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Research Article

SCREENING OF *ACHILLEA MILLEFOLIUM* L (YARROW) FLOWERS FOR ITS ANTITRYPANOSOMAL ACTIVITY

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ABSTRACT
In the current resurgence of trypanosomosis in animals and resistance to limited available classes of trypanocides, and resistant strains of trypanosomes, *Achillea millefolium* flowers were screened for possible antitrypanosomal activity. *A. millefolium* powdered was cold extracted with methanolic solvent. The obtained methanolic plant extract (MPE) was screened against *Trypanosoma evansi* at different concentrations (250-1000 µg/ml) for its antitrypanosomal activity. The screening for antitrypanosomal activity of MPE of *A. millefolium* against trypanosomes was carried out 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 *A. millefolium* flowers at different concentrations (1.56-100 µg/ml) was done on Vero cells but without FCS. The observed antitrypanosmal activity of test extract varied from immobilization, reduction and to the killing of trypanosome*s* in the corresponding ELISA plates. At 250 µg/ml of the test extract, there was marked reduction of average mean trypanosomes count (16.67±0.33) as observed at 9 h of incubation. However, at 1000 µg/ml of the test extract, trypanosomes were not detectable at 9 h of incubation in the corresponding ELISA plates wells. Trypanosomes counts decreased in concentration and time – dependent faction with significant difference ($P<0.05$). *In vitro* cytotoxicity test revealed both MPE of *A. millefolium* flowers and diminazine aceturate, standard drug, were cytotoxic to Vero cells in all concentrations except at 3.13-1.56 and 6.25-1.56 µg/ml. There was considerable *in vitro* antitrypanosomal activity, which was verified via *in vivo* infectivity test.

Key Words: *Medicinal Plant, Achillea millefolium ,flowers, Trypanosoma Evansi, Antitrypanosomal Activity, In Vivo Infectivity, In Vitro Cytotoxicity*

INTRODUCTION
Trypanosoma evansi, blood protozoan parasite, is one of the causative agents of animal trypanosomosis (WHO, 2004). In Africa, the diseases has re-emerged with lots of havoc to livestock industries especially in sub-Saharan Africa where the parasites thrives (Kamuanga, 2003; Shaba *et al.,* 2006). 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 trypanosomosis, resistant strains of trypanosomes have been identified in both domestic and wild animals (Freiburghaus *et al.,* 1998; Shaba *et al.,* 2006).
Chemotherapy and chemoprophylaxis are two means of controlling trypanosomosis. Both methods are bedevilled 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; Nok and Nock, 2002; Shaba *et al.,* 2006).
Many medicinal plants/extracts/compounds with antitrypanosmal activity have been identified (Nok and Nock, 2002; Shaba *et al.,* 2009 and Shaba *et al.,* 2012). More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds (Cragg *et al.,* 1997; Soerjatta, 1996).
Traditionally, *A. millefolium* flowers has been used in catarrhal respiratory infection, neuritis, neuragial, varicose ulcer, hepatic and digestive deficiencies (Tyler, 1998).

From medical point of view, *A. millefolium* has been used as antiallergic, anticatarrhal, haemostatic, emenagogue and uterus sedative. (Nemeth, 2008).
Phytochemical analysis of *A. millefolium* revealed momoterpenes, sesquiterpenes, lactones (achilline) and hydrozulene has been isolated (Orav *et al.*, 2006).
Based on the aforementioned reasons, *Achillea millefolium* flowers were screened for its antitrypanosomal activity.

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

Achillea millefolium flowers 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 *A. millefolium* flowers 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 37oC and stored at 4oC 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 **e**ach 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 Antitrypanonosomal Activity

Extract of *A. mellifolium* flowers 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 1x106 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 12 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 (Yong *et al.,* 2000).

In vivo Infectivity Assessment

After incubation for antitrypanosomal activity of MPE of *A. millefolium 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 37oC 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 (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 37oC 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

In this report, as previously observed in the reported extractions, the usage of methanol as the extraction solvent of *A. millefolium* flowers appeared to be suitable. The extraction of *A. millefolium* flowers is similar to the extraction of *Khaya senegalensis tree* root bark (Shaba *et al.,* 2011a) 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 *A. millefolium* flowers 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 *Ageratum houstonionum* leaves on TLC plates (Shaba *et al.,* 2011b).

The result of *in vitro* antitrypanosomal activity of MPE of *A. millefolium* flowers against *T. evansi* 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 of the test extract, there was considerable reduction of average mean trypanosomes counts (40.00+0.00 to 16.67±0.33). But at 1000 µg/ml, trypanosomes were not detectable in the corresponding ELISA plate wells at 9 h of incubation, which was comparable to 4 h of standard drug (diminazine aceturate) at 50 µg/ml. Result of *in vitro* antitrypanosomal activity of *A. millefolium* is comparable to antitrypanosomal activity of methanolic extract of *Khaya senegalensis* tree bark where trypanosomes were not detectable at 250 a6 h of incubation (Shaba *et al.,* 2011 a); antitrypanosomal potential of methanolic extract of *Ageratum houstonionum* flowers in which trypanosomes were not detectable at 500 µg/ml (Shaba *et al.,* 2011d); i*n vitro* antitrypanosomal activity of methanolic extract of *Plumbago zeylanica* root bark where trypanosomes were completely killed at 750 µg/ml (Shaba *et al.,* 2006); anti-trypanosomal potential of methanolic extract of *Calotropis gigantea* leaves with complete killing of trypanosomes at 750 µg/ml (Shaba *et al.,* 2011c) 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.,* 2011d).

Table 1. *In vitro* trypanocidal activity of methanolic extract of *Achillea millefolium* flowers against *Trypanosma evansi on* Vero cell line.

Conc. of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
250	39.00±0.58	37.33±0.33	35.33±0.33	31.33±0.33	28.67±0.33	25.67±0.33	22.33±0.33	19.33±0.33	16.67±0.33
500	38.33±0.33	34.67±0.33	31.33±0.33	26.00±0.33	24.67±0.33	22.33±0.33	19.33±0.33	15.33±0.33	12.67±0.33
750	37.33±0.33	33.67±0.33	30.67±0.33	28.33±0.33	21.67±0.33	18.67±0.33	14.67±0.33	12.33±0.33	10.67±0.33
1000	37.00±0.0	32.00±1.00	28.67±0.33	23.33±0.33	18.00±0.58	13.33±0.33	10.33±0.33	3.667±0.33	0.0±0.0
Control (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
Diminazen aceturate (50) Positive control	22.33±0.33	9.333±0.33	1.667±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

Bioassay status: significant reduction of trypanosomes counts from concentration of 250 µg/ml and complete killing of parasites at 1000 µg/ml at 9th hour of observation. 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 ($P \leq 0.05$ to 0.01).

Table 2. Cytotoxic effect of methanolic extract of *Achillea millefolium* flowers on Vero cell line compared to diminazen aceturate (Berenil)

Concentration of test material in µg/ml	Effects of test extract at various periods of incubations (24 h, 48 h, 72 h)						
	Achillea millefolium	Berenil	*Achillea millefolium*	Berenil	*Achillea millefolium*	Berenil	Control
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	0	0	100%	100%	100%	100%	0
12.5	0	0	33.3%	0	100%	33.3%	0
6.25	0	0	0	0	66.6%	0	0
3.13	0	0	0	0	0	0	0
1.56	0	0	0	0	0	0	0

Achillea millefolium flowers and dimainazen aceturate were toxic to Vero cell line except at concentrations of 3.13-1.56 and 6.25-1.56 µg/ml. Same concentrations were used for diminazine aceturate (Berenil)

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. ($P \leq 0.05$).

In vitro cytotoxicity test of MPE of *A. millefolium* 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 *A. millefolium* and 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 *Centella asiatica* leaves (Shaba *et al.,* 2012) on Vero cells with similar cytotoxic effects as MPE of *Quercus borealis* leaves and *Zingiber officinale* roots (Shaba *et al.,* 2011e) 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 *A. millefolium* is comparable to antitrypanosomal effect of the aqueous extract of *Brassica oleracea* and antitrypanosomal activity of methanolic extract of *Ageratum houstonionum* flowers (Igweh *et al.,* 2002 and Shaba *et al.,* 2011f) where mice inoculated with apparently killed trypanosomes survived.

In this report, antitrypanosomal activity may be due to isolated compounds from *A. millefolium* flowers as mentioned above.

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