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# *In-vitro* and *In-vivo* Anti-trypanosomal Activity of *Terminalia chebula* Retz Dried Fruits

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**Abstract:** African trypanosomosis had caused lots of havocs to both humans and animals over a century with successes and failure in curtailing it. This study was aimed at screening medicinal plant, *Terminalia chebula* dried fruits against *Trypanosoma evansi* for trypanocidal activity. Twenty grams of powdered *Terminalia chebula* dried fruits was cold extracted with methanol. Obtained MPE (methanolic plant extract) was *in vitro* tested against *Trypanosoma brucei* ($1 \times 10^6$ trypanosomes/mL of the medium in each ELISA plate wells) at concentrations (250~1,000 µg/mL) on Vero cells grown in DMEM (Debecco's Modified Eagle Medium) in appropriate conditions for trypanocidal activity. *In-vitro* cytotoxicity test of MPE of *Terminalia chebula* was conducted on Vero cells grown in DMEM. *In-vivo* assay for trypanocidal activity, each mouse was inoculated with $1 \times 10^4$/mL of trypanosomes and treated (48 h post inoculation) with MPE of *Terminalia chebula* at concentrations (12.5, 25, 50, 100 and 200 mg/kg body weight) were administered at dose rate of 100 µL per mouse via intraperitoneal route to different groups of mice, 6 mice per concentration. *In-vitro* cytotoxicity test was done on Vero cells at concentrations (1.58~100 µg/mL) of MPE of *Terminalia chebula*. Results of *in-vitro* trypanocidal activity varied from immobilization, reduction and to the killing of the trypanosomes. At 250 µg/mL of MPE of *Terminalia chebula* dried fruits, there was significant trypanocidal activity at 4 h of incubation and trypanosomes were not detected in corresponding ELISA plate wells at 5 h of incubation, which was statistically equivalent to reference drug, diminazine aceturate (50 µL/mL) at 4 h of incubation. Results of *in-vivo* trypanocidal activity revealed that at concentrations (12.5~25 mg/kg body weight) of MPE of *Terminalia chebula*, mice in these groups survived for 6 days. While at 50 and 100 to 200 mg/kg body weight, mice in these groups survived up to 7 and 8 days, respectively. *In-vitro* cytotoxicity test showed that all concentrations of MPE of *Terminalia chenula* and diminazine aceturate were cytotoxic to cells except at 1.56 µL/mL and 6.25 µL/mL. In conclusion, MPE of *Terminalia chebula* dried fruits possessed trypanocidal compounds. Further study (bioassay-guided purification) is required to know the full potential of *Terminalia chebula* as future trypanocide candidate.

**Key words:** *Terminalia chebula* dried fruits, *in-vitro* and *in-vivo* trypanocidal activity, *in-vitro* cytotoxicity.

## 1. Introduction

Trypanosomosis is a disease that has devastated certain parts of Sub-Saharan Africa with resultant untold consequences highlighted by different researchers in terms of increased in morbidity and mortality rates in of recent both in animals and humans as a result of resistance to available trypanocides with emergence of resistant strains of trypanosomes [1, 2]. In recent years, the disease has resurfaced and the vectors are climbing greater heights of highlands that hitherto it was not found in such places [2, 3]. The disease had rendered large cultivable fertile land unusable [3-6].

It is a zoonotic disease that affects both animals and humans caused by genus *Trypanosoma* but different species are responsible for its in animals (e.g., *Trypanosoma brucei*) and in humans (*Trypanosoma brucei gambiense*) and transmitted by tsetse flies (e.g., *Glossina morsitan morsitam*) [2].

Trypanosomosis is a menace in certain parts of Africa and Central America where it thrives [1, 7].

Resistance to limited classes of drugs available in the

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market has been reported at different parts of Africa and elsewhere the disease thrives [2, 3, 8].

Lots of local usages of herbs to treat trypanosomosis have been validated and documented [4, 8-11].

The available trypanocides are bedeviled with lots of problems, such as cost, toxicity, pock resistance to different classes of trypanocides and resistant strain of trypanosomes [12].

Lots of research works are ongoing in regard to antitrypanosomal extracts/compounds from medicinal plants for isolation of trypanocidal compounds [8-15].

*Terminalia chebula* Retz (*Combretaceae*) ("black myrobalan" in English) has been referred to as "king of medicines" in India sub-continent and Tibet. It is always listed first in the Ayurvedic materia medica because of its extraordinary powers of healing with a wide spectrum of biological activity [16].

In traditional medicine, *Terminalia chebula* has been used extensively in the indigenous system of medicine (Ayurvedic) for its homeostatic, antitussive, laxative, diuretic and cardiotonic activities [16, 17].

A lot of phytochemical constituents (e.g., tannic acid) have been isolated from *Terminalia chebula* fruits. The chief constituents of tannic acid are chebulic acid, chebulagic acid, corilagin and gallic acid. Tannic acid of *Terminalia chebula* is of pyrogallol (hydrolyzable) type [18-21].

Pharmacological activities, such as anti-ulcerogenic, neuroprotective, anti-convulsant, anti-oxidant and cardioprotective, have been documented [22-26].

On this resounding note, *Terminalia chebula* dried fruits were screened for trypanocidal activity

## 2. Materials and Methods

### *2.1 Plant Material*

*Terminalia chebula* Retz dried fruits of the family combretaceae were obtained from reputable Ayurvedic shop from hilly region of Palampur, Himachal Pradesh. *Terminalia chebula* dried fruits were identified by Institute of Himalaya Biosource Technology, Palampur, Himachal Pradesh, India.

### *2.2 Extraction*

Twenty grammes of *Terminalia chebula* dried fruits were pounded into powder with pestle and mortar and cold extracted twice with 200 mL of methanol (analytical grade) according to Ref. [27]. The filtrates were dried at 37 °C and stored at 4 °C until used.

### *2.3 TLC (Thin Layer Chromatography) Plates*

Aliquots (0.2 mL) of MPE of *Terminalia chebula* dried fruits extract was applied on TLC plates, which were 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 TLC plates were immersed in iodine vapours in a glass jar. Second set of TLC plates were sprayed with vanillin-sulphuric acid spray. Both used media facilitated the detection of bioactive constituents. This was carried out according to the method of Stahl [27].

### *2.4 Solvent Systems Applied*

The following solvent systems were tested to develop the TLC plates according to the method of Stahl [27]:

(1) chloroform/hexane/acetic acid (50:50:1);
(2) chloroform/ethyl acetate/acetic acid (50:50:1);
(3) methanol and chloroform (20: 80).

### *2.5 Test Organism*

*Trypanosoma evansi* was obtained from the Division of Parasitology, IVRI (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 [28, 29].

### *2.6 In-vitro Tryponocidal Activity*

*In-vitro* trypanocidal activity was carried out by modified method of Oliveira et al. [7]. In this method, a

Vero cell line was grown in DMEM (Dulbecco's Modified Eagle Medium) (*Sigma*) in 96-well flat bottom micro culture plates (Nunc, Denmark). Each well received 100 μL of DMEM containing $5 \times 10^5$ cells/mL. The plates were incubated at 37 °C under 5% $CO_2$ for 48 h to complete development of monolayer. After the formation of confluent monolayer, the medium (DMEM) was discarded and replaced with a fresh DMEM. And the medium was supplemented with 20~40% FCS (fetal calf serum), Gibco USA and antibiotics (100 units penicillin, 100 μg streptomycin and 40 μg gentamycin). A high parasitaemic blood from mouse was diluted with DMEM to obtain a final parasite of $1 \times 10^6$ parasites/mL. The suspension (100 mL of medium with trypanosomes was added at rate of 1:1 to MPE of *Terminalia chebula* dried fruits at concentrations (250~1,000 μg/mL). The suspension (100 mL of medium with trypanosomes) was added at rate of 1:1 to test extract and the plates were incubated at 37 °C under 5% $CO_2$. The mixture was incubated for 9 h. The test was repeated at least thrice .and the plate was incubated under the same conditions mentioned above. The test was repeated at least thrice.

Stock of test MPE was solubilized in 1% DMSO (dimethylsuphoxide). The concentration in the experiment had no deleterious effect by itself on host cells or parasites. The 1% DMSO in distilled water was used as control [30].

*2.7 In-vivo Infectivity Assessment*

After incubation for anti-trypanosomal activity was completed, contents of microculture plate wells that contained reduced and apparently killed trypanosomes from methanolic extract of *Terminalia chebula* dried fruits were inoculated (0.1 mL/mouse) into two groups of mice (six groups-1) via intra-peritoneal, and observed for more than 30 days for parasitaemia [31, 32].

*2.8 In-vitro Cytotoxicity Test*

Cytotoxic effects of the methnolic extract of *Terminalia chebula* were determined according to the method described by Sidwell and Hoffman [33]. Vero cell line was grown in DMEM (*Sigma*) Gibco, USA antibiotics (100 units penicillin, 100 μg streptomycin and 40 μg gentamycin) in 96-well flat bottom microculture plates (Nunc, Denmark). Each well received 100 μL of DMEM containing $5 \times 10^5$ cells/mL. The plates were incubated at 37 °C under 5% $CO_2$ for 48 h. After the formation of confluent monolayer, the medium was discarded and replaced with a fresh one. A high parasitaemic blood from mouse was diluted with DMEM to obtain a final parasite of $1 \times 10^6$ parasites/mL of the medium. Confluent monolayer of Vero cell was treated with serial dilutions of test methanolic extract (1.56~100 μg/mL) in triplicate and incubated under the same conditions described previously. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects. The plate was incubated for 72 h and observed daily. It was repeated thrice. In each case, after 72 h of incubation, the culture media 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 h at 37 °C in an ordinary incubator. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects.

*2.9 In-vitro Assay for Tryponocidal Activity*

After the completion of *in-vitro* trypanocidal activity, extract *Terminalia chebula* under investigation was tested in mice for *in-vivo* trypanocidal activity. Solvent in the extract was removed according to the method of Freiburghause et al. [34]. The MPE of *Terminalia chebula* was dissolved in dimethyl sulfoxide at rate of 5 mg/100 mL. Different concentrations of the extract were used to test the efficacy of the trypanocidal activity/clearance of trypanosomes (48 h post inoculation). Mice, each inoculated with $1 \times 10^4$/mL of trypanosomes were divided into 6 animals. Test extract concentrations (12.5, 25, 50, 100 and 200 mg/mL) were

inoculated into mice at dose rate of 100 μL per mouse via intraperitoneal route consecutively for 3 days The mice of positive control group were treated with diminazine aceturate at 10 mg/kg body weight. Negative control group were administered DMSO in distilled water. The efficacy of the test sample was assessed microscopically [29] and mean survival period of mice between the treated and control mice for at least 30 days.

*2.10 Institute Committee on Welfare and Cruelty to Animals*

Indian Veterinary Research Institute Committee on Welfare and Cruelty to Animals received and approved application for the usage of mice in this research.

*2.11 Statistical Analysis*

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

## 3. Results

Results of this investigation are presented in Tables 1-3.

*3.1 Extraction*

Judging from bioactive constituents present in MPE of *Terminalia chebula* as observed on the TLC plates, solvent, methanol was suitable for its extraction.

*3.2 Solvent System*

Out of three solvent systems tested in the analysis of TLC plates with applied aliquots of *Terminalia chebula*, solvent system, methanol/chloroform (20:80), was more suitable than other solvent systems tested (plates not shown). On the TLC plates, different patterns of bioactive constituents were on display from extract of *Terminalia chebula,* which were subsequently responsible for anti-trypanosomal activity.

*3.3 In-vivo Infectivity Test*

Group of mice inoculated with contents of ELISA plate wells with completely killed trypanosomes survived for more than 60 days, while other group inoculated with contents of ELISA plate wells with reduced trypanosomes count died of parasitemia.

*3.4 In-vitro Cytotoxicity Test*

During the *in-vitro* cytotoxicity test of MPE of *Terminalia chebula* dried fruits, and diminazine aceturate at the same concentrations on Vero cells depicted different cytotoxic effects such as distortion, swelling, sloughing and death of Vero cells compared to negative normal cells in control wells (Table 3).

*3.5 In-vitvo Trypanocidal Activity*

Results of *in-vitro* trypanocidal activity showed that at 250 μg/mL, MPE of *Terminalia chebula* exhibited strong trypanocidal activity. Trypanocidal activity varied from immobilization, reduction and to the killing of trypanosomes. At the same concentration, there was complete killing of trypanosomes in corresponding ELISA plate wells at 5 h of incubation, which was statistically the same as diminazine aceturate (50 μg/mL) standard drug at 4 h. Average mean trypanosomes counts from 37.67 ± 0.58 and below is significant between the treatment groups and negative control ($P \leq 0.05$~$0.01$).

*3.6 In-vitvo Assay for Tryponocidal Activity*

*In vivo*, mice treated with MPE of *Terminalia chebula* at concentrations (12.5~25 mg/mL) survived up to Day 6 at the onset of treatment 48 h post inoculation of trypanosomes. Mice in these groups treated with MPE of *Terminalia chebula* concentrations (50, 100 and 200 mg/mL) survived up to Days 7 and 8, respectively.

However, mice treated with MPE of *Terminalia chebula* at a dose of 200 mg/kg body weight had a maximum survival time of 8 days.

**Table 1** ***In-vitro*** **trypanocidal activity of methanolic extract of** ***Terminalia chebula*** **dried fruits against** ***Trypanosma evansi on*** **Vero cell line.**

| Concentration of the plant extract (µg/mL) | 1 h | 2 h | 3 h | 4 h | 5 h | 6 h | 7 h | 8 h | 9 h |
|---|---|---|---|---|---|---|---|---|---|
| 250 | 18.67 ± 0.33 | 11.67 ± 0.33 | 6.667 ± 0.33 | 0.667 ± 0.33 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 500 | 15.00 ± 0.0 | 10.67 ± 0.33 | 5.667 ± 0.33 | 1.000 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 750 | 12.67 ± 0.33 | 9.000 ± 0.0 | 3.333 ± 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 |
| 1,000 | 10.00 ± 0.0 | 4.667 ± 0.33 | 0.6667 ± 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 |
| Berenil (50) | 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 | 40.00 ± 0.0 | 40.00 ± 0.0 | 40.00 ± 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 observation. 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 ($P \leq 0.05$~$0.01$).

**Table 2** ***In-vivo*** **trypanocidal activity of methanolic extract of** ***Terminalia chebula*** **dried fruits in mice.**

| Concentration of test material (mg/kg body weight) | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 |
|---|---|---|---|---|---|---|---|---|
| 12.5 | 6.000 ± 0.26 | 12.33 ± 0.33 | 28.50 ± 0.99 | 39.35 ± 0.55 | - | - | - | - |
| 25 | 6.167 ± 0.31 | 11.50 ± 0.43 | 25.83 ± 0.54 | 35.17 ± 1.35 | - | - | - | - |
| 50 | 6.167 ± 0.31 | 10.67 ± 0.71 | 24.67 ± 0.61 | 37.00 ± 0.64 | 41.67 0.10 | - | - | - |
| 100 | 6.167 ± 0.31 | 6.500 ± 0.56 | 12.17 ± 0.48 | 27.50 ± 0.43 | 37.17 ± 0.87 | 43.67 0.56 | - | - |
| 200 | 6.667 ± 0.49 | 6.667 ± 0.33 | 11.33 ± 0.49 | 17.83 ± 0.40 | 31.83 ± 0.60 | 41.33 ± 1.23 | - | - |
| 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<br>0.0 | 0.0<br>0.0 | 0.0<br>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 6 days post on set of parasitaemia. There was degree of significant difference between treated groups with test material in comparison to negative control that survived for only 3 days.

**Table 3 Cytotoxic effect of methanolic plant extract of** ***Terminalia chebula*** **dried fruits on Vero cell line compared to diminazine aceturate (Berenil).**

| Concentration of test material (µg/mL) | Effects of test extract at various periods of incubation | | | | | | Control |
|---|---|---|---|---|---|---|---|
| | 24 h | | 48 h | | 72 h | | |
| | *Terminalia chebula* | Berenil | *Terminalia chebula* | Berenil | *Terminalia chebula* | Berenil | |
| 100 | 100% | 66.6% | 100% | 100% | 100% | 100% | 0 |
| 50 | 100% | 33.3% | 100% | 100% | 100% | 100% | 0 |
| 25 | 100% | 0 | 100% | 100% | 100% | 100% | 0 |
| 12.5 | 100% | 0 | 100% | 0 | 100% | 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 chebula* and diminazine aceturate were toxic to Vero cell line except at concentrations of 1.56 and 6.25~1.56 µg/mL. Same concentrations were used for diminazine aceturate (Berenil).

## 4. Discussion

*4.1 Extraction*

This method of extraction of MPE of *Terminalia chebula* with methanol is comparable to previous work carried out by the same authors such as extractions of *Plumbago zeylanica* root bark and *Vitex negundo* leaves that result in maximum yield of MPE of extracts [35, 36] and extraction of *Entada Abyssinia* [34].

*4.2 TLC (Thin Layer Chromatography) Plates Analysis*

Development and analysis of TLC plates via solvent system, methanol/chloroform (20:80), with applied aliquots of extract of *Terminalia chebula* is comparable to analysis of TLC plates with applied aliquots of *Plumbago zeylanica* root bark [35] and *Picrorrhiza kurroa* rhizomes [37], respectively.

*4.3 In-vitro Trypanocidal Activity*

In this study, *in-vitro* trypanocidal activity of *Terminalia chebula* with varied *in vitro* activity, which is directly corresponding to distinct MPE concentrations in the microtiter plates is comparable with antitrypanosomal activity of previous work reported by [10, 37-39] and Freiburghause et al. [34] in which different solvents extracted bioactive constituents presence in the extracts of medicinal plants and demonstrated anti-trypanosomal activity at different concentrations.

*4.4 In-vivo Infectivity Test*

*In-vivo* infectivity assessment of trypanocidal activity is comparable to anti-trypanosomal effects of the aqueous extract of *Brassica oleracea* buds (fruits), MPES of *Ageratum houstonionum* flowers and *Moringa oleifera* (bark of the tree and seed pods) where inoculated mice with contents of wells with apparently killed trypanosomes survived, while the other group of mice inoculated with reduced or immobilized trypanosomes died of parasitemia [10, 11, 31].

*4.5 In-vitro Trypanocidal Activity*

In this investigation, *in-vitro* trypanocidal activity of MPE of *Terminalia chebula* dried fruits is comparable with previous work reported by [37, 39] and Igweh et al. [31] whereby distinct medicinal plants and extracted bioactive constituents exhibited marked trypanocidal activities.

It is possible that the trypanocidal activity of *Terminalia chebula* may be due to one of its chemical constituents, gallic acid, which its trypanocidal activity has been reported [40]. This is in addition to chelate of trypanosomes DNA by extracts/purified compounds of medicinal plants that leads to death of the trypanosomes [12].

*4.6 In-vitro Cytotoxicity Test*

In this *in-vitro* cytotoxicity test, both MPE of *Terminalia chebula* and diminazine aceturate were cytotoxic to Vero cells in all concentrations except at 1.56 and 6.25 µg/mL.

These results are in a line with *in-vitro* cytotoxicity tests of extraction of *Ageratum houstonionum* flowers and 50% methanolic extract of *Khaya senegalensis* tree bark in which similar cytotoxic effects were observed [11, 41].

*4.7 In-vitvo Assay for Tryponocidal Activity*

*In-vivo* trypanocidal activity of MPE of *Terminalia chebula* dried fruits was moderate in comparison to *in-vitro* trypanocidal activity. This result is comparable to that of *in-vivo* testing of dichloromethane and methanol extracts of *Doyadis abyssinica* leaves at concentrations (100, 150, 200 and 250 mg/kg body weight) against *Trypanosoma congolense* in which at higher doses (200 and 250 mg/kg body weight) there was reduction in parasitemia and prolongation of albino mice life span, though none cure the mice, was observed in comparison to negative control group [42]. Also, this result is in line with trypanocidal potential of methanolic extract of *Camellia sinensis* leaves against

*Trypanosoma evansi* in which highest dose (200 mg/kg body weight) prolonged the lifespan of the mice for 6 days [38].

Probably the reasons for limited *in-vivo* trypanocidal activity could be attributed to unavailability of the MPE (e.g., protein binding) in reasonable amount to maintain the plasma level to kill the trypanosomes, ease of degrading of MPE and toxicity of MPE as observed in *in-vitro* cytotoxicity test.

## 5. Conclusions

It could be concluded from this study that, MPE of *Terminalia chebula* dried fruits exhibited significant trypanocidal activity as demonstrated in *in-vitro* test, while *in-vivo* test in mice had moderate trypanocidal activity. This indicates the presence of trypanocidal compound(s) in the MPE of test extract. These results pave way for further studies (e.g., bioassay-guided purification, isolation of trypanocidal compound(s) and *in-vivo* testing) to pinpoint trypanocidal compound(s) and possibly, a lead to new trypanocide.

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