

# Therapeutic Activity of Partially Purified Fractions of *Emblica officinalis* (Syn. *Phyllanthus emblica*) Dried Fruits against *Trypanosoma evansi*

Shaba Peter<sup>1</sup>, Sahab Dey<sup>1</sup>, Bhanuprakash Veerakyathappa<sup>2</sup>, Singh Raj Kumar<sup>2</sup> and Chaudary Paulad<sup>3</sup>

1. Division of Medicine, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

2. Indian Veterinary Research Institute, Regional Station, Mukteswar, Uttranchal 263 138, India

3. Division of Bacteriology and Biotechnology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

**Abstract:** *Emblica officinalis* (*E. officinalis*) dried fruits were evaluated for its antitrypanosomal activity and cytotoxic effects. Vero cell line maintained in DMEM (Dubecco's Modified Eagle Medium) and incubated with *Trypanosoma evansi* for more than 12 h. MPE was added to the Vero cell culture medium at different concentrations (250~1,000 µg/mL) with trypanosomes concentration ( $1 \times 10^6$  trypanosomes/mL in each ELISA plate well) and incubated at appropriate conditions for 72 h. *In-vitro* cytotoxicity of MPE of *E. officinalis* was determined on Vero cells at concentrations ((1.56~100 µg/mL). Acute toxicity and *in-vivo* infectivity tests were done in mice. Obtained MPE of *E. officinalis* underwent process of purification via column chromatography, preparative chromatography and HPLC (higher performance liquid chromatography) with bioassay at different strata on Alsever's medium. *In-vivo* assay for trypanocidal activity, MPE and PPFs (partially purified fractions) of *E. officinalis* with two sets of mice, each mouse was inoculated with  $1 \times 10^4$ /mL of trypanosomes and treated (48 h post inoculation) 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 (in treating parassitemic mice) to different groups of mice, 6 mice per concentration. HPLC of partially purified fractions of *E. officinalis* was carried out with mobile phase of acetonitrile: water (40:60) in gradient mode. *In vitro*, MPE induced immobilization and killing of the parasites in concentration-time dependent manner. Significant reduction of trypanosomes counts from concentration of 250 µg/mL and complete killing of trypanosomes at 5th hour of observation, which was statistically equivalent to 4th hour of Diminazine Aceturate (Berenil), standard reference drug used. HPLC of the partially purified fractions revealed two major prominent peaks at retention time of 1~4 min. *In vivo*, both MPE and PPFs of test material did prolong lives of mice by 6~9 days but could not cure them. At concentration of 2,000 kg/kg body weight of MPE in acute test, all mice survived. For *in-vivo* infectivity test, mice injected with immobilized trypanosomes developed parasitemia and died while, the other group survived. MPE, PPFs and Diminazine Aceturate were toxic to Vero cells at all concentrations exception of 1.56, 1.56~3.13 and 1.56~6.25 µg/mL, respectively. From this report, PPFs of *E. officinalis* dried fruits demonstrated potential pathway for a new development of trypanocide in near future if additional investigations are put in place.

**Key words:** *Emblica officinalis* dried fruits, *in vitro* and *in vivo* partially purified fractions trypanocidal activity, *Trypanosoma evansi*, *in vitro* cytotoxicity.

## 1. Introduction

Trypanosomosis, a disease caused by blood protozoan parasites of genus *Trypanosoma*, is on the increase in endemic regions, (e.g., Africa and Latin America), where millions of population and cattle are

affected with considerable morbidity and mortality, which are due to largely emergence of resistant strains of trypanosomes, vectors reaching new heights of highlands and occupying vast lands that are now uncultivable, and in addition to resistant to available trypanocides in endemic areas of the world where trypanosomosis thrives for decades [1, 2]. It is an important parasitic disease of veterinary and medical sciences [2, 3]. The disease is caused by distinct agents,

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**Corresponding author:** Shaba Peter, Ph.D., chief lecturer, research fields: alternative medicine-major, animal health related diseases and basic anatomical structures of domestic animals.

such as *Trypanosoma evansi* in animals as to *Trypanosoma brucei rhodesiense* in humans [2]. Human African trypanosomiasis, for instance, affects more than 60 million people with 300,000~500,000 new cases per annum [4-6]. About 3 billion pounds are lost annually in Africa from animal trypanosomiasis [7].

Reports of resistance to available trypanocides [8] and by trypanosomes [1, 2] had hampered effective treatment and control.

Reports of naturally active extras/fractions/compounds from medicinal plants against trypanosomes have been reported [9-14].

In folk medicine, *Emblica officinalis* (*E. officinalis*) dried fruits (Syn. *Phyllanthus emblica*) (Euphorbiaceae) has been used as anti-inflammatory, anti-stomach ache, antipyretic, and in disease conditions, such as anemia, jaundice, dyspepsin, scabies and itch, nausea and emesis have been documented [15, 16].

Phytochemical compounds, such as triterpene [17] phyllantine, zeatin nucleotide and riboside [18]; Kaempferol-3-O-B-D, quercetin-3-O-B-D and glucose [19] and diterpene [20] have been isolated from *E. officinalis* dried fruits. Also, it includes tannins, alkaloids, polyphenols, vitamins and minerals, gallic acid, ellagic acid, Emblicanin A & B, phyllembein, quercetin and ascorbic acid [21, 22].

Previously, preliminary report of anti-trypanosomal activity of *E. officinalis* dried fruits has been reported [23].

In this report, *Emblica officinalis* dried fruits were extracted with methanol, obtained MPE (methanolic plant extract) was further fractionated via column chromatography, preparative chromatography and its level of purification was determined by HPLC (high performance liquid chromatography) with attended bioassay at different strata with interest on *in-vivo* trypanocidal activity.

## 2. Material and Methods

### 2.1 Plant Material

*Emblica officinalis* dried fruits of the family

euphorbiaceae were obtained from reputable Ayurvedic shop from hilly region of Palampur, Himachal Pradesh. Plant material was identified by Institute of Himalaya Biosource Technology, Palampur, Himachal Pradesh, India.

### 2.2 Extraction

Twenty grammes of *E. officinalis* dried fruits were pounded into powder with pestle and mortar and cold extracted twice with 200 mL of methanol (analytical grade) according to Stahl [24]. 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 and partial purified fractions of *E. officinalis* dried fruits were 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 MPE and PPFs (partially purified fractions). 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 media used facilitated the detection of bioactive constituents. This was carried out according to the method of Stahl [24].

### 2.4 Applied Solvent Systems

The following solvent systems were tested to develop the TLC plates to obtain a more suitable system for both extract and fractions according to the method of Stahl [24]:

- Chloroform/hexane/acetic acid (50:50:1);
- Chloroform/ethyl acetate/acetic acid (50:50:1);
- 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 of Williamson et al. [25].

## 2.6 *In-vitro* Trypanocidal Activity

*In-vitro* trypanocidal activity was carried out on two media:

(1) On modified method of Oliveira et al. [26]: 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  $\mu$ L of DMEM containing  $5 \times 10^5$  cells/mL. The plates were incubated at 37 °C under 5% CO<sub>2</sub> 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  $\mu$ g streptomycin and 40  $\mu$ g gentamycin). A high parasitemic 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 *E. officinalis* dried fruits at concentrations (250~1,000  $\mu$ g/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<sub>2</sub>. 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 (dimethyl sulphoxide);

(2) On Alsever's medium: Trypanosomes were suspended in Alsever's solution with inactivated bovine serum at 58 °C for 1 h. Trypanosomes concentration was  $1 \times 10^6$  parasites/mL. 180  $\mu$ L of the medium was added to the test extract of *E. officinalis* dried fruits (20  $\mu$ L) and incubated at 37 °C with 5%

carbon dioxide for 5 h. On hourly basis, drops of the incubated mixture were observed under inverted microscope for antitrypanosomal activity [27].

The concentration of DMSO in the experiment had no deleterious effect by itself on host cells or parasites. 1% DMSO in distilled water was used as control [28].

## 2.7 *In-vivo* Infectivity Assessment

*In-vivo* infectivity of MPE of *E. officinalis* dried fruits was carried out after successful completion of anti-trypanosomal activity. Contents of microculture plate wells that contained reduced and apparently killed trypanosomes with MPE of the test material were inoculated (0.1 mL per mouse) into two groups of mice (six per group) via intra-peritoneal, and observed for more than 60 days for parasitaemia [29, 30].

## 2.8 *In-vitro* Cytotoxicity Test

Cytotoxic effects of the MPE and pooled PPFs of *E. officinalis* dried fruits were determined according to the method described by Sidwell and Hoffman [31]. Vero cell line was grown in DMEM (Dulbecco's Modified Eagle Medium) (Sigma) Gibco, USA antibiotics (100 units penicillin, 100  $\mu$ g streptomycin and 40  $\mu$ g gentamycin) in 96-well flat bottom micro culture plates (Nunc, Denmark). Each well received 100  $\mu$ L of DMEM containing  $5 \times 10^5$  cells/mL. The plates were incubated at 37 °C under 5% CO<sub>2</sub> 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. Confluent monolayers of Vero cells were treated with serial dilutions of MPE and pooled PPFs of the test material at concentrations (1.56~100  $\mu$ g/mL) in triplicate and incubated under the same conditions described previously. After 24 h of incubation, the culture plates were observed for evidence of cytotoxic effects. The plates were incubated for 72 h and observed daily. It was repeated thrice. In each case, after the 72 h of incubation, the culture media of the

incubated Vero cells were discarded. The adhered cells to ELISA plates were stained with a drop of crystal violet in phosphate buffered solution. The plates were incubated for 24 h at 37 °C in an ordinary incubator. After 24 h of incubation, the culture plates were observed for evidence of cytotoxic effects.

### 2.9 Acute Toxicity Test

Acute toxicity test of *E. officinalis* dried fruits was carried out according to the method of Madubunyi [32]. In this method, powdered test material was dissolved in either distilled water or vegetable oil pending on its solubility at a dose rate of 2,000 mg/kg body weight and administered to six mice according to the body weight. Mice were observed for at least two weeks for any sign of toxicity and mortality.

### 2.10 Column Chromatography of Methanolic Plant Extract of *E. officinalis* Dried Fruits

This was done according to the method of Stahl [24]. Obtained MPE of *E. officinalis* dried fruits were used for bioassay-guided purification. Residues (11.623 g of MPE) obtained from methanol extraction of 60 g *E. officinalis* dried fruits were used for column chromatography. Extract was loaded into already packed glass column with silica gel (60~120 mesh) for column chromatography. The extract was eluted with varied ratios of chloroform/methanol.

### 2.11 In-vivo Trypanocidal Activity of Methanolic Plant Extract and Partially Purified Fractions of *E. officinalis* Dried Fruits

This was carried out as per the method of Freiburghaus et al. [9]. Six mice in a group were inoculated with trypanosomes ( $1 \times 10^4$  /mL). Infected mice were treated with both MPE and pooled PPFs of *E. officinalis* dried fruits at concentrations (12.5~200 mg/kg body weight) intraperitoneally 48 h post on set of parasitemia. 1% of DMSO was added to the extract, PPFs 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.

### 2.12 HPLC (Higher Performance Liquid Chromatography) Analysis of Representative Pooled Partially Purified Fractions from *E. officinalis* Dried Fruits

HPLC analysis was done according to Sharma et al. [33]. HPLC (Waters) analysis by injecting 20 µL of dissolved representative fractions of *E. officinalis* in HPLC graded methanol via 18 columns. Gradient of methanol: water (40:60) to methanol (100%) for 30 min was used. At a zero minute, the ratio of acetonitrile to water percentages was 10:90. But at 25 min, the ratio percentages were 64:36, respectively.

### 2.13 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.

### 2.14 Statistical Analysis

Results of trypanocidal activity were expressed as “mean  $\pm$  SEM”. Statistical significance was determined by Sigma Stat (Jandel), USA.

## 3. Results

In this current research, results are presented in Tables 1-9 and Figs. 1-3 accordingly.

### 3.1 Extraction

Solvent, methanol, was used in extraction of *E. officinalis* dried powdered fruits. It appeared that methanol was suitable for its extraction as per the bioactive constituents present in the MPE of sample material observed on the TLC plates.

### 3.2 Solvent System

Out of four solvent systems tested in the analysis of TLC plates with applied aliquots of MPE and fractions of the test material, solvent systems,

methanol/chloroform (20:80) and chloroform/ethyl acetate/acetic acid (50:50:1), were suitable than other solvent systems tested (plates not shown) for both in that order. On the TLC plates, different patterns of bioactive constituents were on display from MPE and pooled fractions of sample material, which were subsequently responsible for anti-trypanosomal activity.

### 3.3 In-vivo Infectivity Test

One group of mice inoculated with contents of ELISA plate wells with completely killed trypanosomes survived for more than 60 days as to the other group inoculated with contents of ELISA plate wells with reduced trypanosomes count that died of parasitemia.

### 3.4 In-vitro Trypanocidal Activity of Methanolic Plant Extract of *E. officinalis* Dried Fruits

Results of *in-vitro* trypanocidal activity of MPE of *E. officinalis* dried fruits at different concentrations (250~1,000 µg/mL) were as given in Fig. 1. In this result, strong trypanocidal activity was observed with

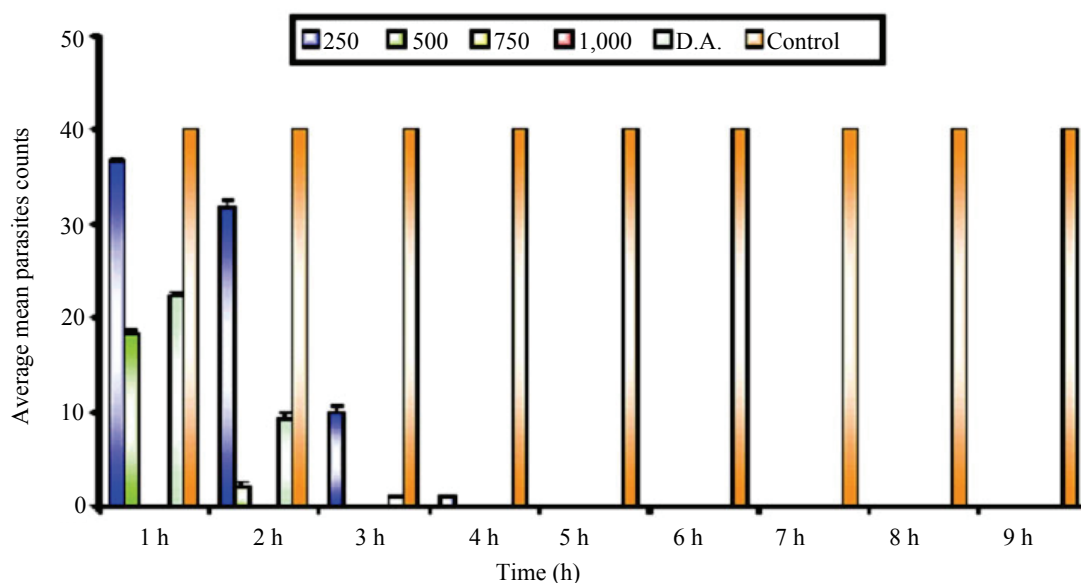
complete killing of the trypanosomes at 5 h of incubation at 250 µg/mL, which was statistically the same as Diminazine Aceturate (50 µg/mL) standard drug at 4 h of incubation (Fig. 1). Trypanocidal activity was concentration-time dependent faction. Average mean trypanosomes counts from  $37.67 \pm 0.58$  and below is significant between the treatment groups and negative control ( $P \leq 0.05$  to  $0.01$ ).

### 3.5 In-vitro Cytotoxicity Test

As shown in Tables 1 and 2, MPE and PPFs of *E. officinalis* dried fruits, and Diminazine Aceturate were cytotoxic to Vero cells in all concentrations except at 1.56, 1.56~3.13 and 1.56~6.25 µg/mL, respectively. Cytotoxic effects, such as distortion, sloughing, swelling and dead of the affected cells, were observed compared to normal cells.

### 3.6 Acute Toxicity Test

At concentration of 2,000 kg/kg body weight, MPE of *E. officinalis* was not toxic to mice in different groups. All mice survived in acute toxicity test as given in Table 3.



**Fig. 1** *In-vitro* trypanocidal activity of methanolic extract of *E. officinalis* on Vero cell line.

Test extract: concentrations (250~1,000 µg/mL);

DA: Diminazine Aceturate (50 µg/mL).

Control: parasites + medium;

$P$  value:  $P \leq 0.05$  to  $0.01$ .

**Table 1** Cytotoxic effect of methanolic plant extract of *E. officinalis* 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		
	<i>Emblica officinalis</i>	Berenil	<i>Emblica officinalis</i>	Berenil	<i>Emblica officinalis</i>	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	100%	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

MPE of *E. officinalis* 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 and Berenil.

**Table 2** Cytotoxic effect of representative pooled partially purified fractions of *E. officinalis* 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		
	<i>Emblica officinalis</i>	Berenil	<i>Emblica officinalis</i>	Berenil	<i>Emblica officinalis</i>	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	0	0	0
1.56	0	0	0	0	0	0	0

PPFs of *E. officinalis* and diminazine acetate 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 acetate (Berenil).

**Table 3** Acute toxicity test of methanolic extract of *E. officinalis* dried fruits in mice.

Number of mice per extract	Body weight of mice (g)	Type of plant extract	Concentration used (2,000 mg/kg body weight)	Observation (toxicity signs and mortality)
1	27	<i>Emblica officinalis</i>	0.27 mL	The mice Survived without any apparent toxic signs observed
2	28		0.28 mL	-
3	29		0.29 mL	-
4	26		0.26 mL	-
5	28		0.28 mL	-
6	27		0.27 mL	-

### 3.7 Column Chromatography of Methanolic Plant Extract of *E. officinalis* Dried Fruits with Corresponding Trypanocidal Activity

Elution of MPE of *E. officinalis* dried fruits with ratios of chloroform/methanol yielded 581 fractions of 25 mL each. Solvent systems, chloroform/hexane/acetic acid (50:50:1) and chloroform/ethyl acetate/acetic acid (50:50:1), were

used to analyzed the TLC plates. However, chloroform/ethyl acetate/acetic acid (50:50:1) was more suitable for resolutions of the TLC plates. Subsequently, TLC plates were detected in iodine vapors. Pooling of fractions was done according to similarity of TLC profiles and were given as follows: (1) Pooled Fractions I (1~7 and 11~40); (2) Pooled Fractions II (8~10); (3) Pooled Fractions III (41~96); (4) Pooled Fractions IV (97~581).

Fractions I (1~7 and 11~40) did depict presence of small amount of bioactive components and were combined as a result of similarity of TLC profile. Fractions II (8~10) displayed three layers of broad bands on TLC plates and nothing remained at the origin of applications. In Fractions III (41~96), there was a little mobility of bioactive components from the origin of applications. But, Fractions IV (97~581) mostly did

not move from origin of applications of aliquots as depicted on TLC plates, which gradually increased in intensity. Corresponding *in-vitro* trypanocidal activities were given in Tables 4-7.

### 3.8 *In-vitro Trypanocidal Activity of PPFs (Partially Purified Fractions) of E. officinalis Dried Fruits*

As shown in Table 8, distinct pooled fractions

**Table 4 Pooled Fractions I (1~7 and 11~40).**

Concentration of pooled plant extract (µg/mL)	1 h	2 h	3 h	4 h	5h
250	40.00 ± 0.0	40.00 ± 0.0	31.33 ± 0.33	23.67 ± 0.68	12.67 ± 0.68
500	40.00 ± 0.0	40.00 ± 0.0	28.67 ± 0.33	18.67 ± 0.33	8.667 ± 0.68
750	40.00 ± 0.0	40.00 ± 0.0	18.67 ± 0.33	9.667 ± 0.33	3.667 ± 0.33
1000	34.67 ± 0.33	20.67 ± 0.68	11.33 ± 0.33	0.0 ± 0.0	0.0 ± 0.0
Diminazine Aceturate (50) (positive control)	22.33 ± 0.33	9.000 ± 0.58	1.333 ± 0.33	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

Bioassay status: Significant reduction of parasites counts started from concentration of 250 µg/mL and complete killing of parasites at 1,000 µg/mL at 4th hour of incubation. An average mean parasites count of 37.67 ± 0.58 is statistically critical value. Average mean from 37.67 ± 0.58 and below is significant between the treatment groups and negative control ( $P \leq 0.05$  to 0.01).

**Table 5 Pooled Fractions II (8~10).**

Concentration of pooled plant extract (µg/mL)	1 h	2 h	3 h	4 h	5h
250	40.00 ± 0.0	22.67 ± 0.68	10.67 ± 0.68	5.667 ± 0.33	25.00 ± 0.25
500	40.00 ± 0.0	19.67 ± 0.33	8.667 ± 0.33	4.667 ± 0.33	0.0 ± 0.0
750	40.00 ± 0.0	16.33 ± 0.33	7.333 ± 0.33	0.0 ± 0.0	0.0 ± 0.0
1000	33.67 ± 0.33	14.67 ± 0.33	0.0 ± 0.00	0.0 ± 0.0	0.0 ± 0.0
Diminazine Aceturate (50) Positive control	22.33 ± 0.33	9.000 ± 0.58	1.333 ± 0.33	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

Bioassay status: Significant reduction of parasites counts started from concentration 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 meanparasites 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 6 Pooled Fractions III (41~96).**

Concentration of pooled plant extract (µg/mL)	1 h	2 h	3 h	4 h	5h
250	40.0 ± 0.0	40.00±0.0	28.33±0.88	18.67±0.33	10.33±0.33
500	40.00 ± 0.0	39.33±0.88	24.33±0.58	14.33±0.33	6.667±0.33
750	40.00 ± 0.0	30.67±0.88	14.67±0.33	6.667±0.33	0.0±0.0
1,000	38.33 ± 0.33	24.67 ± 0.58	9.333 ± 0.33	0.0 ± 0.0	0.0 ± 0.0
Diminazine aceturate (50) (positive control)	22.33 ± 0.33	9.000 ± 0.58	1.333 ± 0.33	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

Bioassay status: Significant reduction of parasites counts from concentration of 250 µg/mL and complete killing of parasites at 750 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 ( $P \leq 0.05$  to 0.01).

**Table 7 Pooled Fractions IV (97~581).**

Concentration of pooled plant extract (µg/mL)	1 h	2 h	3 h	4 h	5h
250	39.67 ± 0.33	20.00 ± 0.0	10.67 ± 0.68	4.667 ± 0.33	0.3333 ± 0.33
500	38.67 ± 0.68	18.67 ± 0.33	9.000 ± 0.0	3.333 ± 0.33	0.0 ± 0.0
750	36.33 ± 0.68	16.67 ± 0.33	7.667 ± 0.33	2.667 ± 0.33	0.0 ± 0.0
1,000	33.33 ± 0.33	14.67 ± 0.33	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Diminazine Aceturate (50) (positive control)	22.33 ± 0.33	9.000 ± 0.58	1.333 ± 0.33	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

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 ( $P \leq 0.05$  to 0.01).

**Table 8 *In-vivo* trypanocidal activity of methanolic extract of *E. officinalis* 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
12.5	7.100 ± 0.25	26.67 ± 0.88	39.67 ± 0.33			
25	8.667 ± 0.33	26.67 ± 0.88	38.00 ± 0.59			
50	7.333 ± 0.88	23.00 ± 0.68	34.33 ± 0.72			
100	6.667 ± 0.33	14.67 ± 0.33	22.33 ± 0.33	32.67 ± 0.67		
200	5.0000 ± 0.58	10.67 ± 0.33	19.00 ± 0.58	29.00 ± 0.58	39.67 ± 0.33	52.34 ± 0.33
Diminazine Aceturate (10) (positive control)	6.167 ± 0.3073	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 6 days post on set of parasitemia. There was significant difference ( $P < 0.05$ ) between treated groups with test material in comparison to negative control that survived for only three days.

exhibited significant trypanocidal activity in all concentrations (250~1,000 µg/mL) with significant difference ( $P < 0.01$ ).

### 3.9 *In-vivo* Trypanocidal Activity of Methanolic Plant Extract of *E. officinalis* Dried Fruits

*In-vivo* trypanocidal activity of MPE of *E. officinalis* dried fruits at different concentrations were given in Table 8. Mice in distinct groups treated with MPE of the test material at concentrations (12.5, 25, 50, 100 and 200 mg/mL) after the onset of parasitemia survived up to Days 4, 5 and 6, respectively, in comparison to Day 4 of untreated control with significant difference ( $P < 0.05$ ).

### 3.10 *In-vivo* Trypanocidal Activity of Partially Purified Fractions of *E. officinalis* Dried Fruits in Mice

Partially purified fractions of *E. officinalis* fruits

with high content of desired unidentified compounds with maximum *in-vitro* trypanocidal activity were used to treat parasitemic mice, which exhibited different levels of trypanocidal activity, were as given in Table 9 and Fig. 2. Mice treated with the obtained pooled fractions of bioactive constituents of interest survived for maximum 9 days post infection as to 4 days of untreated control with significant difference ( $P \leq 0.05$  to 0.01).

### 3.11 HPLC (Higher Performance Liquid Chromatography) Analysis of Pooled Fractions of *E. officinalis* Dried Fruits

HPLC analysis of representative pooled fractions of *E. officinalis* dried fruits that contained bioactive constituents of interest revealed two prominent peaks depicting more than one compounds at two detections (210 and 320 nm), as given in Fig. 3. Detection at more than one wavelength depicted glaringly impurity of the



Table 9 *In-vivo* trypanocidal activity of partially purified fractions of *E. officinalis* dried fruits in mice.

Concentration of test materials	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
12.5	6.923 ± 0.33	14.33 ± 0.33	29.33 ± 0.33	42.67 ± 0.67					
25	6.667 ± 0.33	13.00 ± 0.58	27.67 ± 0.33	41.00 ± 0.58					
50	7.333 ± 0.33	12.00 ± 0.58	24.33 ± 0.33	37.00 ± 0.58	44.00 ± 0.58				
100	7.333 ± 0.33	11.33 ± 0.33	22.33 ± 0.33	20.00 ± 1.0	21.67 ± 0.33	26.33 ± 0.33	40.00 ± 0.58		
200	7.000 ± 0.22	9.333 ± 0.33	20.00 ± 0.58	17.67 ± 0.58	17.33 ± 0.33	17.00 ± 0.58	18.67 ± 0.33	29.00 ± 0.58	41.00 ± 0.58
Diminazine aceturate (10) (positive control)	7.00 ± 0.58	0.33 ± 0.33	0.00 ± 0.0						
Control (negative control)	7.00 ± 0.58	22.33 ± 0.45	43.00 ± 1.52						

At doses of 12.5, 25, 50, 100 and 200 mg/kg body weight, the mice in each group survived for 5, 5, 6, 8 and 10 days post on set of parasitemia. There was degree of significant difference between treated groups with test material compared to negative control that survived for only three days ( $P \leq 0.05$  to  $0.01$ ).

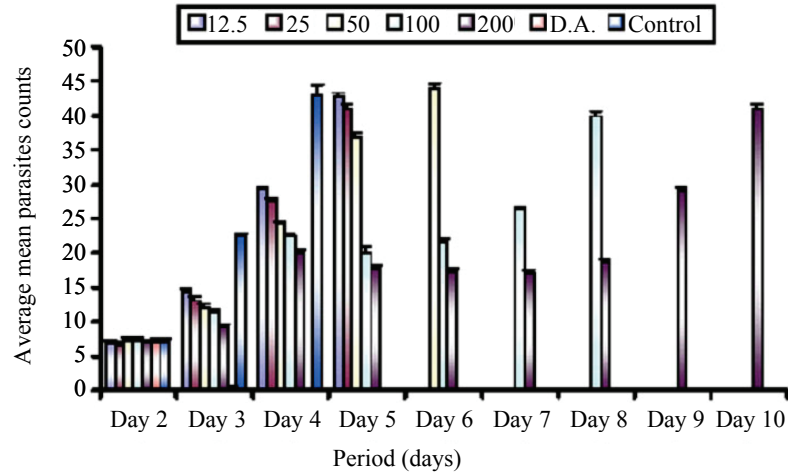
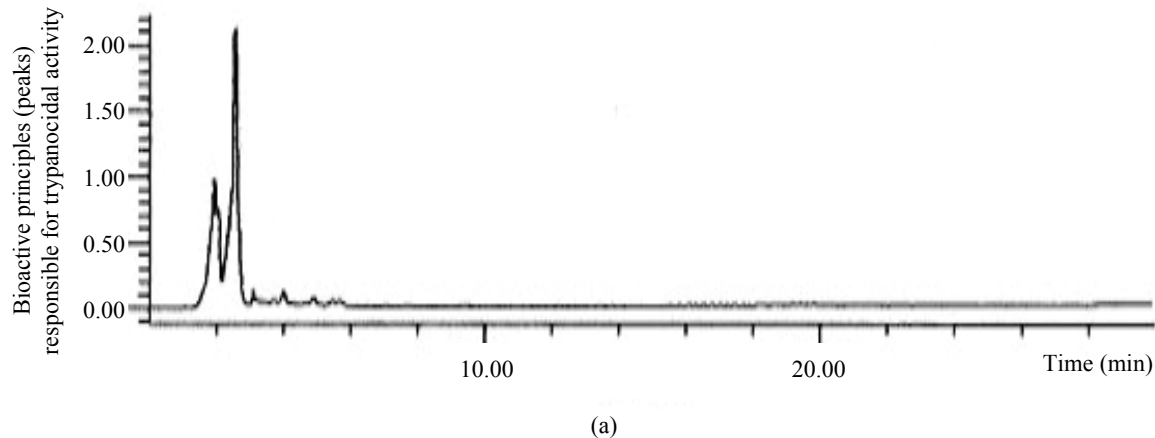


Fig. 2 *In-vivo* trypanocidal activity of partially purified fractions of *E. officinalis* dried fruits in mice.

Test extract: 250–1,000 µg/mL;  
DA: Diminazen Aceturate (50 µg/mL);  
Control: parasites + medium.



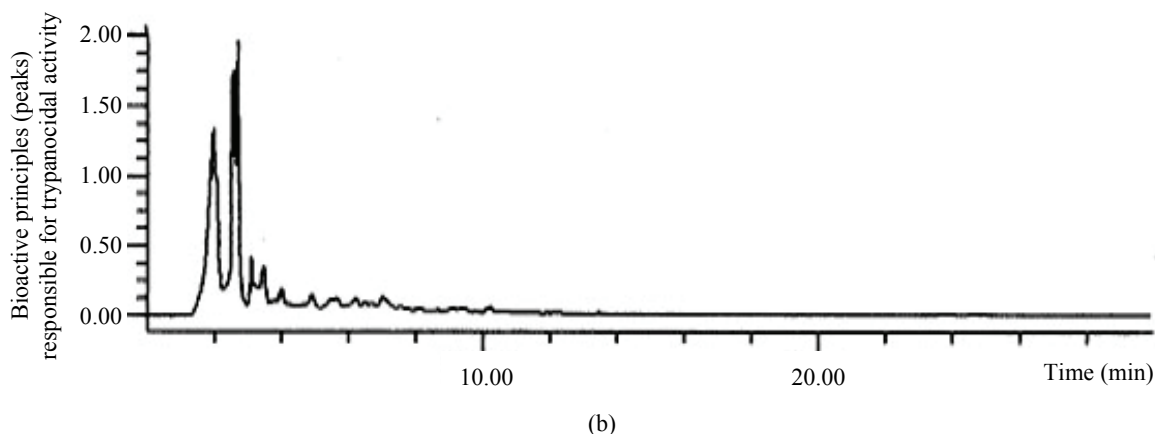


Fig. 3 HPLC profile of pooled fractions of column chromatography of *E. officinalis*: (a) detection: 210 nm, flow rate: 1 mL/min; (b) injection: 20  $\mu$ L, detection at: 320 nm, flow rate: 1 mL/min.

representative pooled fractions of *E. officinalis* dried fruits.

## 4. Discussion

### 4.1 Extraction

In this report, methanol used in the extraction of *E. officinalis* dried fruits is similar to previous work documented by Shaba et al. [12, 30, 34].

### 4.2 Solvent System

Solvents, methanol and chloroform/ethyl acetate/acetic acid (50:50:1) used in analysis of TLC plates applied with MPE and fractions of *E. officinalis* dried fruits are comparable to that used in bioassay guided isolation of a diastereoisomer of kolavenol from *Entada absyssinica* [9].

### 4.3 In-vivo Infectivity Test

*In-vivo* infectivity test of MPE of the test is in line with work done by Igweh et al. [29] and Shaba et al. [35] where groups of mice inoculated with contents of ELISA plate wells with apparent killed trypanosomes survived.

### 4.4 In-vitro Cytotoxicity Test

Cytotoxic effects of *E. officinalis* dried fruits are comparable to cytotoxic effects of *Terminalia arjuna* bark extract with distortion and apoptosis of human hepatoma cell line (HEPG2) [36] and *Terminalia*

*belirica* dried fruits with similar cytotoxic effects observed in this report [37].

### 4.5 Acute Toxicity Test

Acute toxicity test of MPE of the test material is comparable to that of *Nuclea latifolia*, in which no toxic sign was observed in rats at concentrations of 100~300 mg/kg body weight. But, fatalities were observed at 400 and 800 mg/kg body weight [32].

### 4.6 Column Chromatography of Methanolic Extract of *E. officinalis* Dried Fruits

Fractionation of MPE of *E. officinalis* dried fruits via column chromatography is similar to fractionation of *Cannabis sativa* with two fractions active against *T. brucei rhodesiense* [38, 39].

### 4.7 In-vitro Trypanocidal Activity of Methanolic Extract of *E. officinalis* Dried Fruits

This result is comparable to *in vitro* antitrypanosomal activity of some medicinal plants used in northern Nigeria against trypanosomosis with different level of antitrypanosomal activity at concentration of 8.3 mg/mL [40] and *in-vitro* screening of American plants extracts on *Trypanosoma cruzi* and *Trichomonas vaginalis* [41]. Trypanocidal activity, in this report, could be due to already isolated compounds, such as gallic acid from test material. Trypanocidal activity of gallic acid has been documented [42].

#### 4.8 *In-vitro* Trypanocidal Activity of PFs (Pooled Fractions) of *E. officinalis* Dried Fruits

Results of varied trypanocidal activity of PFs of *E. officinalis* dried fruits is in line with that of fractionation of *Cannabis sativa* with two fractions active against *T. brucei rhodesiense* [39] and bioassay guided isolation of a diastereoisomer of kolavenol from *Entada absyssinica* active on *Trypanosoma brucei rdesiense* [9].

#### 4.9 *In-vivo* Trypanocidal Activity of Methanolic Plant Extract of *E. officinalis* Dried Fruits in Mice

Distinct levels of *in-vivo* trypanocidal activity of MPE of test material is in line with trypanocidal activity of *Nuclea latifolia* in which treated mice exhibited decreased in parasitemia but could not cure the infected rat with trypanosomes [32] and *in-vivo* anti-trypanosomal activity of dichloromethane and methanol crude leaf extracts of *Dovyalis abyssinica* (Salicaceae) against *Trypanosoma congolense* with limited *in-vivo* result [43].

#### 4.10 Higher Performance Liquid Chromatography Analysis of Pooled Fractions of *E. officinalis* Dried Fruits

HPLC analysis of pooled PPFs of *E. officinalis* dried fruit is comparable to fractionation and purification of bioassay guided isolation of a diastereoisomer of kolavenol from *Entada absyssinica* [9].

#### 4.11 *In-vivo* Trypanocidal Activity of PPFs (Partially Purified Fractions) of *E. officinalis* Dried Fruits in Mice

*In-vivo* trypanocidal activity of PPFs of *E. officinalis* dried fruits is in line with fractionation of *Cannabis sativa* with two fractions active against *T. brucei rhodesiense* [39] and bioassay guided isolation of a diastereoisomer of kolavenol from *Entada absyssinica* active on *Trypanosoma brucei rdesiense* [9].

Mechanism of action of *E. officinalis* dried fruits may be due to gallic acid that has been isolated already

from it and its corresponding trypanocidal activity has been documented [40]. Also, it could be due to intercalation of obtained extracts/fractions/isolated compounds of *E. officinalis* with the DNA of trypanosomes of which such actions have been reported [10].

Representative pooled fractions of PPFs of *E. officinalis* could not cure the mice but prolonged its lifespan to Day 9 post infection at maximum dose of 200 mg/kg body weight. This may be due to inability of the PPFs to sustain sufficient blood plasma to kill the trypanosomes and possibly, the ease of its being degraded in the body of the mice, which is common to such fractions/isolated compounds [10].

## 5. Conclusions

In conclusion, *E. officinalis* dried fruits exhibited significant *in-vitro* antitrypanosomal activity. Even though strong results of *in-vitro* trypanocidal activity was not completely transformed during *in-vivo* testing due to differences in physiological status, attained level bioassay-guided purification decreased cytotoxic effects level and increased trypanocidal activity as shown during *in-vivo* testing with PPFs of the test material. In near future, this could lead to development of urgently needed new trypanocide against menacing trypanosomes in both animals and humans.

Further purification of PPFs of *E. officinalis* dried fruits is needed to determine its maximum trypanocidal status.

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