 48hours at 37°C with 5% carbon dioxide.
4. After 48 hours incubation the above media was replaced with 100 µl of fresh DMEM with out FBS and to this 10 µl of MTT (5 mg dissolved in 1ml of Phosphate Buffer Saline) was added and incubated for three hours at 37°C with 5% carbon dioxide.
5. After 3 hours incubation the above media was removed with multi channel pipette, then 200 µl of DMSO was added to each well and the incubated at 37°C for 15minutes.
6. Finally the plate was read at 570 nm using spectrophotometer (Spectra Max, Molecular devices).

2.5 Antiprotozoal assay

2.5.1 In vitro assay for Trypanosoma 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 [15] with 2-mercapto-ethanol 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°C under a 5% CO_2 atmosphere for 72 hours. 10 µL of resazurin solution [16,17] (12.5 mg resazurin dissolved in 100mL distilled water) was then added to each well and incubation continued for a further 2-4 hours. 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 [18]. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic program Softmax Pro (Molecular Devices) which calculated IC_{50} values.

2.5.2 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-glutamine. After 24 hours 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) [17]. Forty-eight hours later 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 µg/mL to 0.123 µg/mL. Each drug was tested in duplicate. After 96 hours 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 hours 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.

2.5.3 In vitro assay for Leishmania donovani (axenic amastigote assay):

50 µL of culture medium, a 1:1 mixture of SM medium [19] and SDM-79 medium [20] 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 37ºC under a 5% CO_2 atmosphere for 72 hours. 10 µL of resazurin solution [17] (12.5 mg resazurin dissolved in 100mL distilled water) were added to each well and incubation continued for a further 2-4 hours. 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 [18]. 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.

2.5.4 In vitro assay for Plasmodium falciparum

Antiplasmodial activity was determined using the KI strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay was used [21]. Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax II were exposed to serial drug dilutions in micro titer

plates. After 48hours of incubation at 37ºC in a reduced oxygen atmosphere, 0.5 µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated at 37ºC in a reduced oxygen atmosphere, 0.5 µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 hours before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Beta plate 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.

2.5.5 Cytotoxicity assay

The cytotoxicity assay was determined by using the method of Page [22] with the modification of Ahmed [23]. Cell line L-6 (rat skeletal muscle myoblasts) were seeded in 96-well Costar micro titer plates at 2x103 cells/100 ml, 50 ml per well in MEM supplemented with 10% heat inactivated Fetal Bovine Serum (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 37ºC for 72 hours in a humidified incubator containing 5% CO_2. Resazurin was added as viability indicator according to Ahmed [23]. After additional 2hours incubation, the plate was measured with a fluorescence scanner using as excitation wavelength of 536 nm and an emission wavelength of 588 nm (Spectrs-Max Gemini XS, Molecular Devices). Podophyllotoxin (Polysciences INC., USA) was used as a positive reference.

3. Results and Discussion

The compound pulcherrimin showed high toxicity towards RAW and HT-29 cell lines at 19.95 and 10.71 µg/ml concentrations. The compounds candidone, millettone and spinoflavanone B exhibited toxicity at 30.1, 36.03 and 37.15 µg/ml, against RAW cell lines but tephcalostan displayed moderate toxicity at 64.5 µg/ml concentrations. When tested on RAW cell lines the compounds 7-methyl glabranin and pongachin showed less toxicity at 177 and 117 µg/ml. Whereas the compounds candidone, pongachin and millettone showed high toxicity towards HT-29 cell lines at low concentrations 10.96, 12.02 and 16 µg/ml like pulcherrimin. Further the compounds tephcalostan, 7-methyl glabranin and spinoflavanone B showed toxicity at concentrations 24.54, 35.48 and 58.8 µg/ml. However the toxicity is lower when compared to pulcherrimin and candidone.

The compounds spinoflavanone B and dehydrovillosin were highly active towards parasite *P. falciparum*, but showed moderate activity against *T. b. rhodesiense*, *T. cruzi* and *L. donavani* parasites. The compound candidone also displayed significant activity towards *L. donovani* parasite, but less activity against *T. b. rhodesiense*, *T. cruzi* and *P. falciparum*. The compound pumilanol showed considerable activity towards *T. b. rhodesiense* and *T. cruzi*, but exhibited moderate activity against the parasite *P. falciparum*. The compounds pulcherrimin and pongachin exhibited significant activity towards all the tested parasites. The compound 7-methyl glabranin showed activity towards parasites *T. cruzi*, *L. donovani* and *P. falciparum* and mild activity against the parasite *T. b. rhodesiensen*. The compound millettone showed activity towards the parasites *T. b. rhodesiensen*, *L. donovani* and *P. falciparum*, but moderate activity against parasite *T. cruzi*. The compounds tephcalostan, tephcalostan C and 2-methoxymaackianin have not displayed any activity towards the tested parasites. In order to compare the effect of the compounds on parasites with toxicity towards rat skeletal myoblasts (L-6 cells), the cytotoxic activities of the isolated

compounds against the L-6 cell lines were evaluated and the compounds pulcherrimin and candidone exhibited high toxicity at low concentrations at 6.1 and 7.5 µg/ml respectively, but the isolates spinoflavanone B, 7-methyl glabranin, millettone, pongachin, pumilanol, dehydrovillosin displayed IC_{50} of 24.3, 19.9, 20.1, 23.5, 17.13, 28.5 respectively. From the above observations the compounds that contain prenyl side chain exhibited the antiprotozoal activity and no activity was observed to the compounds without prenyl side chain. So these findings suggest that prenyaltion enhances the antiprotozoal activity.

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