

In vitro trypanocidal activity of comparative extraction of Terminalia bellirica (Combretaceae) dried fruits with solvents of different polarities against Trypanosoma evansi.

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Citation

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Abstract

Terminalia belirica (Combretaceae) dried fruits was comparatively extracted with solvents of different polarities (hexane, chloroform, methanol and aqueous), screened against Trypanosoma evansi at different concentrations (250-1000 µg/ml) on Vero cell line grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal calf serum (FCS) 40% and incubated at appropriate conditions. In vitro cytotoxicity test was carried out on Vero cells but not supplemented with FCS. T. belirica dried fruits extracts exhibited antitrypanosomal activity both in forms of immobilization and killing of trypanosomes. Order of trypanocidal activity was methanol, aqueous, chloroform and hexane. At 250 µg/ml, trypanosomes could not be detected in methanolic and aqueous extracts after 4 h and 5 h of incubation, which was comparable to diminazine aceturate at 50 µg/ml (standard drug). Trypanosomes counts decreased in concentration and time –dependent manner with significant difference ($P < 0.05$) Both methanolic plant extract of T. Belirica and diminazine aceturate (Berenil) were cytotoxic to Vero cell line in all concentrations except at 1.56 and 6.25 µg/ml.

INTRODUCTION

In Africa, the estimated losses as a result of trypanosomiasis in agricultural production amounted to 3 billion pounds annually (Hursey, 2000). In addition, it is estimated that 50 million cattle are at risk of being infected with trypanosomes leading to more than 3 million livestock deaths yearly, losses in calving, reduction in livestock number, drop in meat and milk off take and reduced work efficiency of draft animals and profitability of mixed farming (Mahmoud and Gray, 1980; Hursey, 2000; Seed, 2001)..

Human African trypanosomiasis (HAT) is caused by Trypanosoma brucei rhodesiense and other species and transmitted by tsetse flies, which are equally spreading to other part of Africa (.Doua and Yapo 2001). At present, over 60 million people living in 36 sub-Saharan countries are at risk of contacting the disease (WHO, 2000). It is estimated that currently about 300-500,000 people are infected with 50,000 deaths annually (WHO, 2000). The above mentioned facts stress the zoonotic importance of trypanosomiasis.

Chemotherapy and chemoprophylaxis are the only means of combating the menace of the disease. Chemotherapy of

trypanosomiasis 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 (Freiburghaus et al., 1996a; Denise and Barret, 2001). Recent ethnopharmacology and ethnomedicine revealed that several medicinal plants possess trypanocidal compounds, which may hold the key for a future potential trypanocides (Lopez et al. 1998;; Wurochekke and Nok, 2004; Shaba et al., 2006) More so, several semi-synthetic and synthetic drug derivatives were originally isolated from natural compounds (Cragg et al., 1997; Soerjatta, 1996)

Terminalia belirica (Combretaceae) dried fruits have been used immemorial in traditional medicinal herb of India. It is an ingredient of Indian Ayurvedic drug 'triphala' used for the treatment of digestion and liver disorders (Nadkarni, 1954). Active principle such as gallic acid (3,4,5-trihydroxybenzoic acid) has been isolated. T. belirica has been evaluated against carbon tetrachloride I (Jando et al., 2006)

Thus, new approach and trypanocides are highly needed to combat trypanosomes. Based on this, Terminalia belirica

***In vitro* trypanocidal activity of comparative extraction of Terminalia bellirica (Combretaceae) dried fruits with solvents of different polarities against Trypanosoma evansi.**

dried fruits were screened for possible antitrypanosomal activity.

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.

PLANT MATERIAL

Dried fruits of Terminalia belirica (Combretaceae) were collected in September, 2004 and identified at Institute of Himalayan Biosource and Technology, Palampur, India.

PREPARATION OF EXTRACT

Twenty grams of Terminalia belirica dried fruits was pounded into powder with pestle and mortar and cold extracted twice with 200 ml of ethanol (analytical grade) according to Stahl (1969). The filtrates were dried at 37 °C and stored at 4 °C until used.

SOLVENT SYSTEM

The following solvent systems were tested for a suitable solvent to be used in developing 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)

THIN LAYER CHROMATOGRAPHY (TLC) PLATES

This was done according to the method of Stahl, 1969. Aliquots from extractions were applied on TLC plates and developed in appropriate solvent system.

ANIMALS

Swiss albino mice (20-30g) of either sex were obtained from Animal Research Laboratory Section of Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly 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.

TEST ORGANISM

Trypanosoma evansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute, Izatnaga, Bareilly and was maintained in the laboratory by serial sub-passage in Swiss albino mice. The strain was routinely tested for virulence following the method Williamson et al., (1982)

IN VITRO TRYPONOCIDAL ACTIVITY

Extracts at concentrations (250-1000 µg/ml) were added to a high parasitemic 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 extracts with inactivated bovine serum at 58 °C for 1 h. incubated at 37 °C under 5% CO₂ for 5 h (Talakai et al., 1995). Each test was repeated at least thrice and tested in vitro for trypanocidal activity. Dried plant extracts were 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).

INFECTIVITY ASSESSMENT

After incubation for antitrypanosomal activity was completed, contents of wells with reduced and apparently killed parasites from MPE of Terminalia belirica dried fruits extract (0.1ml/mouse) was inoculated into mice (six/group) intraperitoneally and observed for more than 30 days for parasitemia (Petama, 1964; Woo, 1971a).

:: Stock of test MPE was solubilized in 1% dimethylsulphoxide (DMSO) The concentration in the experiment had no deleterious effect by it self on host cells or parasites. 1% DMSO in distilled water was used as control (Young, 2000)

CYTOXICITY TEST

Cytotoxic effect of the plant extract was determined according to the method described by Sidwell and Hoffman (1997). Vero cell line was grown as stated above but was not supplemented with fetal calf serum. Confluent monolayer of Vero cell was treated with serial dilutions of test MPE (1.56-100 µg/ml) in triplicate and incubated under same conditions described previously. After 24h of incubation, the culture plate was observed for evidence of cytotoxic effects such as distortion, detachment, swelling and sloughing of cells. The plate was incubated for 72 h and observed daily. It was repeated thrice.

In each case, after the 72 h of incubation, the culture media

***In vitro* trypanocidal activity of comparative extraction of Terminalia bellirica (Combretaceae) dried fruits with solvents of different polarities against Trypanosoma evansi.**

of the incubated Vero cells were discarded. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. The plate was incubated for 24 hours at 37°C in an ordinary incubator. The plate was observed for cytotoxic effects under inverted microscope.

STATISTICAL ANALYSIS

Results of trypanocidal activity were expressed as mean ± SEM. Statistical significance was determined by Sigma Stat (Jandel), USA.

RESULTS

The results of trypanocidal activity of T. bellirica dried fruits using different solvents of polarities were as shown in Tables 1-4. The order of trypanocidal activity was methanol, aqueous, chloroform and hexane. Bioassay status of all solvents used indicated that an average mean parasites 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 tested extracts and negative control. (P ≤ 0.05). In vitro cytotoxicity test on Vero cell line, Table 5, indicated that the concentrations of MPE of T. bellirica and diminazine acetate (Berenil) were cytotoxic to Vero cells except at 1.56 and 6.25 µg/ml .

Figure 1

Table 1: trypanocidal activity of comparative extraction of dried fruits with solvents of different polarities against Alsevier medium.

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h
250	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	37.33±0.0
500	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	37.00±0.0
750	40.00±0.0	40.00±0.0	38.00±0.0	34.33±0.33	29.33±0.33
1000	40.00±0.0	40.00±0.0	37.33±0.33	30.67±0.33	22.67±0.33
Control (Negative control)	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0
Diminazine acetate (50) Positive control	23.00±0.58	9.667±0.67	1.333±0.33	0.0±0.0	0.0±0.0

HEXANE EXTRACT

Bioassay status: significant reduction of trypanosomes average counts from concentrations (250-1000 µg/ml and but no complete killing of trypanosomes in any well throughout hours of observation. An average mean trypanosomes count of 37.67± 0.58 is statistically critical value.

Figure 2

Table 2: Chloroform extract

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h
250	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	37.67±0.33
500	40.00±0.0	40.00±0.0	39.00±0.0	35.67±0.33	29.67±0.33
750	40.00±0.0	40.00±0.0	38.00±0.0	32.33±0.33	23.33±0.33
1000	40.00±0.0	40.00±0.0	37.33±0.33	28.33±0.33	20.33±0.33
Control (Negative control)	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0	40.00±0.0
Diminazine acetate (50) Positive control	23.00±0.58	9.667±0.67	1.3±0.33	0.0±0.0	0.0±0.0

Bioassay status: significant reduction of trypanosomes counts from concentrations (250-1000 µg/ml and but no complete killing of trypanosomes in any well through out hours of observation. An average mean trypanosomes parasites count of 37.67± 0.58 is statistically critical value.

Figure 3

Table 3: Methanolic extract

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h
250	17.67±0.33	11.67±0.33	5.667±0.33	0.0±0.0	0.0±0.0
500	15.33±0.33	8.667±0.33	2.667±0.33	0.0±0.0	0.0±0.0
750	12.67±0.33	6.667±0.33	0.6767±0.33	0.0±0.0	0.0±0.0
1000	10.00±0.0	3.000±0.0	0.0±0.0	0.0±0.0	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
Diminazine acetate (50) Positive control	23.00±0.58	9.667±0.67	1.333±0.33	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 trypanosomes at same concentration at 4th hour of observation. An average mean trypanosomes count of 37.67± 0.58 is statistically critical value.

Figure 4

Table 4: Aqueous extract

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h
250	20.00±0.0	13.00±0.0	7.667±0.33	1.667±0.33	0.0±0.0
500	17.00±0.0	11.00±0.0	7.000±0.58	1.667±0.33	0.0±0.0
750	13.67±0.33	9.667±0.33	4.667±0.33	0.0±0.0	0.0±0.0
1000	9.333±0.33	4.333±0.33	0.0±0.0	0.0±0.0	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
Diminazine acetate (50) Positive control	23.00±0.58	9.667±0.67	1.333±0.33	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 trypanosomes at same concentration at 5th hour of

***In vitro* trypanocidal activity of comparative extraction of Terminalia bellirica (Combretaceae) dried fruits with solvents of different polarities against Trypanosoma evansi.**

observation. An average mean trypanosomes count of 37.67 ± 0.58 is statistically critical value.

Figure 5

Table 5: Cytotoxic effect of methanolic extract. of dried fruit on Vero cell line compared to diminazine aceturate (Berenil)

Concentration of material in $\mu\text{g/ml}$	Effects of test extract at various periods of incubation						
	24 h		48 h		72 h		Control
	<i>Terminalia bellirica</i>	diminazine aceturate	<i>Terminalia bellirica</i>	diminazine aceturate	<i>Terminalia bellirica</i>	diminazine aceturate	
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	66.6%	100%	100%	100%	0
25	100%	0	66.6%	66.6%	100%	100%	0
12.5	100%	0	33.3%	0	66.6%	33.3%	0
6.25	0	0	33.3%	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

Terminalia belirica and diminazine aceturate were toxic to Vero cell line except at concentrations of 1.56 and 6.25-1.56 $\mu\text{g/ml}$

Same concentrations were used for both extracts and diminazine aceturate (Berenil)

Methanolic extract was chosen for cytotoxicity test due to its maximum trypanocidal activity

DISCUSSION

Methanol extracted more of bioactive constituents than other solvents as observed on TLC plates in accordance to the mobility of applied aliquots of extracts on the plates (plates not shown) Solvent system methanol/chloroform (20: 80) was suitable in development of TLC plates. Also, methanolic extract exhibited antitrypanosomal activity than other solvents. Antitrypanosomal activity ranged from immobilization and killing of trypanosomes. At 250 $\mu\text{g/ml}$, trypanosomes in methanolic extract were apparently killed at 4 h which was equivalent to diminazine atceturate (50 $\mu\text{g/ml}$). This results are comparable to comparative antitrypanosomal activity of *Terminalia chebula* against *T. evansi* with minimum activity at 250 $\mu\text{g/ml}$ in methanolic extract (Shaba et al., 2007), in vitro trypanocidal activity of some screened American plants extracts against *T. cruzi* at 250 $\mu\text{g/ml}$ (Muellas-Serrano et al. 2000) and comparative in vitro extraction of some Nigeria medicinal plants with most higher activities in methanolic extracts (Atawodi, 2005). Mice inoculated with contents of wells with complete killing of trypanosomes, methanolic extract, survived for more than 60 days, while other group died of parasitemia. Infectivity assessment of antitrypanosomal activity is comparable to antitrypanosomal effect of the aqueous extract of *Brassica oleracea* (Igweh et al, 2002). Both MPE and diminazine aceturate were cytotoxic to Vero cells in all concentrations

with effects such as distortion, swelling and sloughing of cells in the affected wells. These effects are comparable to cytotoxic effects of in vitro comparative antitrypanosomal activity of methanolic extract of *Terminalia chebula* dried fruits against *T. evansi* on Vero cells with almost same pattern of effects (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)..Antrypanosomal activity could be due to intercalation of extracts with DNA leading to death of trypanosomes, blockage of glycolysis pathway and interference with flagella which temporally immobilizes trypanosomes (.Madubunyi, 1995; Sepulveda- Boza et al. 1996, Nok and Nock, 2002)

In conclusion, results of current investigation shows a positive promise of trypanocidal compounds depicted by different degrees of extraction of bioactive constituents by solvents with corresponding antitrypanosomal activity. Further studies (e.g. bioassay-guided purification and in vivo testing) are required.

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***In vitro* trypanocidal activity of comparative extraction of Terminalia bellirica (Combretaceae) dried fruits with solvents of different polarities against Trypanosoma evansi.**

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