

## THERAPEUTIC ACTIVITY OF METHANOLIC EXTRACT OF *EUPATORIUM ADENOPHORUM* LEAVES AGAINST *TRYPANOSOMA* *EVANSI*

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### ABSTRACT

In search of trypanocidal compounds from medicinal plants, *Eupatorium adenophorum* leaves were cold extracted with methanol. The methanolic plant extract (MPE) at different concentrations (250-1000 µg/ml) was screened against *Trypanosoma evansi* for its therapeutic activity against trypanosomes. The therapeutic activity of MPE against trypanosomes was conducted on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with foetal calf serum (20-40%) at appropriate conditions. *In vitro* cytotoxicity test of MPE of *E. adenophorum* leaves at concentrations (1.56-100 µg/ml) was done on Vero cells but without FCS. Therapeutic activity of *E. adenophorum* leaves extract against trypanosomes ranged from immobilization, reduction and to the killing of trypanosomes in the corresponding ELISA plates. At 250 µg/ml of the test extract, there was considerable reduction of average mean trypanosomes count (19.67±0.33) as observed after 9 h of incubation. At 1000 µg/ml of the test extract, there was complete killing of trypanosomes at 7 h of incubation in the corresponding ELISA plates wells. Trypanosomes counts decreased in concentration and time –dependent manner with significant difference (P<0.05). *In vitro* cytotoxicity test revealed both MPE of *E. adenophorum* leaves and diminazine aceturate, standard drug, were cytotoxic to Vero cells in all concentrations except at 1.56 and 6.25-1.56 µg/ml. Moderate therapeutic activity of MPE of *E. adenophorum* was observed.

**Key Words:** Medicinal Plant, *Eupatorium Adenophorum*, Leaves, *Trypanosoma Evansi*, Therapeutic Activity, *In Vivo* Infectivity, *In Vitro* Cytotoxicity

### INTRODUCTION

Trypanosomiasis, a zoonotic disease of blood protozoan origin, has re-emerged in recent years with lots of havoc in both animals and humans caused by various strains of genus *Trypanosoma* (WHO, 2004; (Nok and Nock, 2002). At present, over 60 million people are living in 36 sub-Saharan countries are at risk of contracting the disease with resultant consequences (WHO, 2001).

As result of limited classes of trypanocides available for use against the menace of trypanosomiasis, resistant strains of trypanosomes have been identified in both domestic and wild animals (Freiburghaus *et al.*, 1998; Shaba *et al.*, 2006). The menace of the disease can only be combated by judicious usage of trypanocides in chemotherapy and chemoprophylaxis means. But both methods are confronted with challenges 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; Nok and Nock, 2002; Shaba *et al.*, 2006).

With the re-insurgence of ethno pharmacology and ethno medicine research, several medicinal plants possess trypanocidal compounds, which may pave way for a future potential trypanocides (Wurocheke and Nok, 2004; Shaba *et al.*, 2009 and Shaba *et al.*, 2012a; Shaba *et al.*, 2012b). More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds (Cragg *et al.*, 1997; Soerjatta, 1996).

Traditionally, *E. adenophorum* leaves decoction has been applied on cut wounds to abate bleeding, and used against infection of gum and tooth ache (Dahanukar *et al.*, 2000). Pharmacologically, antibacterial and antioxidant activities has been reported (Dahanukar *et al.*, 2000)

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On the basis of discovery new trypanocidal compounds from medicinal plants, *Eupatorium adenophorum* leaves were evaluated for its therapeutic activity against *Trypanosoma evansi*.

**MATERIALS AND METHODS**

**Chemicals**

Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol ethyl acetate and acetic acid) 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.

**Plant Material**

*Eupatorium adenophorum* 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 of *E. adenophorum* leaves was powdered using laboratory pestle and mortar, and cold extracted with 200 ml of methanol (analytical grade). Residues obtained were extracted twice in the same medium. The filtrates were combined, dried at 37°C and stored at 4°C until used.

**Thin Layer Chromatography (TLC) Plate**

Aliquot (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 Indian Veterinary Research Institute, (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).

**Trypanosomes Counts**

Estimation of trypanosomes 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 trypanosomes in triplicate were counted using glass slides under inverted microscope (400X). An average mean trypanosomes count was taken as number of trypanosomes per field.

**In Vitro Trypanocidal Activity**

Extract of *E. adenophorum* leaves 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 1x10<sup>6</sup> 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% CO<sub>2</sub> for 12 h (Talakai *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

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effect of the DMSO was noticed on host cells or trypanosomes with the given concentration (Yong *et al.*, 2000).

**In vivo Infectivity Assessment**

After incubation for therapeutic activity of MPE of *E. adenophorum* was completed, contents of wells with reduced and apparently killed trypanosomes from test extract were inoculated into mice intraperitoneally and observed for more than 30 days for parasitaemia (Igweh *et al.*, 2002).

**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 seeded with 500,000 cells/ml. The plates were incubated at 37°C with 5% CO<sub>2</sub> 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) 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 AND DISCUSSION**

The result of this present study indicated that methanol was suitable for the extraction of *E. adenophorum* leaves. The extraction of *E. adenophorum* leaves is similar to the extraction of *Zanthoxylum alatum* leaves and *Eugenia caryophyllata* buds (fruits) (Shaba *et al.*, 2012a) and *Centella asiatica* leaves (Shaba *et al.*, 2012b) where methanol was used in extraction of medicinal plants. 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 *E. adenophorum* leaves as observed on TLC plate (plate not shown). The development of TLC plates in the solvent system is similar to the development of TLC plates of bioassay-guided isolation of a diastereoisomer of kolavenol from *Entada absyssinica* (Freiburghaus *et al.*, 1998), comparative extractions of *Terminalia chebula* dried fruits (Shaba *et al.*, 2007) and *Calotropis gigantea* leaves on TLC plates (Shaba *et al.*, 2011a).

The result of *in vitro* therapeutic activity of MPE of *E. adenophorum* leaves against *T. evansi* was as given in Table 1. Therapeutic activity varied from immobilization, reduction and to the killing of trypanosomes on the Vero cells medium. At 250 µg/ml of the test extract, there was considerable reduction of average mean trypanosomes counts (40.00±0.00 to 11.67±0.33). But at 1000 µg/ml, trypanosomes were not detectable in the corresponding ELISA plate wells at 7 h of incubation, which was comparable to 4 h of standard drug (diminazine aceturate) at 50 µg/ml. Result of *in vitro* therapeutic activity of *E. adenophorum* is comparable to antitrypanosomal potential of methanolic extract of *Ageratum houstonianum* flowers in which trypanosomes were not detectable at 500 µg/ml (Shaba *et al.*, 2011d); *in vitro* antitrypanosomal activity of methanolic extract of *Plumbago zeylanica* root bark where trypanosomes were completely killed at 750 µg/ml (Shaba *et al.*, 2006); antitrypanosomal potential of methanolic extract of *Calotropis gigantea* leaves with complete killing of trypanosomes at 750 µg/ml (Shaba *et al.*, 2011a) and trypanocidal potential of *Camellia sinensis* leaves where trypanosomes were not detected in the corresponding ELISA plate wells at 250 µg/ml of the test extract at 4 h of incubation (Shaba *et al.*, 2011c). An average mean trypanosomes count of 37.67± 0.58 is statistically critical value. Average mean trypanosomes counts from 37.67± 0.58 and below is significant between the treatment groups and negative control. (P ≤ 0.05).

*In vitro* cytotoxicity test of MPE of *E. adenophorum* and diminazine aceturate exhibited different cytotoxic effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in the control ELISA plate wells (Table 2). Both MPE of *E. adenophorum* and

**Table 1: *In vitro* trypanocidal activity of methanolic extract of *Eupatorium adenophorum* leaves against *Trypanosoma evansi* on Vero cell line**

Concentration of plant extract in $\mu\text{g/ml}$	1 h	2 h	3 h	4 h	5h	6 h	7 h	8 h	9 h
<b>250</b>	40.00 $\pm$ 0.0 0.0	37.67 $\pm$ 0.33 0.33	35.33 $\pm$ 0.33 0.33	31.67 $\pm$ 0.33 0.33	29.67 $\pm$ 0.33 0.33	27.33 $\pm$ 0.33 0.33	25.33 $\pm$ 0.33 0.67	22.00 $\pm$ 0.58 0.58	19.67 $\pm$ 0.33 0.33
<b>500</b>	40.00 $\pm$ 0.0	37.33 $\pm$ 0.33	31.67 $\pm$ 0.33	27.00 $\pm$ 0.58	23.67 $\pm$ 0.33	20.67 $\pm$ 0.33	18.00 $\pm$ 0.58	15.00 $\pm$ 0.58	12.00 $\pm$ 0.58
<b>750</b>	33.00 $\pm$ 4.04	21.00 $\pm$ 0.67	26.00 $\pm$ 1.00	21.67 $\pm$ 0.33	16.67 $\pm$ 0.67	12.33 $\pm$ 0.88	9.333 $\pm$ 0.88	5.333 $\pm$ 0.33	0.6667 $\pm$ 0.33
<b>1000</b>	35.33 $\pm$ 0.33	27.67 $\pm$ 0.33	21.33 $\pm$ 0.33	16.33 $\pm$ 0.33	11.67 $\pm$ 0.33	3.667 $\pm$ 0.33	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
<b>Control (Negative control)</b>	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
<b>Diminazen aceturate (50) Positive control</b>	23.00 $\pm$ 0.54	9.333 $\pm$ 0.33	1.333 $\pm$ 0.33	0.0 $\pm$ 0.0					

*Bioassay Status:* significant reduction of parasites counts from concentration of 750  $\mu\text{g/ml}$  and complete killing of parasites at 1000  $\mu\text{g/ml}$  at 7<sup>th</sup> hour of observation. Average mean parasites counts 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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diminazine aceturate 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 cytotoxic effects of *Terminalia chebula* dried fruits on Vero cells with similar cytotoxic effects as MPE of *Quercus borealis* leaves and *Zingiber officinale* roots (Shaba et al., 2011b) and extract of *Terminalia arjuna* bark with distortion and apoptosis of cells on human hepatoma cell line (HEPG2) (Sarveswaran et al., 2006).

During *in vivo* infectivity assessment, mice inoculated with contents of ELISA 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. *In vivo* infectivity assessment of MPE of *E. adenophorum* is comparable to antitrypanosomal effect of the aqueous extract of *Brassica oleracea* and antitrypanosomal activity of methanolic extract of *Khaya senegalensis* root bark (Igweh et al., 2002 and Shaba et al., 2011e) where mice inoculated with apparently killed trypanosomes survived.

From this report, there is likelihood of therapeutic compound(s) from the *E. adenophorum* leaves against *Trypanosoma evansi*. This is the first attempt to determine its therapeutic activity against *T. evansi*. To know its fullest therapeutic activity against trypanosomes, studies such as bioassay-guided isolation and *in vivo* testing must be carried out.

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