ADVANCES IN PHARMACOGNOSY AND PHYTOMEDICINE

Journal homepage: http://www.journals.wsrpublishing.com/index.php/APP

Print ISSN: 2423-706X *Online ISSN:* 2423-7078

Original Article

Trypanocidal Activity of Methanolic Extracts (50 and 100%) of *Emblica officinalis* (*Phyllanthus emblica* L.) Dried Fruits against *Trypanosoma evansi*

P. Shaba[1,*], S. Dey[1], N.P. Kurade[2], and R.K. Singh[3]

[1] Division of Medicine, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India
[2] Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India
[3] Indian Veterinary Research Institute, Regional Station, Mukteswar, Uttranchal, India

ARTICLE INFO

Corresponding Author:
P. Shaba
shabamine@gmail.com

How to Cite this Article
Shaba, P., Dey, S., Kurade, N.P., & Singh, R.K. (2016). Trypanocidal Activity of Methanolic Extracts (50 and 100%) of *Emblica officinalis* (*Phyllanthus emblica* L.) Dried Fruits against *Trypanosoma evansi*. *Advances in Pharmacognosy and Phytomedicine*, 2(1), 1-8.

Article History:
Received: 19 Nov. 2015
Revised: 22 Dec. 2015
Accepted: 25 Dec. 2015

ABSTRACT

Trypanosomosis is on the increase in the endemic regions of the world and resistant strains of trypanosomes had defiled few limited classes of readily available trypanocides. There is a need to search for new drug. Because of this, *Emblica officinalis* dried fruits were extracted with methanolic solvent (50 and 100%). The test extracts of *E. officinalis* dried fruits at different concentrations (250-1000 µg.ml^{-1}) were screened against *Trypanosoma evansi* on Alsever's medium. Trypanosomes were suspended in Alsever's solution with inactivated bovine serum at 58°C for 1h. Trypanosomes concentration was 1×10^6 parasites/ml. 180 µl of the medium was added to the test extract of *E. officinalis* (20 µl) and incubated at 37°C with 5% carbon dioxide for 5h. On hourly basis, drops of the incubated mixture were observed under inverted microscope for antitrypanosomal activity. *In vitro* cytotoxicity test of methanolic plant extract (MPE) of *E. officinalis* at concentrations (1.56-100 µg/ml) was done on Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) but without fetal calf serum at appropriate conditions. Marked trypanocidal activity was observed at 250 µg/ml of MPE (100%) of *E. officinalis* and trypanosomes were not detected after 5h of incubation, which was statistically equivalent to diminazine aceturate (50µg/ml) standard drug at 4h. For 250 µg/ml of MPE of *E. officinalis* (50%), there was drastic reduction at the end of 5h incubation but not complete killing of trypanosomes. However at 500 µg/ml, trypanosomes were not detectable at 4h of incubation. Extract of *E. officinalis* and diminazine aceturate were cytotoxic to Vero cells in all concentrations except at 1.56-6. 25 µg/ml. MPE of *E. officinalis* exhibited marked trypanocidal activity with significant difference ($P\leq0.05$ to 0.01). These results pave way for further research (e.g. bioassay-guided purification) for isolation of trypanocidal compound, which will give a lead for development of new trypanocide.

Keywords: *Emblica officinalis*, Cytotoxicity test, *Trypanosoma evansi*, Trypanocidal activity.

INTRODUCTION

Trypanosomosis, a zoonotic disease, is caused by group of blood protozoan parasites under the genus *Trypanosoma,* which is causing a lot of havocs in both animals and humans in the regions of the world where the disease thrives especially in Sub-Saharan Africa and Latin America (Freiburghause *et al*., 1996; WHO, 2010).

Direct losses due to trypanosomosis are estimated to amount to between US$ 1-1.2 billion each year whereas the indirect impact of AAT on agriculture in sub-Saharan Africa exceeds this amount. A pondered evaluation extrapolated for the total tsetse-infested lands values the total losses, in terms of agricultural Gross Domestic Product, at US$ 4.75 billion per year (FAO, 2004). The overall impact extends to the restricted access to fertile and cultivable areas, imbalances in land use and exploitation of natural resources and compromised growth and diversification of crop-livestock production systems (Mattioli *et al*., 2004).

A lot of research have been documented on medicinal plants with antitrypanosomal activity (Nok and Nock, 2002; Kubuta *et al*., 2005; Shaba *et al*., 2006; 2007; 2008; 2011(ab); Wube *et al*., 2012; Obbo *et al*., 2013; Shaba *et al*., 2014).

Emblica officinalis (family: Euphorbiaceae) dried fruits popularly referred to as Indian gooseberry or Amla is one of the most important medicinal plants in Indian traditional systems of medicine (Ayurveda, Unani and Siddha) (Srivasuki *et al*., 2012).

E. officinalis dried fruits are found throughout India, Pakistan, Uzbekistan, Sri Lanka, South East Asia, China and Malaysia. Amla fruits are widely used in Indian sub-continent (Udupa, 1985).

It is widely used in the system of medicine as diuretic, laxative, liver tonic, refrigerant, stomachic, restorative, anti-pyretic, hair tonic, ulcer preventive and also, for common cold, fever; as alone or in combination with other plants (Udupa, 1985; Maurya and Srivastava, 2011).

Traditional uses of *E. officinalis* dried fruits in disease conditions such as anemia, jaundice, dyspepsia, scabies and itch, nausea and emesis have been documented (Maurya and Srivastava 2011; Srivasuki, 2012).

Pharmacological activities of *E. officinalis* dried fruits such as analgesic, anti-tussive, anti-atherogenic, adaptogenic, cardio, gastro, nephro and neuroprotective, chemopreventive, radio and chemo modulatory and anticancer properties have been reported (Bhattacharya *et al*., 2002; Zhang *et al*., 2003; Nosal ova *et al*. 2003; Yi-Fei *et al*., 2009).

Phytochemical studies on *E. officinalis* dried fruits revealed many chemical constituents such as tannins, alkaloids, polyphenols, vitamins and minerals, gallic acid, ellagic acid, emblicanin A & B, phyllembein, quercetin and ascorbic acid (Sharma *et al*., 2004; Rehman *et al*.,2007; Choudhary and Malik, 2007).

Previously, we reported preliminary trypanocidal activity of the *E. officinalis* dried fruits using different medium and a concentration (Shaba *et al*., 2012a). Present report attempted comparing two concentrations and different medium to demonstrate ability of the medium to sustain the trypanosomes and observed trypanocidal activity.

Due to above mentioned problems militating against available trypanocides, *Emblica officinalis* dried fruits were extracted and obtained MPES of *E. officinalis* were screened against *Trypanosoma evansi*

MATERIALS AND MEMTHODS

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 were used which were purchased from E. Merck, India.

Plant Material

Emblica officinalis dried fruits were purchased from a reputable Ayurveda shop from Palampur, Himachal Pradesh, India. The dried fruits were subsequently identified at Institute of Himalayan Biosource and Technology, Palampur, Himachal Pradesh, India.

Preparation of Extracts

The extraction was carried out according to the method of Stahl (1969). 20 g each of dried *E.*

officinalis dried fruits were powdered using laboratory pestle and mortar, and cold extracted with 200 ml of methanol (50% and 100%)(Analytical grade). Residues obtained were extracted twice in the same medium. The filtrates were combined, dried at 37°C and stored at 4°C 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 extracts were 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 extracts. This was done in accordance with 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 and regulations of committee on Ethics and Cruelty to Animals of the institute.

Test Organism

Trypanosom aevansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar and was maintained in the laboratory by serial sub-passages in Swiss albino mice. The strain was routinely tested for virulence as per the method of Williamson *et al*., (1982).

Trypanosomes Count

Counting of trypanosomes 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**

In vitro trypanocidal activity was carried out according to the method of Talakal *et al*., (1995). A high parasitemic blood of a mouse was diluted with Alsever's solution to obtain final trypanosomes concentration of $1x10^6$ parasites/ml. The medium consist of Alsever's solution and inactivated bovine serum at 58°C for 1 h. Suspension (180 µl of medium with trypanosomes) was added to 20µl of the test MPES of *E. officinalis* dried fruits and the plates were incubated at 37°C under 5% CO_2. The test was repeated at least thrice.

Stock of MPES of *E. officinalis* dried fruits was solubilized in 1% dimethylsuphoxide (DMSO). The concentration of DMSO in the experiment had no deleterious effect by itself on host cells or trypanosomes. 1% DMSO in distilled water was used as control (Young *et al*., 2000).

***In Vivo* Infectivity Assessment**

In vivo infectivity assessment of MPES of *E. officinalis* (50% and 100%) was carried out after completion of incubation for anti-trypanosomal activity. Contents of ELISA plate wells with reduced and apparently killed trypanosomes from MPES of *E. officinalis* dried fruits were inoculated (0.1ml per mouse) into two groups of mice (six per group) via intra-peritoneum, and observed for more than 60 days for parasitemia (Woo, 1970; lgweh *et al*., 2002).

***In Vitro* Cytotoxicity Test**

It was done according to the method of Sidwell and Hoffman. (1997). Vero cell line (SIGMA) was grown in DMEM in 96-wells micro culture plates. Each well was seeded with 500,000 cells ml-1 and plates were incubated at 37°C with 5% CO2 for 48 h. After the formation of confluent monolayer, the supernatant was discarded and replaced with fresh medium. Confluent monolayer of Vero cell lines was treated with serial dilutions (1.56-100 µg/ml) of MPES of *E. officinalis* dried fruits in triplicate and incubated for 72h consecutively under the same conditions described previously. At 24h interval, ELISA plate was observed under inverted microscope for cytotoxic effects as compared to untreated normal cells that served

as control. In each case, after 72h of incubation, the culture media of the incubated Vero cells was discarded. Adhered cells were stained with a drop of crystal violet in phosphate buffered solution. ELISA plates were then incubated for 24h at 37°C in ordinary incubator. Plates were later observed under inverted microscope for cytotoxic effects. It was repeated thrice.

Statistical Analysis

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

RESULTS

Results of this investigation are presented in Tables (1-4).

Extraction

The solvent, methanol, used in this extraction was suitable in the extracting bioactive constituents present in *E. officinalis* dried fruits as depicted on the TLC plates.

Solvent System

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. *E. officinalis* extracts (50% and 100%). TLC plates (plates not shown) showed different patterns of bioactive constituents from extracts of *E. officinalis,* which were subsequently responsible for anti- trypanosomal activity.

***In vivo* Infectivity Test**

Group of mice inoculated with contents of ELISA plate wells withcompletely killed trypanosomes (50 and 100% MPES of *E. Officinalis* dried fruits)survived for more than 60 days, while other group of mice inoculated with contents of ELISA plate wells with reduced trypanosomes count(50 and 100% MPES of *E. officinalis* dried fruits) died of parasitemia.

***In Vitro* Cytotoxicity Test**

In two separate ELISA plate wells containing MPES (50 and 100%) of *E. officinalis* dried fruits and diminazineaceturate at the same concentration 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 (Tables 3 and 4).

***In Vitro* Trypanocidal Activity**

For *in vitro* trypanocidal activity of 100% MPE of *E. officinalis* at 250 µg/ml, trypanosomes counts were completely undetectable after 5 h of incubation. At 250 µg/ml 50% of MPE of *E. officinalis*, there was drastic reduction in trypanosomes count in corresponding ELISA plate wells but no complete killing of trypanosomes in any of the ELISA plate well throughout the 5 h incubation period as observed. However, at 500 µg/ml of MPE (50%) of *E. officinalis*, trypanosomes were not detected in the corresponding ELISA plate wells at 4 h of incubation. Complete killing of trypanosomes in corresponding ELISA plate wells at 250 and 500 of µg/ml of 100 and 50% MPES of *E. officinalis* were statistically equivalent to diminazine aceturate (50µg/ml) standard drug at 4 h. Marked trypanocidal activity was observed with significant difference (P≤0.05 to 0.01).

Table 1: In vitro trypanocidal activities of 100% methanolic extractof *Emblica officinalis* dried fruits against Trypanosma evansi

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h
250	37.67±0.33	32.00±0.58	11.33±0.33	2.000±0.58	0.0±0.0
500	19.67±0.33	1.667±0.33	0.0±0.0	0.0±0.0	0.0±0.0
750	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
1000	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Diminazineaceturate 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 trypanosomes counts from concentration of 250 µg/ml and complete killing of parasites at the concentration at 5th hour of observation. Average mean trypanosomes counts of 37.67± 0.58 are statistically critical value. Average mean from 37.67± 0.58 and below is significant between the treatment groups and negative control. (P ≤ 0.05 to 0.01).

Table 2: In vitro trypanocidal activities of 50% methanolic extract of *Emblica officinalis* dried fruits against Trypanosma evansi

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5 h
250	40.00±0.0	35.00±0.58	17.67±0.67	10.33±0.33	4.667±0.67
500	40.00±0.0	13.67±0.33	4.667±0.33	0.0±0.0	0.0±0.0
750	38.33±0.33	10.67±0.67	0.0±0.0	0.0±0.0	0.0±0.0
1000	19.33±0.33	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Diminazineaceturate 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 trypanosomes counts from concentration of 250 µg/ml and complete killing of parasites at 500 µg/ml at 4th hour of observation. Average mean trypanosomes counts of 37.67± 0.58 are statistically critical value. Average mean from 37.67± 0.58 and below is significant between the treatment groups and negative control

Table 3: Cytotoxic effect of methanolic extract (100%) of *Emblica officinalis* on Vero cell line compared to diminazineaceturate (Berenil)

Concentration of test material (µg/ml)	Effects of test extract at various periods of incubation (24 h, 48 h, 72 h)						
	Emblica officinalis	Berenil	*Emblica officinalis*	Berenil	*Emblica officinalis*	Berenil	Control
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	0	0	100%	0	100%	33.3%	0
6.25	0	0	0	0	100%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

Emblica officinalis and diminazineaceturate (Berenil) were toxic to Vero cell line except at concentrations of 1.56 and 6.25-1.56 µg/ml.
Same concentrations were used for diminazineaceturate.

Table 4: Cytotoxic effect of methanolic extract (50%) of *Embilica officinalis* on Vero cell line compared to diminazineaceturate (Berenil).

Concentration of test material (µg/ml)	Effects of test extract at various periods of incubation (24 h, 48 h, 72 h)						
	Emblica officinalis	Berenil	*Emblica officinalis*	Berenil	*Emblica officinalis*	Berenil	Control
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	0	0	60%	0	100%	33.3%	0
6.25	0	0	0	0	66.6%	0	0
3.13	0	0	0	0	33.3%	0	0
1.56	0	0	0	0	0	0	0

Embilica officinalis and diminazineaceturate (Berenil) were toxic to Vero cell line except at concentrations of 1.56 and 6.25-1.56 µg/ml.
Same concentrations were used for diminazineaceturate.

DISCUSSION

Extraction

This method of extraction, which yields MPES of *E. officinalis* dried fruits, is comparable to previous work carried out by the same authors such as extraction of *Plumbago zeylanica* root bark and *Vitex negundo* leaves (Shaba *et al*., 2006 and 2008) and extraction of *Entada Abyssinia* Freiburghause *et al*., (1996).

Thin Layer Chromatography (TLC) Plates Analysis

The solvent system, methanol/chloroform (20: 80), used in development of the TLC plates with applied aliquots of extracts of *E. officinalis* is comparable to the same medium used in analysis of TLC plates with applied extracts of *Plumbago zeylanica* root bark (Shaba *et al*., 2006) and *Picrorrhiza kurroa* rhizomes (Shaba *et al*., 2007) with maximum depictions of bioactive principles contained in the MPES of *E. officinalis* on TLC plates.

In Vitro Trypanocidal Activity

In this investigation, *in vitro* trypanocidal activity of *E. officinalis* dried fruits was concentration and time dependent manner in both extracts (50 and 100% MPES of *E. officinalis*) used. *In vitro* results are comparable with anti-trypanosmal activity of previous work reported by Freiburghause *et al*. (1998); Igweh *et al*. (2002) and Shaba *et al*. (2007, 2011a, 2012a and 2014) in which different solvents extracted bioactive constituents' presence in the extracts of medicinal plants tested and demonstrated anti-trypanosomal activities in gradation at different concentrations.

Also, trypanocidal activity of *E. officinalis* may be due to one of its chemical constituents, gallic acid, which its trypanocidal activity has been reported (Koide *et al*., 1999). This is in addition to chelate of trypanosomes DNA by extracts/purified compounds of medicinal plants that led to death of the trypanosomes (Sepulveda- Boza, 1996).

In Vivo Infectivity Test

In vivo Infectivity assessment of trypanocidal activity demonstrates the effectiveness of different MPES of *E. officinalis* against *T. evansi*. This effectively assessment 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 (Igweh *et al*., 2002; Shaba *et al*., 2011b and 2014).

In Vitro Cytotoxicity Test

For *in vitro* cytotoxicity test, both MPES of *E. officinalis* (50 and 100%) and diminazineaceturate were cytotoxic to Vero cells except at concentrations of 1.56 and 6.25 µg/ml. These results are comparable to *in vitro* cytotoxicity tests of extraction of *Ageratum houstonionum* flowers in which similar cytotoxic effects were observed (Shaba *et al*., 2011b).

CONCLUSION

Both MPES (50 and 100%) of *E. officinalis* dried fruits exhibited varied trypanocidal activity but the later MPE of *E. officinalis* extracted more of the bioactive constituents that were responsible for marked trypanocidal activity. This gives a clear picture of usage of solvent (100% methanol over 50%) in extraction of bioactive principles from the medicinal plants, in most cases, irrespective of method used in the extraction. Alsever's medium supported the incubation of trypanosomes and observed trypanocidal activity but there was no multiplication of trypanosomes. This paves way for further research (e.g. bioassay-guided purification) for isolation of compound(s) responsible for trypanocidal activity with ultimate goal of a lead to developing new trypanocide.

ACKNOWLWDGEMENT

Financial contributions by Indian and Nigerian governments towards the research, invaluable advice/inputs by Dr. A. K Mishra, former Principal Scientist, contributions by other scientists and technical staff, Division of Medicine, Parasitology IVRI, IVRI Regional stations at Palampur and Mukteswar are is highly acknowledged.

REFERENCES

Bhattacharya, S. K., Bhattacharya, D., Sairam, K., & Ghosal, S. (2002). Effect of bioactive tannoid principles of *Emblica officinalis* on ischemia-reperfusion-induced oxidative stress in rat heart. *Phytomedicine*, 9(2), 171-174.

Food and Agricultural Organization (FAO). (2004). Interaction of food security and Human African trypanosomiasis.

Freiburghaus, F., Kaminsky, R., Nkunya, M. H. H., & Brun, R. (1996). Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. *Journal of Ethnopharmacology*, 55(1), 1-11.

Habib-ur-Rehman, Yasin, K. A., Choudhary, M. A., Khaliq, N., Atta-ur-Rahman, Choudhary, M. I., & Malik, S. (2007). Studies on the chemical constituents of *Phyllanthus emblica*. *Natural Product Research*, 21(9), 775-781.

Igweh, A. C., Aguiyi, J. C., & Okwuasaba, F. K. (2002). Antitrypanosomal effect of the aqueous extract of *Brassica oleracea*. *Fitoterapia*, 73(1), 17-21.

Koide, T., Nose, M., Inoue, M., Ogihara, Y., Yabu, Y., & Ohta, N. (1998). Trypanocidal effects of gallic acid and related compounds. *Planta Medica*, 64(1), 27-30.

Kubata, B. K., Nagamune, K., Murakami, N., Merkel, P., Kabututu, Z., Martin, S. K., & Kinoshita, T. (2005). *Kola acuminata*

proanthocyanidins: a class of anti-trypanosomal compounds effective against *Trypanosoma brucei*. *International Journal for Parasitology*, 35(1), 91-103.

Lumsden, W. H. R., Herbert, W. J., Mc Neilage, G. J. C. (1973). *In vitro* Techniques with Trypanosomes. Churchil Livingstone, London, United of Kingdom.

Nok, A. J., & Nock, I. H. (2002). Transferrin coupled azanthraquinone enhances the killing effect on trypanosomes. The role of lysosomal mannosidase. *Parasite*, 9(4), 375-379.

Nosal'ova, G., Mokrý, J., & Hassan, K. T. (2003). Antitussive activity of the fruit extract of *Emblica officinalis* Gaertn (Euphorbiaceae). *Phytomedicine*, 10(6), 583-589.

Obbo, C. J. D., Makanga, B., Mulholland, D. A., Coombes, P. H., & Brun, R. (2013). Antiprotozoal activity of *Khaya anthotheca* (Welv.) CDC a plant used by chimpanzees for self-medication. *Journal of Ethnopharmacology*, 147(1), 220-223.

Perianayagam, J. B., Sharma, S. K., Joseph, A., & Christina, A. J. M. (2004). Evaluation of anti-pyretic and analgesic activity of *Emblica officinalis*. *Journal of Ethnopharmacology*, 95(1), 83-85.

Sepúlveda-Boza, S., & Cassels, B. K. (1996). Plant metabolites active against *Trypanosoma cruzi*. *Planta Medica*, 62(2), 98-105.

Shaba ,P., Pandey, N.N., Sharma, O.P,, Rao, J.R. and Singh, R.K.(2008). *In Vitro* Trypanocidal and cytotoxicity effects of methanolic extract of *Vitex negundo* leaves against *Trypanosoma evansi.* The 13th Congress of the Federation of Asian Veterinary Associations, 43-44. FAVA-OIE Joint Symposium on Emerging Diseases, Bangkok, Thailand.

Shaba, P., Pandey, N. N., & Singh, R. K. (2014). Evaluation of anti-trypanosomal activity of methanolic extracts of *Moringa oleifera* tree bark and seed pods. *Research Journal of Engineering and Applied Sciences*, 3, 43-49.

Shaba, P., Pandey, N. N., Sharma, O. P., Rao, J. R., & Singh, R. K. (2011a). *In vitro* trypanocidal activity of methanolic extracts of *Quercus borealis* leaves and *Zingiber officinale* roots. *Greener Journal of Agriculture Sciences*, 1(1), 41-47.

Shaba, P., Pandey, N. N., Sharma, O. P., Rao, J. R., & Singh, R. K. (2007). *In vitro* trypanocidal activity of methanolic extract of *Picrorrhiza kurroa* rhizomes against *Trypanosoma evansi*. *Planta Medica*, 73(09), 997-1034.

Shaba, P., Pandey, N. N., Sharma, O. P., Rao, J. R., & Singh, R. K. (2012). Preliminary studies of trypanocidal activity of *Embilica Officinalis* fruits (Syn: *Phyllantus Emblica*). *Journal of Veterinary Advances*, 2(5), 248-253.

Shaba, P., Pandey, N. N., Sharma, O. P., Rao, J. R., & Singh, R. K. (2012b). Therapeutic Effects of *Zanthoxylum Alatum* Leaves and *Eugenia Caryophyllata* Buds (Fruits) Against *Trypanosoma Evansi*. *Journal of Veterinary Advances*, 2(2), 91-97.

Shaba, P., Pandey, N. N., Sharma, O. P., Rao, J. R., & Singh, R. K. (2011b). Anti-trypanosomal potential of methanolic extract of *Ageratum houstonionum* flowers against *Trypanosoma evansi*. *International Journal of Basic Applied and Medical Sciences.1 (1), 149-154.*

Shaba, P., Sharma, O. P., Kurade, N. P., Rao, J. R., Singh, R. K., Pandey, N. N., & Bhanuprakash, V. (2006). *In vitro* antitrypanosomal activity and cytotoxicity of methanolic extract of *Plumbago zeylanica* against *Trypanosoma evansi*. *Journal of Veterinary Public Health*, 4(1), 31-36.

Sidwell, R. W., & Huffman, J. H. (1997). Antiviral Drug Resistance. *Research Virology*, 148, 353-365.

Srivasuki, K. P. (2012). Nutritional and health care benefits of Amla. *Journal of Pharmacognosy*, 3(2), 147-151.

Stahl, E. (1969). Thin layer chromatography. A Laboratory Handbook Springer, New York, USA.

Talakal, T. S., Dwivedi, S. K., & Sharma, S. R. (1995). *In vitro* and *in vivo* antitrypanosomal activity of *Xanthium strumarium* leaves. *Journal of Ethnopharmacology*, 49(3), 141-145.

Udupa, K. N. (1985). Ayurveda for promotion of health. *Journal of Ayurveda*, 3, 3.

Umashanker, M., & Shruti, S. (2011). Traditional Indian herbal medicine used as antipyretic, antiulcer, anti-diabetic and anticancer: A review. *International Journal of Research in Pharmacy and Chemistry*, 1(4), 1152-1159.

Williamson, J., March, J. C., & Scott-Finning, J. J. (1982). Drug Synergy in Experimental African Trypanosomiasis. *Tropennmedizin und Parasitologie*, 33, 76-82.

Woo, P. T. K. (1971). Evaluation of the haematocrit centrifuge and other techniques for the field diagnosis of human trypanosomiasis and filariasis. *Acta tropica*, 28(3), 298-303.

World Health Organization (2010). Working to overcome the global impact of neglected tropical diseases: First WHO report on neglected tropical diseases. No. 1.2010, WHO, Geneva, Switzerland

World Health Organization (WHO). (2004). Communicable Disease Surveillance and Rsponse, Geneva, Switzerland.

Wube, A. A., Bucar, F., Gibbons, S., Asres, K., Rattray, L., & Croft, S. L. (2010). Anti-protozoal activity of drimane and coloratane sesquiterpenes towards *Trypanosoma brucei* rhodesiense and *Plasmodium falciparum in vitro*. *Phytotherapy Research*, 24(10), 1468-1472.

Yi-Fei, W., Ya-Fenga, W., Xiao-Yana, W.,Zhea, R.,Chui-Wena, Q., Yi- Chenga L., Kitazatoc, K., Qing-Duan, Q., Yan, W., Li-Yun, Z., Jin-Hua, Z., Chong-Rene, Y., Qinge L and Ying-June, Z. (2009). Phyllaemblicin B inhibits Coxsackie virus B3 induced apoptosis and myocarditis, *Antiviral Research*, 84, 150-58.

Yong, V., Schmitz, V., Vannier-Santos, M. A., Lima, A. P. C. D. A., Lalmanach, G., Juliano, L., & Scharfstein, J. (2000). Altered expression of cruzipain and a cathepsin B-like target in a *Trypanosoma cruzi* cell line displaying resistance to synthetic inhibitors of cysteine-proteinases. *Molecular and Biochemical Parasitology*, 109(1), 47-59.

Zhang, L. Z., Zhao, W. H., Guo, Y. J., Tu, G. Z., Lin, S., & Xin, L. G. (2003). Studies on chemical constituents in fruits of Tibetan medicine *Phyllanthus emblica*. *China Journal of Chinese Materia Medica*, 28(10), 940-943.