Evaluation of Methanolic Extract of *Centella asiatica* Leaves against *Trypanosoma evansi*

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Abstract

This is *in vitro* ethno veterinary medicine screening of *Centella asiatica* leaves dissolved in methanolic solvent at concentrations (250-1000 µg ml^{-1}). The methanolic plant extract (MPE) obtained was tested against *Trypanosoma evansi* for trypanocidal activity. It was done on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with foetal calf serum (FCS) 20-40% at appropriate conditions. *In vitro* cytotoxicity test of *C. asiatica* extract at concentrations (1.56-100 µg ml^{-1}) was done on Vero cells but without FCS. Mice were administered with *C. asiatica* leaves dissolved in either water or vegetable oil at concentration (2000 mg kg^{-1} body weight) to determine its acute toxicity test. *In vitro* trypanocidal activity varied from immobilization, reduction and to the killing of trypanosomes. At 250 µg ml^{-1} of MPE, there was drastic reduction of average mean trypanosomes count as observed. At 750 µg ml^{-1} of the test extract, there was complete killing of trypanosomes at 9 h of incubation. Parasites counts decreased in concentration and time-dependent manner with significant difference (p<0.05). Both MPE of *C. asiatica* and diminazine aceturate, standard drug, were cytotoxic to Vero cells except at concentrations of 1.56 and 1.56-6. 25 µg ml^{-1}. Groups of mice that were subjected to acute toxicity test survived without exhibiting acute toxic effect.

1. Introduction

Trypanosomosis is a disease caused by blood protozoan parasites belonging to the genus *Trypanosoma*. The disease is zoonotic in nature. At present, over 60 million people are living in 36 sub-Saharan countries are at risk of contracting the disease (WHO, 2001). It is estimated that currently about 300-500,000 people are infected with 50,000 deaths annually (WHO, 2001). Estimated losses in agricultural production as a result of the disease are 3 billion pounds annually (Hursey, 2000). Chemotherapy and chemoprophylaxis are the only means of combating the menace of the disease. Chemotherapy of trypanosomosis is faced with problems such as limited choice of trypanocides in the market, high cost, toxicity, and emergence of drug-resistant trypanosome strains that have been reported (Gutteridge, 1985; WHO, 2004). Recent ethno pharmacology and ethno medicine revealed that several medicinal plants possess trypanocidal compounds, which may hold the key for a future potential trypanocides (Wurocheke and Nok, 2004; Shaba et al., 2007; Shaba et al., 2009a; Shaba et al., 2011abc). More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds (Cragg et al., 1997; Soerjatta, 1996). More than 300 year ago, *C. asiatica* leaves has been used in treating skin diseases, wounds and revitalizing of nerves and brain cells (often referred to as brain food in India) (Sakshi, 2010). Ethnopharmarcological activities such as antioxidant and cytotoxicity activities of *C. asiatica* leaves have been documented. Constituents such as phenolic compound and flavonoids have been isolated from *C. asiatica* leaves (Rafael et al., 2009). In this paper, antitrypanosomal activity of *Centella asiatica* is reported.

2. Materials and Methods

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

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bioactive constituents These were purchased from E. Merck, India. *Centella asiatica* leaves at matured stages were collected in September, 2006 and identified at Institute of Himalayan Biosource and Technology, Palampur, India. The extraction was carried out according to the method of Stahl (1969). Twenty gram of *C. asiatica* 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. Aliquots (0.2 ml) of extract were applied on TLC plates, dried under room temperature and immersed inside the solvent systems in glass jar listed in the next subsection. This was done to detect, if any, the presence of bioactive constituents in applied extract. After full development of plates in solvent systems, plates were dried at room temperature. Then, one set of plates were immersed in iodine vapors in a glass jar. Second set of plates were sprayed with Vanillin-sulphuric acid spray. Both media used facilitated the detection of bioactive constituents. This was carried out according to the method of Stahl (1969). The solvent systems tested to develop the TLC plates according to the method of Stahl (1969.) were chloroform/hexane/acetic acid (50: 50: 1), chloroform/ethyl acetate/acetic acid (50: 50: 1), methanol and chloroform (20: 80). Swiss albino mice (20-30 g) of either sex were obtained from Animal Research Laboratory Section of Indian Veterinary Research Institute (IVRI), Izatnagar, India 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 animal of the institute. *T. evansi* was obtained from the Division of Parasitology, IVRI, Izatnagar and was maintained in the laboratory by serial sub-passage in Swiss albino mice. The strain was routinely tested for virulence following the method of Williamson et al. (1982). Estimation of parasite counts was carried out according to Lumsden et al. (1973). A number of fields (10-15) of each drop of a blood or incubated media and parasites in triplicate were counted using glass slides under inverted microscope (400X). An average mean parasites count was taken as number of parasites per field. Extract of *C. asiatica* at concentrations (250-1000 µg ml^{-1}) were added to a high parasitaemic blood from mouse diluted with Alsever solution to obtain a final parasite concentration of 1x10^{6} parasites ml^{-1}. The suspension (100 ml of medium with trypanosomes) was added at rate of 1: 1 to test extract with inactivated bovine serum at 58^{0}C for 1 h, and incubated at 37^{0}C under 5% CO_2 for 5 h (Talakal et al., 1995). Each test was repeated at least thrice and tested *in vitro* for trypanocidal activity. The extract was solubilized in 1% dimethylsulphoxide (DMSO). No deleterious effect of the DMSO was noticed on host cells or parasites with the given concentration (Young et al., 2000). After incubation for antitrypanosomal activity was completed, contents of wells with reduced and apparently killed trypanosomes from MPE of *C. asiatica* were inoculated into mice intraperitoneally and observed for more than 30 days for parasitaemia (lgweh et al., 2002). This was done according to the method of Sidwell and Huffman (1997). Vero cell line (Sigma) was grown in Dulbecco's Modified Eagle Medium (DMEM) in 96-well microculture plates. Each well was seeded with 500,000 cells ml^{-1}. The 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 test extract in triplicate and incubated for 72 h consecutively under the same conditions described previously. At 24 h interval, plates were observed under inverted microscope for cytotoxic effects 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. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. The plate was then incubated for 24 h at 37^{O}C in ordinary incubator. Plates were later observed for cytotoxic effects. Acute toxicity test of *C. asiatica* leaves was based on the results of both *in vitro* trypanocidal activity and *in vitro* cytoxicity effects on Vero cells. This was carried out according to the improved designed methods of acute toxicity test by Madubunyi (1995). In this method, the powdered leave sample of *C. asiatica* was dissolved either in distilled water or vegetable oil at a dose rate of 2000 mg kg^{-1} body weight, and administered to six mice per each group. The volume of dissolved powdered leave sample in the suitable medium was administered orally to the mice according to their body weights. Mice were observed for at least two weeks for any sign of toxicity and mortality. Results of trypanocidal activity were expressed as mean ±SEM. Statistical analysis was done using Sigma stat (Jandel, USA).

3. Results and Discussion

In this current report, methanol appeared to be suitable for the extraction of *C. asiatica* leaves. Solvent system, methanol/ chloroform (20: 80) was more suitable in development of TLC plates than other solvent systems tested. It extracted bioactive constituents present in the *C. asiatica* leaves as observed on TLC plate (plate not shown). This is similar to extraction and development of *Calotopis gigantea* leaves on TLC plates (Shaba et al., 2011a) and extraction of some African plants for their trypanocidal activity (Freiburghaus et al., 1996a). The result of *in vitro* evaluation of trypanocidal activity of *C. asiatica* leaves was as given in Table 1.

Antitrypanosomal activity varied from immobilization,

reduction and to the killing of trypanosomes on the Vero cells medium. At 250 µg ml^{-1} of the test extract, there was drastic reduction of average mean trypanosomes counts (40.00+0.00 to 14.00+0.33), and at 500 µg ml^{-1} (40.00+0.00 to 11.00+0.33) were observed. But at 750 µg ml^{-1}, there was complete killing of trypanosomes at 8 h of incubation, which was comparable to 4 h of standard drug (diminazine aceturate) at 50 µg ml^{-1}. Result of antitrypanosomal activity of *C. asiatica* leaves is comparable to *in vitro* antitrypanosomal activity of methanolic extract of *Plumbago zeylanica* root bark where trypanosomes were completely killed at 750 µg ml^{-1} (Shaba et al., 2006); antitrypanosomal potential of methanolic extract of *Calotropis gigantea* leaves with complete killing of trypanosomes at 750 µg ml^{-1} (Shaba et al., 2011a) and trypanocidal potential of *Camellia sinensis* leaves where trypanosomes were completely killed at 250 µg ml^{-1} (Shaba et al., 2011c). An average mean parasites count of 37.67±0.58 is statistically critical value. Average mean parasites counts from 37.67±0.58 and below is significant between the treatment groups and negative control. ($p \leq 0.05$). *In vitro* cytotoxicity test of MPE of *C. asiatica* leaves and diminazine aceturate exhibited different cytotoxic effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in control ELISA plate wells (Table 2).

Both MPE of *C. asiatica* and diminazine aceturate were cytotoxic to Vero cells in all concentrations except at 1.56 and 6.25-1.56 µg ml^{-1}. These *in vitro* cytotoxic effects are comparable to cytotoxic effects of *in vitro* trypanocidal activity of methanolic extracts of *Quercus borealis* leaves and *Zingiber officinale* roots against *T. evansi* with similar cytotoxic effects as MPE of *C. asiatica* (Shaba et al., 2011b) and extrac*t of Terminalia arjuna* bark with distortion and apoptosis of cells on human hepatoma cell line (HEPG2) (Sarveswaran et al., 2006). During infectivity assessment, mice inoculated with contents of ELISA

Table 1: *In vitro* trypanocidal activity of methanolic extract of *Centella asiatica* leaves against *Trypanosma evansi* on Vero cell line

	1 h	2 h	3 h	4 h	5h	6 h	7 h	8 h	9 h
250	40.00±0.0	40.00±0.0	37.67±0.33	37.33±0.33	35.67±0.33	31.00±0.58	27.33±0.67	21.67±0.58	14.00±0.33
500	40.00±0.0	40.00±0.0	37.67±0.33	36.33±0.33	33.67±0.33	29.00±0.58	24.33±0.33	19.00±0.58	11.33±0.33
750	40.00±0.0	37.67±0.33	36.33±0.33	34.67±0.33	30.00±0.58	26.33±0.33	20.33±0.33	14.00±0.58	0.0±0.0
1000	38.33±0.67	36.00±0.58	35.33±0.33	31.00±0.58	23.67±0.33	16.67±0.33	11.67±0.33	0..0±0. 0	0.08±0.00
B*	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
C**	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

*Berenil (50)- Concentration of plant extract in µg ml^{-1}; **C: Control- Negative control; Trypanocidal activity was expressed as mean ±SEm; Significant difference ($p<0.05$)

Table 2: Cytotoxic effects of methanolic extract of *C. asiatica* leaves on Vero cell line compared to diminazine aceturate (BERENIL)

Concentration of test material in µg ml^{-1}	Cytotoxic effects of extract at various time intervals of incubation						Control
	24 h		48 h		72 h		
	C. asiatica	DA	*C. asiatica*	DA	*C. asiatica*	DA	
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	66.6%	0	100%	33.3	100%	66.6	0
12.5	66.6%	0	100%	0	100%	33.3%	0
6.25	66.6%	0	100%	0	100%	0	0
3.13	33.3%	0	66.6%	0	66.6%	0	0
1.56	0	0	0	0	0	0	0

plate wells with completely killed trypanosomes survived for more than 30 days, while other group of mice inoculated with contents of ELISA plate wells with reduced trypanosomes died of parasitaemia. Infectivity assessment of antitrypanosomal activity is comparable to antitrypanosomal effect of the aqueous extract of *Brassica oleracea* and methanolic extract of *Vitex negundo* leaves (Igweh et al., 2002; Shaba et al., 2008) where mice inoculated with apparently killed trypanosomes survived. Mice in two groups were subjected to acute toxicity test of *C. asiatica* leave sample at concentration of 2000 mg kg^{-1} body weight survived (Table 3).

This is similar to acute toxicity test of *Nuclea latifolia* bark in rats, in which the rats survived the test (Madubunyi, 1995).

Table 3: Acute toxicity test of *Centella asiatica* leaves in mice

A	B		C		D
	G1	G2	G1	G2	
1	24	22	0.24	0.22	The mice Survived without any apparent toxic signs observed
2	22	22	0.22	0.22	
3	21	23	0.21	0.23	
4	23	24	0.23	0 24	
5	25	23	0.25	0.23	

A: Number of mice extract^{-1}; B: Body weight of mice (g); C: Concentration used in ml (2000 mg kg^{-1} body weight); D: Observation (toxicity signs and mortality)

4. Conclusion

In conclusion, the current research findings indicate a possible antitrypanosomal compound(s) from the *C. asiatica* leaves. This is a preliminary result of inherent antitrypanosomal activity of *C. asiatica* leaves. To ascertain its complete antitrypanosomal status, studies such as bioassay-guided purification and *in vivo* testing must be undertaken.

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