Anti-trypanosomal Potential of Methanolic Extract of *Calotropis gigantea* Leaves against *Trypanosma evansi* and its Cytotoxicity

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Abstract

In an attempt to find anti-trypanosomal extract/compound(s) from medicinal plant for the treatment of trypanosomosis, *Calotropis gigantea* leaves were extracted with methanol. The obtained extract was screened at concentrations (250-1000 µg ml^{-1}) against *Trypanosoma evansi* for an *in vitro* trypanocidal activity on Vero cell line (SIGMA) maintained in Dubecco's Modified Eagle Medium (DMEM). 500,000 cells ml^{-1} $well^{-1}$ was seeded in flat bottom ELISA plates supplemented with 20-40% fetal calf serum (FCS), and incubated at appropriate conditions for more than 12 h. *In vitro* cytotoxic effects of methanolic plant extract (MPE) of *C. gigantea* was done on same medium, without FCS, at concentrations (1.56-100 µg ml^{-1}), and incubated for 72 h. Adhered cells in the plate were stained with a drop of crystal violet in phosphate buffered solution. During *in vitro* testing, at concentrations of 250-500 µg ml^{-1}, there was immobilization, reduction of average mean trypanosomes count (40.00±0.0 to 13.33±0.67), and complete killing of trypanosomes at 750 µg ml^{-1} in 9 h of incubation, which was equivalent to diminazine aceturate (Berenil) 50 µg ml^{-1} (standard drug) at 4 h. *In vitro* cytotoxic effects such as distortion, swelling, sloughing and death of Vero cells from the bottom of affected wells were observed. MPE of *C. gigantea* and diminazine aceturate were cytotoxic to Vero cells in all concentrations except at 1.56 and 6.25-1.56 µg ml^{-1}, respectively. MPE of *C. gigantea* demonstrated presence of trypanocidal compounds.

1. Introduction

Trypanosomosis is a disease caused by blood protozoan parasites (*Trypanosoma* sp.) that affect both animals and humans (Soulsby, 1982). Reports of resistance to currently used trypanocides are on the increase in endemic regions of the world where the disease thrives (Freiburghaus et al., 1996a; Denise and Barret, 2001; Wurochekke and Nok, 2004; and Shaba et al., 2006). Ethno-pharmacology and ethno-medicine of extracts and isolated compounds of medicinal plants against trypanosomes have been reported by Lopes et al., 1999; Nok and Nock, 2002; Shaba et al., 2006; Shaba et al., 2007; and Shaba et al., 2009a. In folk medicine, different parts of *C. gigantea* have been used in treatment of diseases such as fever, diarrhea, swellings, etc. (Nadkarni, 1954). Also its pharmacological activity as antimicrobial (Samy et al., 1998) and anti-diarrheal (Chitmen et al., 2004) has been documented. Pharmacologically active agents such as 20-epoxy-cardenolides, calotropin, calatin, and calotrogenin have been isolated from leaves, stem, root, and flowers of *C. gigantea* (Thitima and Somyote, 2006). The present study was designed to investigate the possibility of detecting anti-trypanosmal activity from *C. gigantea* leaves extract, which may pave way for anti-trypanosmal compounds that might be a target in drug discovery against trypanosomosis in both animals and humans. Therefore, *C. gigantea* leaves were extracted with methanol and tested against *Trypanosoma evansi* to confirm its trypanocidal activity status.

2. Materials and Methods

2.1. Chemicals

Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol, acetic acid and ethyl acetate) for extraction of plant materials and development/analysis of TLC plates, vanillin for spray, and iodine for detection of bioactive constituents were used which were purchased from

E. Merck, India.

2.2. Plant material

C. gigantea leaves at matured stages were collected in the month of September from within the Indian Veterinary Research Institute (IVRI) campus at Izatnagar in Bareily district of Uttar Pradesh state in India, and subsequently were used.

2.3. Preparation of extract

The extraction was carried out according to the method of Stahl (1969). 20 g *C. gigantea* leaves was powdered using laboratory pestle and mortar, and cold extracted with 200 ml of ethanol (analytical grade). Residues obtained were extracted twice in same medium. The filtrates were combined, dried at 37^{O}C and stored at 4^{O}C until used.

2.4. Solvent systems

The following solvent systems were tested to develop the TLC plates according to the method of Stahl (1969).
Chloroform/hexane/acetic acid (50:50:1)
Chloroform/ethyl acetate/acetic acid (50:50:1)
Methanol and chloroform (20: 80)

2.5. TLC plates

Aliquots (0.2 ml) of extract were applied on TLC plates, dried under room temperature and immersed inside the appropriate solvent systems in a glass jar. It was done to detect the presence of bioactive constituents in applied extract. This was also done following the method of Stahl (1969).

2.6. Animals

Swiss albino mice (20-30 g) of either sex were obtained from Animal Research Laboratory Section of IVRI, Izatnagar, maintained in standard environmental conditions and fed on a standard diet prepared by the institute with water *ad libitum*. Usage of mice in the experiment was strictly guided by laid down rules of committee on Ethics and Cruelty to Animals of the institute.

2.7. Test organism

T. evansi was obtained from the Division of Parasitology, IVRI, Izatnagar and was maintained in the laboratory by serial sub-passages in Swiss albino mice. The strain was routinely tested for virulence following the method of Williamson et al. (1982).

2.8. Parasite count

Counting of parasite was carried out following the method of Lumsden et al. (1973). A number of fields (10-15) of each drop of blood or incubated media and parasites in triplicate were counted using glass slides under inverted microscope (400X). An average mean parasite count was taken as number of parasites per field.

2.9. In vitro trypanocidal activity

In vitro trypanocidal activity was carried out with modified method of Oliveira et al. (2004). A Vero cell line (SIGMA) was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20-40% fetal calf serum (FCS), GIBCO USA and antibiotics (100 iu penicillin, 100 µg streptomycin and 40 µg gentamycin) in 96-wells flat bottom microculture plates (NUNC, Denmark). Each well received 100 µl of DMEM containing 5x105 cells ml^{-1}. Plates were incubated at 37^{0}C under 5% CO_2 for 12h. After the formation of confluent monolayer, the medium was discarded and replaced with a fresh one. Finally, a high parasitaemic blood from mouse was diluted with DMEM to obtain 1x10^{6} parasites ml^{-1}. Suspension (100 ml of medium with parasites) was added at the rate of 1: 1 to test MPE of *C. gigantea*, and the plate was incubated under the same conditions mentioned above. The test was repeated at least thrice.
Stock of test MPE of *C. gigantea* leaves was solubilized in 1% dimethylsuphoxide (DMSO) The concentration in the experiment had no deleterious effect by itself on host cells or parasites. 1% DMSO in distilled water was used as control (Young et al., 2000).

2.10. Infectivity assessment

When incubation for anti-trypanosomal activity was completed, contents of wells with reduced and apparently killed trypanosomes from MPE of *C. gigantea* leaves were inoculated (0.1ml mouse^{-1}) into two groups of mice (six group^{-1}) intra-peritoneal, and observed for more than 30 days for parasitemia (Petama, 1964 and Woo, 1971).

2.11. In vitro cytotoxicity test

It was done according to the method of Sidwell and Huffman. (1997). Vero cell line (SIGMA) was grown in DMEM in 96-wells microculture plates without FCS. Each well was seeded with 500,000 cells ml^{-1} and plates were incubated at 37^{O}C with 5% CO_2 for 48 h. After the formation of confluent monolayer, the supernatant was discarded and replaced with fresh medium. Confluent monolayer of Vero cell lines was treated with serial dilutions (1.56-100 µg ml^{-1}) of MPE of *C. gigantea* of test materials in triplicate and incubated for 72 h consecutively under the same conditions described previously. At 24 h interval, plate was observed under inverted microscope for cytotoxic effects as compared to untreated normal cells that served as control. In each case, after 72 h of incubation, the culture media of the incubated Vero cells was discarded. Adhered cells were stained with a drop of crystal violet in phosphate buffered solution. Plate was then incubated for 24 h at 37^{O}C in ordinary incubator. Plates were later observed under inverted microscope for cytotoxic effects.

2.12. Statistical analysis

Results of trypanocidal activity were expressed as mean ± SEM. Statistical analysis was done using Sigma stat (Jandel, USA).

3. Results and Discussion

In the present study methanol was suitable in extraction of bioactive constituents as observed on TLC plates (plates not shown). Solvent system chloroform methanol^{-1} (80:20) was more suitable in development of TLC plates than other solvent systems tested. Presence of bioactive constituents from MPE of *C. gigantea* leaves was detected on TLC plates. These solvents used in extraction and development of TLC are comparable to extraction and development of MPE of *Picrorrhiza korroa* rhizomes on TLC plates (Shaba et al., 2007).

Results of *in vitro* anti-trypanosomal activity of *C. gigantea* leaves are presented in Table 1. Anti-trypanosomal activity varied from immobilization, reduction and killing of trypanosomes at different concentrations used. At a concentration of 250-500 µg ml^{-1}), there was drastic reduction in trypanosome population (40.00±0.0 to 13.33±0.67) and complete killing of trypanosomes occurred at 750 µg ml^{-1} in 9 h of incubation, which is equivalent to 4 h of diminazine aceturate (Berenil, a standard drug, at 50 µg ml^{-1}). Anti-trypanosomal activity is comparable to *in vitro* trypanocidal activity of MPE of medicinal plants used in treatment of trypanosomosis in northern Nigeria at an effective concentration of 8.3 mg ml^{-1} (Wurochekke and Nok, 2004) and MPE of *Picrorrhiza korroa* rhizomes where trypanosomes were completely killed at 500 µg ml^{-1} concentration. (Shaba et al., 2007). An average mean parasite count of 37.67±0.58 is statistically critical value. Average mean parasite count from 37.67±0.58 and below was significant between the treatment groups and negative control ($p \leq 0.05$).

Group of mice inoculated with contents of wells with completely killed trypanosomes survived for more than 30 days, while other group died of parasitemia. Infectivity assessment of anti-trypanosomal activity is comparable to anti-trypanosomal effect of the aqueous extract of *Brassica oleracea* and MPE of *Terminalia belirica* dried fruits where inoculated mice with contents of wells with apparently killed trypanosomes survived (Igweh et al., 2002 and Shaba et al., 2009a).

In vitro cytotoxic effects of MPE of *C. gigantea* leaves and diminazine aceturate at same concentrations on Vero cells depicted different effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in control wells (Table 2). MPE of *C. gigantea a*nd diminazine aceturate were

Table 1: Cytotoxic effect of methanolic extract of *C. gigantea* leaves on Vero cell line as compared to diminazine aceturate (Berenil)

Conc. of test material (µg ml^{-1})	Cytotoxic effects of extract at various time intervals of incubation (%)						
	24 h		48 h		72 h		Control
	C. gigantea	DA	*C. gigantea*	DA	*C. gigantea*	DA	
100.00	100.0	66.6	100.0	100.0	100.0	100.0	0.0
50.00	100.0	33.3	100.0	100.0	100.0	100.0	0.0
25.00	66.6	0.0	100.0	33.3	100.0	66.6	0.0
12.50	66.6	0.0	100.0	0.0	100.0	33.3	0.0
6.25	66.6	0.0	100.0	0.0	100.0	0.0	0.0
3.13	33.3	0.0	66.6	0.0	66.6	0.0	0.0
1.56	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DA=Diminazine aceturate							

Table 2: *In vitro* trypanocidal activity of methanolic extract of *C. gigantea* leaves against *Trypanosma evansi* on Vero cell line

Conc.of test material (µg ml^{-1})	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	40.00± 0.0	38.33± 0.33	36.67± 0.33	35.00± 0.58	33.00± 0.58	30.33± 0.33	27.67± 0.33	24.67± 0.67	21.00± 0.58
500	37.33± 0.33	35.67± 0.33	32.67± 0.33	30.33± 0.33	26.67± 0.67	22.33± 0.33	19.33± 0.33	16.67± 0.33	13.33± 0.67
750	35.67± 0.33	27.67± 0.33	21.67± 0.33	14.00± 0.58	10.33± 0.33	8.67± 0.33	6.667± 0.33	2.333± 0.33	0.0± 0.0
1000	32.67± 0.33	24.00± 0.0	19.67± 0.33	12.33± 0.33	6.33± 0.33	1.33± 0.33	0.0± 0.0	0.0± 0.0	0.0± 0.0
Berenil (50)	22.00± 0.0	9.333± 0.33	1.333± 0.33	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
Negative control	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0

cytotoxic to Vero cells at all concentrations except 1.56 and 6.25-1.56 µg ml^{-1}, respectively. Cytotoxic effects of *C. gigantea* on Vero cell line are comparable to cytotoxic effects of MPE of *Terminalia arjuna* bark on human hepatoma cell line ($HEPG^{2)}$ with distortion and apoptosis of cells (Sarveswaran et al., 2006), and MPE of *Plumbago zeylanica* root bark with distortion, swelling, sloughing and death of cells (Shaba et al., 2006). Mechanism of MPE of *C. gigantea* leaves is difficult to determine at this point, and any of the active principles mentioned above could be responsible for its anti-trypanosomal activity. However; anti-trypanosomal activity may be due to intercalation of test extract with DNA leading to death of trypanosomes, blockage of glycolysis pathway and interference with flagella which temporarily immobilizes trypanosomes (Sepulveda-Boza and Cassels, 1996; and Denise and Barret, 2001).

4. Conclusion

Results of the present study indicate presence of anti-trypanosomal compound(s) from the extract of *C. gigantea* leaves tested. Moderate anti-trypanosomal activity was observed due to the nature of the extract, which was laden with potent toxic contents of the leave sample. Further research is required, e.g. bioassay-guided purification/*in vivo* test, to isolate the compound (s) responsible for its anti-trypanosomal activity. This will, also, reduce the potent toxic contents of the leaves. After all the applicable processes of purification are done, the full status of *C. gigantea* leaves' anti-trypanosomal activity would be realized.

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