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THERAPEUTIC EVALUATION OF METHANOLIC EXTRACT OF *AGERATUM HOUSTONIONUM* LEAVES AGAINST *TRYPANOSOMA* *EVANSI*

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ABSTRACT

Methanolic plant extract (MPE) of *Ageratum houstonionum* leaves at concentrations (250-1000 µg/ml) was screened against *Trypanosoma evansi* for its trypanocidal activity. It was carried out on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with fetal calf serum (20-40%) at appropriate conditions. *In vitro* cytotoxicity of MPE of *A. houstonionum* at concentrations (1.56-100 µg/ml) was done on Vero cells but without FCS. *In vitro* trypanocidal activity ranged from immobilization to the killing of trypanosomes. At 250 µg/ml of the test MPE, there was drastic reduction of average mean trypanosomes count (.11.3±0.33) as observed. At 500µg/ml of the test extract, there was complete killing of trypanosomes at 7h of incubation. Trypanosomes counts decreased in concentration and time –dependent manner with significant difference (P<0.05). Both MPE of *A. houstonionum* and diminazine aceturate, standard drug, were cytotoxic to Vero cells in all concentrations except at concentrations of 1.56 and 6. 25-1.56 µg/ml.

Key Words: Medicinal Plant, *Ageratum Houstonionum*, *Trypanosoma evansi*, Antitrypanosomal, *In Vitro* Cytotoxicity

INTRODUCTION

Trypanosomiasis is an important blood protozoan parasites belonging to the genus *Trypanosoma*. (Mahmoud and Gray, 1980). 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). 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. But the chemotherapy of trypanosomiasis is beset with problems including limited choice of trypanocides in the market, high cost, toxicity, and emergence of drug-resistant trypanosome strains that have been reported (Gutteridge, 1985). 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. 2009 and Shaba et al. 2011). More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds (Cragg *et al.*, 1997; Soerjatta, 1996). In this present paper, antitrypanocidal activity of *Ageratum houstonionum* is reported.

MATERIALS AND METHODS

Chemicals

Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol and aqueous) for extraction of plant materials and development /analysis of TLC plates, vanillin for spray and iodine for detection of bioactive constituents These were purchased from E. Merck, India.

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Plant material

Ageratum houstonionum leaves at matured stages were collected in September, 2006 and identified at Institute of Himalayan Biosource and Technology, Palampur, India.

Preparation of extract

The extraction was carried out according to the method of Stahl, (1969). 20 g *A. houstonionum* 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°C and stored at 4°C until used.

Thin layer chromatography (TLC) plate

Aliquots (0.2ml) 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 vapours 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).

Solvent system applied.

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).

Animals

Swiss albino mice (20-30g) 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 animal of the institute.

Test organism

T. evansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute, 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).

Parasite counts

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.

In vitro trypanocidal activity

Extract of *A. houstonionum* at concentrations (250-1000 µg/ml) were added to a high parasitaemic blood from mouse diluted with Alsever solution to obtain a final parasite concentration of 1×10^6 parasites/ml. The suspension (100 ml of medium with trypanosomes) was added at rate of 1: 1 to test extract with inactivated bovine serum at 58 °C for 1 h, and incubated at 37 °C under 5% CO₂ 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 trypanosomes with the given concentration (Young et al., 2000).

Infectivity assessment

After incubation for antitrypanosomal activity was completed, contents of wells with reduced and apparently killed trypanosomes from MPE of *A. houstonionum* were inoculated into mice intraperitoneally and observed for more than 30 days for parasitaemia (Petama, 1964).

In vitro cytotoxicity Test

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

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seeded with 500,000 cells/ml. The plates were incubated at 37°C with 5% CO₂ 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 (0.56-100 µg/ml) 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°C in ordinary incubator. Plates were later observed for cytotoxic effects.

Statistical analysis

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

RESULTS

In this current report, methanol appeared to be suitable for the extraction of *A. ageratum* 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 *A. houstonionum* leaves as observed on TLC plate (plate not shown).

The result of *in vitro* therapeutic activity of trypanocidal activity of *A. houstonionum* leaves is given in Table 1. Antitrypanosomal activity ranged from immobilization to the killing of trypanosomes on the Vero cells medium. *In vitro* cytotoxicity test of MPE of *A. houstonionum* leaves and diminazine acetate exhibited different cytotoxic effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in control ELISA wells (Table 2). During infectivity assessment, mice inoculated with contents of ELISA wells with completely killed trypanosomes survived for more than 30 days, while other group of mice inoculated with contents of ELISA wells with reduced trypanosomes died of parasitaemia.

DISCUSSION

The extraction and subsequent development of TLC plates is similar to extraction and development of *Calotropis gigantea* leaves on TLC plates (Shaba et al 2011).

At 250 µg/ml of the test extract, there was drastic reduction of average mean trypanosomes counts (40.00±0.00 to 14.00±0.33). But at 500 µg/ml, there was complete killing of trypanosomes at 7 h of incubation, which was equivalent to 4 h of standard drug (diminazine acetate) at 50 µg/ml. Result of antitrypanosomal activity of *A. houstonionum* is comparable to *in vitro* antitrypanosomal activity of methanolic extract of *Plumbagozeylanica* root bark where trypanosomes were completely killed at 750 µg/ml (Shaba et al. 2006); *in vitro* extraction of some Nigeria medicinal plants with most higher activities in methanolic extracts (Atawodi et al. 2003) and anti-trypanosomal potential of methanolic extract of *Calotropis gigantea* leaves with complete killing of trypanosomes at 750 µg/ml (Shaba et al. 2011). 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 ≤ 0.05).

Both MPE of *A. houstonionum* and diminazine acetate were cytotoxic to Vero cells in all concentrations except at 1.56 and 6.25-1.56 µg/ml. These *in vitro* cytotoxic effects are comparable to

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Table 1. *In vitro* trypanocidal activity of methanolic extract of *Ageratum houstonianum* leaves against *Trypanosoma evansion* Vero cell line.

Concentration of the plant extract in $\mu\text{g/ml}$	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	37.33 \pm 0.33	34.67 \pm 0.33	33.00 \pm 0.0	29.00 \pm 0.58	27.6 \pm 0.33	26.0 \pm 0.0	21.6 \pm 0.33	16.6 \pm 0.33	11.3 \pm 0.33
500	34.33 \pm 0.33	\pm 0.33	24.67 \pm 0.67	19.33 \pm 0.33	13.00 \pm 0.0	5.333 \pm 0.33	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
750	33.00 \pm 0.0	24.00 \pm 0.0	17.67 \pm 0.33	11.00 \pm 0.0	1.333 \pm 0.33	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
1000	30.00 \pm 0.58	19.33 \pm 0.33	12.00 \pm 0.0	1.000 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Diminazenaceturate (50) Positive control	22.33 \pm 0.33	9.333 \pm 0.67	1.000 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
Control (Negative control)	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0	40.00 \pm 0.0

Bioassay status: significant reduction of parasites counts from concentration of 250 $\mu\text{g/ml}$ and complete killing of parasites at 500 $\mu\text{g/ml}$ at 7th hour of observation. An average mean parasites count of 37.67 \pm 0.58 is statistically critical value. Average mean from 37.67 \pm 0.58 and below is significant between the treatment groups and negative control ($P \leq 0.05$ to 0.01).

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Table 2. Cytotoxic effects of methanolic extract of *Ageratum houstonionum* leaves on Vero cell line compared to diminazine acetate (BERENIL).

Concentration of test material in µg/ml	Cytotoxic effects of extract at various time intervals of incubation						
	24 h		48 h		72 h		Control
	<i>A.houstonionum</i>	DA	<i>A.houstonionum</i>	DA	<i>houstonionum</i>	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

cytotoxic effects of *in vitro* comparative antitrypanosomal activity of *Terminalia chebula* against *T. evansi* with similar cytotoxic effects as MPE of *A. houstonionum* (Shaba et al.2007) and extract 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 wells with completely killed trypanosomes survived for more than 30 days, while other group of mice inoculated with contents of ELISA 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 and Shaba et al. 2008) where mice inoculated with apparently killed trypanosomes survived.

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

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