Trypanocidal Activity of Comparative Extractions of Embilical Officinalis (Syn: Phyllantus Fruits With Solvent of Different Polarities


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Trypanocidal Activity of Comparative Extractions of Embilica Officinalis (Syn: Phyllanthus emblica) Fruits With Solvent of Different Polarities.


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Abstract

Previously, preliminary studies of trypanocidal activity of Embilica officinalis fruits were reported. In continuation of the search for trypanocidal compounds, E. officinalis fruits were comparatively extracted with solvents of different polarities (hexane, chloroform, methanol and aqueous) and screened against Trypanosoma evansi. A high parasitemic blood of a mouse was diluted with Alsever solution to obtain a final parasite concentration of 1x10^6 parasites/ml. The medium consist of Alsever solution and inactivated bovine serum at 37°C for 1 h. The suspension (100 ml of medium with parasites) was added at rate of 1:1 to test extracts and the plates were incubated at 37°C under 5% CO₂. Infectivity assessment of incubated aqueous and hexane extracts of E. officinalis fruits and medium with trypanosomes was done in mice. The order of trypanocidal activity was aqueous, methanol, chloroform and hexane extracts. Extracts of E. officinalis exhibited trypanocidal activity, which ranged from immobilization to the killing of trypanosomes At 250 µg/ml, trypanosomes were not detected in aqueous and methanol extracts at 4 h and 5 h of incubation. At 500 µg/ml, no trypanosome was detected in chloroform and hexane extracts. Trypanosomes counts decreased in concentration and time – dependent manner with significant difference (P<0.05). In infectivity assessment, group of mice inoculated with contents of wells with apparently killed trypanosomes (aqueous extract) survived for more than 60 days. There was marked trypanocidal activity.

Keywords: iEmbilica officinalis (fruits), comparative extractions, trypanocidal activity

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Abbreviations

DMSO  Dimethysulfoxide  
MPE   Methanolic plant extract  
DMEM  Dulbecco’s Modified Eagle Medium  
DA    Diminazine aceturae

Introduction

Trypanosoma evansi is one of the species of trypanosomes that causes trypanosomosis in animals (WHO, 2004). Trpanosomosis has been a serious parasitic disease and of zoonotic importance of which its resurgence in both animals and humans is worrisome (WHO, 2004). The disease is on the increase in endemic regions of the world such as Africa and Latin America (WHO, 2002). About 3 billion pounds are lost annually in Africa from animal trypanosomosis interms of lost in different aspects of animal production and draught (Hursey, 2000).

Available trypanocides are faced with resistance, which is one of the major problems militating against its control (Doua and Yapo, 1993) and by parasites (Ross and Sunderland, 1994) had hampered effective treatment and control.

Reports of naturally active extracts/compounds against trypanosomes from medicinal plants have been reported (Atawodi et al., 2005; Shaba et al., 2009 and Shaba et al., 2012ab).

From folk medicine perspective, E. officinals fruits (Syn. Phyllanthus embilica) (Euphorbiaceae) has been used as anti-inflammatory, stomach ache and antipyretic by rural population in India (Prakash et al., 2002). Phytochemical compounds such as triterpene; Kaempherol-3-0-B-D, quercentin-3-0-B-D, glucose, gallic acid-benzenoid and emblicol phylemblic (El.Mekkay et al., 1995; ascorbic acid and astragal (Prakash et al., 2002) were isolated from E. officinalis fruits.

Due to aforementioned problems bedeviling the affected animals and the limited classes of drugs in use for nearly a century with new drug insight, E. officinalis fruits were comparatively evaluated for its trypanocidal activity.

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 materials and development/analysis of TLC plates, vanillin for spray, and iodine for detection of bioactive constituents were used which were purchased from E. Merck, India.

Plant materials
Embilica officinalis L (Phyllantus embilica) fruits were purchased from reputable Ayurveda shop at Palampur 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 dried E. officinalis fruits were obtained from a reputable Ayurveda shop at Palampur. The dried fruits of E officinalis were powdered using laboratory pestle and mortar. The powdered E. officinalis fruits were first cold extracted with 200 ml of hexane. Then, the obtained residue from first extraction was subsequently extracted with 200 ml of chloroform, methanol, and aqueous (analytical grade) consecutively in that order. This was repeated at least thrice. The filtrates were separately combined, dried at 37oC and stored at 4oC until used.

Solvent systems
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)

Thin Layer Chromatography (TLC) plates
Aliquots (0.2 ml) of each extract was applied on TLC plates, dried under room temperature and immersed inside the appropriate solvent systems in a glass jar. It was done to detect the presence of bioactive constituents in applied extract. This was, also, done following the method of Stahl (1969).
Animals
Swiss albino mice (20-30 g) 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 Animals of the institute.

Test organism
*T. evansi* was obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar and was maintained in the laboratory by serial sub-pasages in Swiss albino mice. The strain was routinely tested for virulence following the method of Williamson et al., (1982).

Parasite count
Counting of parasite was carried out following the method of Lumsden et al., (1973). A number of fields (10-15) of each drop of blood or incubated media and parasites 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
A high parasitemic blood of a mouse was diluted with Alsever solution to obtain a final parasite concentration of 1x10⁶ parasites/ml. The medium consist of Alsever solution and inactivated bovine serum at 58°C for 1 h. The suspension (100 ml of medium with trypanosomes) was added at rate of 1:1 to test different extracts of *E. officinalis* fruits and the plates were incubated at 37°C under 5% CO₂ for 5 h of incubation. The test was repeated at least thrice.

Stock of test MPE of *E. officinalis* 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 et al., 2000).

In vivo infectivity assessment
When incubation for anti-trypanosomal activity was completed, contents of wells with aqueous and hexane extracts with apparently killed and reduced trypanosomes with MPE of *E. officinalis* fruits were inoculated (0.1ml mouse-1) into two groups of mice (six group-1 intra-peritoneal, and observed for more than 30 days for parasitemia (Woo, 1970; Igweh et al., 2002).

Statistical Analysis
Results of trypanocidal activity were expressed as mean ± SEM. Statistical analysis was done using Sigma stat (Jandel, USA).

Results and Discussion

Extraction
In this report, different solvents (hexane, chloroform and methanol) and aqueous extract of *E. officinalis* fruits at different levels of bioactive constituents were observed on TLC plates (plates not shown). These solvents of different polarities used in extraction of *E. officinalis* fruits consecutively are comparable to *in vitro* trypanocidal activity of comparative extraction of *Terminalia belirica* dried fruits with solvent of different polarities (Shaba et al., 2009) and effects of solvent type on phenolics and flavonoids content and antioxidant activities in two varieties of young ginger (*Zingiber officinale* Roscoe) extracts (Ali et al., 2011).

Thin Layer Chromatography Plates Analysis
Solvent system, methanol/chloroform (20:80), was more suitable than other solvent systems tested in the analysis of thin layer chromatography (TLC) plates with applied aliquots of plant extracts. Iodine and vanilli-sulphuric acid were suitable in detecting the presence of bioactive constituents in test extract as observed on developed TLC plates. TLC plates (plates not shown) showed different patterns of bioactive constituents of *E. officinalis*, which was subsequently responsible for trypanocidal activity in distinct extracts. This method is comparable to that used by Freiburghaus et al., (1998) in bioassay-guided isolation of a diasterolisomer of kolavenol from *Entada Abyssinia* active on *T. brucei rhodesiense*, extractions of *Picrorrhiza kurroa* rhizomes (Shaba et al., 2012a) and *Zanthoxylum alatum* leaves and *Eugenia caryophyllatum* buds (fruits) (Shaba et al., 2012b) .
In Vitro Trypanocidal Activity

Results of in vitro trypanocidal activity of extracts of E. officinalis fruits at different concentrations (250-1000 µg/ml) are as given in Tables 1-4. The order of trypanocidal activity was aqueous, methanol, chloroform and hexane extracts. Extracts of E. officinalis exhibited trypanocidal activity, which ranged from immobilization to the killing of trypanosomes. At 250 µg/ml, trypanosome was not detected in aqueous and methanol extracts of E. officinalis, at 4 and 5 h of incubation. At 500 µg/ml, no trypanosome was detected in chloroform and hexane extracts. Trypanosomes counts decreased in concentration and time –dependent manner with significant difference (P<0.05). At the same concentration, there was reduction and complete killing of the trypanosomes in corresponding ELISA plates wells at 5 h of incubation, which was equivalent to diminazine aceturate (50 µg/ml) at 4 h. Trypanocidal activity of MPE of E. officinalis fruits has a wide range. Results of trypanocidal activity of different extracts of E. officinalis are comparable to in vitro trypanocidal activity of comparative extractions of Terminalia belirica dried fruits with solvent of different polarities against Trypanosoma evansi, in which the order of trypanocidal activity was methanol, aqueous, hexane and chloroform extracts, respectively (Shaba et al., 2009) and in vitro extraction of some Nigeria medicinal plants with petroleum ether, chloroform, methanol and aqueous of which most higher activity was observed in methanolic extract (Atawodi, 2005). An average mean trypanosomes 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 ≤ 0.05).

Table 1: Hexane extract

<table>
<thead>
<tr>
<th>Concentration of test material in µg/ml</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>37.33 ± 0.33</td>
<td>31.00 ± 0.58</td>
<td>21.67 ± 0.33</td>
<td>11.33 ± 0.67</td>
<td>1.00 ± 0.58</td>
</tr>
<tr>
<td>500</td>
<td>37.67 ± 0.33</td>
<td>13.67 ± 0.88</td>
<td>7.33 ± 0.33</td>
<td>0.67 ± 0.33</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>750</td>
<td>12.00 ± 0.98</td>
<td>4.67 ± 0.88</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1000</td>
<td>7.33 ± 0.88</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Diminazine aceturate (50 )</td>
<td>22.33± 0.33</td>
<td>9.000±0.58</td>
<td>1.333±0.33</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Negative)</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
</tr>
</tbody>
</table>

Bioassay status: significant reduction of parasites counts from concentration of 250 µg/ml and complete killing of parasites at 500 µg/ml at 5th 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 ≤ 0.05 to 0.01).

Table 2: Chloroform extract

<table>
<thead>
<tr>
<th>Concentration of test material in µg/ml</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>36.33 ± 0.33</td>
<td>33.33 ± 0.67</td>
<td>20.33 ± 0.33</td>
<td>8.33 ± 0.98</td>
<td>1.00 ± 0.58</td>
</tr>
<tr>
<td>500</td>
<td>24.00 ± 0.58</td>
<td>13.33 ± 0.88</td>
<td>4.33 ± 0.33</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>750</td>
<td>12.33 ± 0.33</td>
<td>4.00 ± 0.98</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1000</td>
<td>6.33 ± 0.33</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Diminazine aceturate (50 )</td>
<td>22.33± 0.33</td>
<td>9.000±0.58</td>
<td>1.333±0.33</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Negative)</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
</tr>
</tbody>
</table>

Bioassay status: significant reduction of parasites counts from concentration of 250 µg/ml and complete killing of parasites at 500 µg/ml at 4th 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 ≤ 0.05 to 0.01).
During the preliminary screening of *E. officinalis* fruits against *T. evansi*, which was published by the same journal (Shaba et al., 2012c), methanol appeared to be suitable in the extraction. But with the usage of solvents of different polarities, the aqueous medium appeared to be more suitable than methanol solvent in extraction of bioactive constituents as observed on the TLC plates. This stressed the importance of comparative extractions using different solvents of polarities at this point; it is difficult to determine the exact bioactive constituents responsible for its antitypanosomal activity and mechanism of action. However, trypanocidal activity of MPE of *E. officinalis* could be due to any of already isolated bioactive constituents mentioned above. For instance, gallic acid benzenoid may be partly responsible for antitypanosomal activity of *E. officinalis.* This is because gallic acid was reported to possessed anttrypanosomal activity (Koide et al., 1998).

Mechanism of action may be due to intercalation of MPE of *E. officinalis* with DNA leading to death of trypanosomes, blockage of glycolysis pathway and interference with flagella, which temporarily immobilizes trypanosomes (Denise and Barret, 2001).

**In vivo Infectivity Test**

*In vivo* infectivity assessment showed that group of mice inoculated with contents of wells of aqueous extract with apparently killed trypanosomes survived for more than 60 days. While, the other group of mice inoculated with hexane extract died of parasitaemia. This illustrates the potential trypanocidal activity of *E. officinalis* fruits. *In vivo* infectivity assessment results are comparable to that of aqueous extract of *in vitro* trypanocidal activity of comparative extraction of *Terminalia belirica* dried fruits with solvent of different polarities against *Trypanosoma evansi,* where mice inoculated with aqueous extract survived and that of hexane extract died of parasitaemia (Shaba et al., 2009).

In acute toxicity test, all the mice inoculated with the aqueous extract survived.

### Table 3: Methanolic extract

<table>
<thead>
<tr>
<th>Concentration of plant extract in µg/ml</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>37.67±0.33</td>
<td>32.00±0.58</td>
<td>11.33±0.33</td>
<td>2.00±0.58</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>500</td>
<td>19.67±0.33</td>
<td>1.667±0.33</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>750</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>1000</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Diminazine aceturate (50 µg/ml) Positive control</td>
<td>22.33±0.33</td>
<td>9.000±0.58</td>
<td>1.333±0.33</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Control (Negative control)</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
</tr>
</tbody>
</table>

Bioassay status: significant reduction of parasites counts from concentration of 250 µg/ml and complete killing of parasite at same concentration at 5th 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 ≤ 0.05 to 0.01).

### Table 4: Water extract

<table>
<thead>
<tr>
<th>concentration of test material in µg/ml</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>38.67 ±0.33</td>
<td>26.33 ±0.58</td>
<td>9.33 ±0.58</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>500</td>
<td>35.33 ± 0.33</td>
<td>10.33 ± 0.33</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>750</td>
<td>21.34 ± 0.33</td>
<td>0.67 ± 0.33</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1000</td>
<td>11.033 ± 0.34</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Diminazine aceturate (50 µg/ml) Positive control</td>
<td>22.33±0.33</td>
<td>9.000±0.58</td>
<td>1.333±0.33</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Control (Negative)</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
<td>40.00±0.0</td>
</tr>
</tbody>
</table>

Bioassay status: significant reduction of parasites counts from concentration of 250 µg/ml and complete killing of trypanosomes at 4th 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 ≤ 0.05 to 0.01).
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Table 5: Acute toxicity test of methanolic extract of *Emblica officinalis* fruits in mice

<table>
<thead>
<tr>
<th>Number of mice per extract</th>
<th>Body weight of mice in gms</th>
<th>Type of plant extract</th>
<th>Concentration used (2000mg/kg body weight)</th>
<th>Observation (Toxicity signs and mortality)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td><em>Emblica officinalis</em></td>
<td>0.27 ml</td>
<td>The mice Survived without any apparent toxic signs observed</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td></td>
<td>0.28 ml</td>
<td>- do-</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td></td>
<td>0.29 ml</td>
<td>- do-</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td></td>
<td>0.26 ml</td>
<td>- do-</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td></td>
<td>0.28 ml</td>
<td>- do-</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td></td>
<td>0.27 ml</td>
<td>- do-</td>
</tr>
</tbody>
</table>

Conclusion

It can be concluded from these findings that there is (are) presence of trypanocidal compound(s) from *E. officinalis* fruits at different levels as per the contents of bioactive constituents as observed from TLC plates, *in vitro* trypanocidal activity and *in vivo* infectivity tests. Acute toxicity test proved that the extract at doses used were not toxic to the mice. Further investigation will be carried out (e.g. bioassay-guided purification/*in vivo* test, etc.) to isolate the compound(s) responsible for its trypanocidal activity.

Acknowledgement

Financial contributions towards research by India and Nigeria governments are highly acknowledged.

References


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