# Therapeutic evaluation of Terminalia bellirica (Combretaceae) dried fruits against Trypanosoma evansi.

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**Citation**

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**Abstract**

African trypanosomosis has re-emerged in the recent years. Limited classes of chemotherapy for the treatment of the disease is marred by resistance to available trypanocides coupled with trypanosomes resistant-strains. In this study, methanolic plant extract (MPE) of Terminalia belirica dried fruits were evaluated in vitro 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) 20-40% and incubated at appropriate conditions. Infectivity assessment was done in two groups of mice (0.1 ml/mouse). In vivo test of MPE of T. belirica dried fruits at concentrations (12.5-200 mg/kg body weight) was administered in mice (six/concentration) intraperitoneally. MPE of T. belirica dried fruits extract exhibited antitrypanosomal activity in forms of immobilization and killing of trypanosomes. At 250 µg/ml, trypanosomes could not be detected in wells 4 h after incubation, which was comparable to diminazine aceturate at 50 µg/ml (standard drug) in 4 h. Trypanosomes counts decreased in concentration and time–dependent manner with significant difference ($p<.05$). In infectivity assessment, group of mice inoculated with contents of wells with apparently killed trypanosomes survived while, the other group of mice with reduced trypanosomes died of parasitemia. In vivo, infected extract treated mice overtly demonstrated trypanostatic effect. At 200 mg/kg body weight of the test extract, infected treated mice in this group survived for 7 days in comparison to 3 days in untreated negative control group 48 h post on set of paraitemia and commencement of treatment .

## INTRODUCTION

Trypanosomosis, an important blood protozoan zoonotic disease, is caused by flagellate parasites of the genus Trypanosoma. Many causative agents are responsible for trypanosomosis in both animals and humans with devastating consequences in all its ramifications (Stich et al., 2002; Seed,2001; WHO, 2004)..

Chemotherapy and chemoprophylaxis are the only means of combating the menace of the disease. Chemotherapy of trypamosomosis 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., 2009). 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 in traditional medicinal herb of India. It is an ingredient of Indian Ayurvedic drug 'triphala' used for the treatment of digestion and liver disorders (Nadkami, 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).

This study is a follow-up to the previous reported trypanocidal potential of T. belirica dried fruits in this journal (Shaba et al. 2009). In this present paper, MPE of T. belirica dried fruits was evaluated for its in vitro (on different medium) and in vivo antitrypanosomal effects.

## MATERIALS AND METHODS

### CHEMICALS

Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol, acetic acid and ethyl acetate) for extraction of plant material 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 laboratory pestle and mortar and cold extracted twice with 200 ml of methanol (analytical grade) according to Stahl (1969). The filtrates were dried at 37 C and stored at 4 C until used.

## THIN LAYER CHROMATOGRAPHY (TLC) PLATES

This was done according to the method of Stahl, 1969. 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 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.

## 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)

## ANIMALS

Swiss albino mice (20-30 g) 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, Izatnagar, 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)

## PARASITE COUNTS

Estimation of parasite counts was carried out according to Lumsden et al. (1973).. 10 -15 fields of each drop of a blood or incubated media and parasites in triplicate were counted using glass slide under inverted microscope (400X) and an average means parasites count was taken as number of parasites per field.

## IN VITRO TRYPONOCIDAL ACTIVITY

In vitro trypanocidal activity was carried out by modified method of Oliveira et al. (2004). In this method, a Vero cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) in 96-well flat bottom microculture plates (Nunc, Denmark). Each well received 100 µl of DMEM containing 5x105 cells/ml. The plates were incubated at 37 C under 5% $CO_2$ for 48h to complete development of monolayer. After the formation of confluent monolayer, the medium (DMEM) was discarded and replaced with a fresh DMEM.. The medium was supplemented with 20-40% fetal calf serum (FCS) Gibco USA, and antibiotics (100 units penicillin, 100 µg streptomycin and 40 µg gentamycin). A high parasitemic blood from mouse was diluted with DMEM to obtain a final 1x106 parasites/ml. The suspension (100 ml of medium with parasites) was added at rate of 1: 1 to test MPE and the plate was incubated under the same conditions mentioned above. The test was repeated at least thrice.

Stock of test MPE of T. belirica dried fruits was solubilized in 1% dimethylsuphoxide (DMSO). The concentration in the experiment had no deleterious effect by itself on host cells or parasites. 1% DMSO in distilled water was used as control (Young, 2000)

## INFECTIVITY ASSESSMENT

After incubation for antitrypanosomal activity was completed, contents of wells with reduced and apparently killed parasites from MPE of T. 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% dimethylsuphoxide (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 et al.

2000).

## IN VIVO ANTITRYPANOSOMAL ACTIVITY

This was done according to the method of Freiburhgaus et al., (1998). Six mice per group were inoculated with trypanosomes (1x104 /ml). Mice were treated with both extract and fractions at concentrations (12.5-200 mg/kg body weight) of T. belirica dried fruits intraperitoneally 48 h post on set of parasitemia. 1% of DMSO was added to the test extract and diluted with DMEM. A drop of blood was taken from the tail-end of the mice daily and parasites were counted as previously described.

## STATISTICAL ANALYSIS

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

## RESULTS

Methanol was suitable (as previously reported by the same group on different medium during preliminary screening of the T. belirica dried fruits) in detecting the presence of bioactive constituents as observed on TLC plates. This is in accordance to the mobility of applied aliquots of extract on the plates (plates not shown). Solvent system methanol/chloroform (20: 80) was suitable in development of TLC plates. In vitro antitrypanosomal activity of MPE of T. belirica dried fruits were as given in Table 1. There was immobilization, reduction and complete killing of trypanosomes at different concentrations. At 250 µg/ml, trypanosomes were apparently killed at 4 h of incubation. Bioassay status of MPE extract 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 extract and negative control. ($P \leq 0.05$). In infectivity assessment, group of mice inoculated with contents of wells with apparently killed trypanosomes survived while, the other group of mice with reduced trypanosomes died of parasitemia. In vivo, mice treated with test extract of T. belirica dried fruits demonstrated trypanostatic effect (Table 2). At 200 mg/kg body weight test extract, mice in this group survived for 7days in comparison to 3 days in untreated negative control group 48 h post onset of paraitemia and commencement of treatment. .

**Figure 1**

Table 1. trypanocidal activity of methanolic extract of dried fruits on Vero cell line.

| Concentration of the plant extract in µg/ml | 1 h | 2 h | 3 h | 4 h | 5 h | 6 h | 7 h | 8 h | 9 h |
|---|---|---|---|---|---|---|---|---|---|
| 250 | 15.67±0.58 | 9.67±0.33 | 2.667±0.33 | 0.000±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| 500 | 12.33±0.67 | 5.667±0.58 | 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 |
| 750 | 10.67±0.33 | 4.667±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 | 0.0±0.0 |
| 1000 | 9.00±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 | 0.0±0.0 | 0.0±0.0 |
| Diminazine aceturate (50 ) Positive control | 22.33±0.33 | 9.333±0.67 | 1.000±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±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 | 40.00±0.0 | 40.000.0 | 40.000.0 | 40.000.0 |

Antitrypanosomal activity were expressed as mean ± SEM with significant difference ($P<0.05$)

**Figure 2**

Table 2 trypanocidal activity of methanolic extract of dried fruits in mice

| Concentration of test material in mg/kg body weight | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 |
|---|---|---|---|---|---|---|---|---|
| 12.5 | 5.833±0.70 0.70 | 13.00 ± 0.37 | 27.00 ± 0.58 | 42.83 ± 1.20 | | | | |
| 25 | 6.333± 0.42 | 12.00 ± 0.58 | 26.33 ± 0.33 | 38.83 ± 1.47 | | | | |
| 50 | 6.500± 0.43 | 11.50 ± 0.34 | 21.83 ± 0.31 | 32.50 ± 0.81 | 41.00 0.58 | | | |
| 100 | 6.500± 0.34 | 5.667 ± 0.49 | 15.17 ± 0.79 | 24.17 ± 0.70 | 40.80 0.97 | | | |
| 200 | 6.000± 0.26 | 5.500 ± 0.43 | 8.667 ± 0.33 | 12.67 ± 0.23 | 22.67 ± 0.80 | 33.33 ± 0.49 | 43.17 ± 0.70 | |
| Diminazine aceturate (10 ) Positive control | 6.167± 0.31 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| Control (Negative control) | 6.167± 0.31 | 13.50 ± 0.56 | 39.50 ± 0.43 | | | | | |

At dose rate of 200 mg/kg body weight, the mice in this group survived for 7 days post on set of parasitaemia. There was degree of significant difference ($P \leq 0.05$ ) between treated groups with test material in comparison to negative control that survived for only 3 days

## DISCUSSION

African trypanosomosis (either in animals or humans) has been described as neglected disease in our generation. Antitrypanosomal drugs currently in used were developed before 1950 when stringent laws on drugs development and uses were not in placed as today. These drugs are very toxic to the body system (Denis and Barret, 2001).

In this report, Presence of bioactive constituents on TLC plates and subsequent suitable solvent system used in

development of TLC plates is comparable to extraction and development of Terminalia chebula dried fruits (Shaba et al., 2007). At 250 µg/ml, trypanosomes in MPE of test extract were apparently killed at 4 h ,which was equivalent to diminazine aceturate (50 µg/ml) at 4 h after incubation. Sluggish movements and clumps formation of trypanosomes at the bottom of the wells indicated impact of the test extract. This result is comparable to in vitro trypanocidal activity of some screened American plants extracts against T. cruzi at 250 µg/ml (Muellas-Serrano et al., 2000) and. MPE of Picrorrhiza korroa rhizomes where trypanosomes were completely killed at 500 µg/ml. (Shaba et al., 2007). Mice inoculated with contents of wells with apparently killed trypanosomes during infectivity assessment 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) and MPE of Picrorrhiza korroa rhizomes (Shaba et al., 2007). 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). Gallic acid, which has been isolated from extract of T. belrica dried fruits may be partly responsible for the antitrypanosomal activity observed. This is because gallic acid has been reported to possessed anttrypanosomal activity (Koide et al., 1998). Trypanostatic effect was observed during in vivo testing of MPE of T. belirica dried fruits in mice. The in vitro antitrypanosomal activity result was not repeated during in vivo testing of extract in mice. There could be release of trypanosomes lodged in the body tissues that were not affected by the extract. Those trypanosomes would have been relapsed after blood plasma level of the test extract might have waned. This observation was previously reported by Benson, 1988 and Madubunyi, 1995, respectively. The differences in physiological state of mice body system compare to artificial in vitro medium may account for disparity in results.. Also, there may be possibility of ease of degradation of the test extract thereby unable to attain sustained maximum therapeutic blood plasma level for effective antitrypanosomal activity. There could be possibility of test extract binding to proteins in the mice leading to no availability of it for effective antitrypanosomal activity. This result is comparable to trypanocidal activities of the ethanolic extract of Nauclea latifolia root bark at 40-100 mg/kg body weight with decreased in parasitemia in infected- extract treated mice at dose-dependent manner (Madubunyi, 1995). Significant maximum trypanocidal activity of N. latifolia extract was recorded at 100 mg/kg body weight but later rise in parasitemia of mice was observed (Madubunyi, 1995).

It can be concluded from the present study that T. belirica dried fruits possess antitrypanosomal compound(s). Further studies (e.g. bioassay-guided purification to isolate pure compound(s) responsible for its antitrypanosomal activity is needed. The pure compound(s) to be obtained may prolong and enhance antitrypanosomal activity

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