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Original Research Article

Antigiardial, Amoebicidal and Cytotoxic activity of the plant *Prosopis juliflora* leave extracts

Mohammed I. Garbi[1*], Elbadri E. Osman[2], Mahmoud M. Dahab[3], Waleed S. Koko[4], Ahmed S. Kabbashi [4], Awatif A. Elegami[4] and Sheema Y. Hamed[4]

[1]Department of Microbiology, Faculty of Medical Laboratory Sciences, International University of Africa. P.O. Box 2469 Khartoum, Sudan.

[2]Elsheikh Abdallah Elbadri University, Berber, Sudan.

[3]Department of Microbiology, Faculty of Pure and Applied Sciences, International University of Africa. P.O. Box 2469 Khartoum, Sudan.

[4]Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Centre for Research P.O. Box 2404 Khartoum, Sudan.

***Corresponding Author's E-mail: mogh511@gmail.com**

Abstract

The present study was carried out to evaluate antigiardial, amoebicidal activity and explore the cytotoxicity of *Prosopis juliflora* (Leaves) Variety supreme court Leaves petroleum ether and methanolic extracts *in vitro*. Tested were performed using four concentrations: (1000 ppm, 500 ppm, 250 ppm and 125 ppm). The highest activity against *Giardia lamblia*, with respect to time, was obtained from petroleum ether extract which exhibited 78.91% mortality within 72 h with a concentration of 500 ppm followed by the methanolic extract which exhibited 77.48% mortality within 72 h with a concentration of 1000 ppm. On the other hand the lowest antigiardial activity was recorded by petroleum ether extract 38.55 % mortality with 1000 ppm concentration in 24 hours. The highest activity against *Entamoba histolytica*, with respect to time, was obtained from methanolic extract which exhibited 71.97% mortality within 72 h with a concentration of 1000 ppm. On the other hand the lowest antiamobic activity was recorded by petroleum ether extract 31.88% mortality with 125 ppm concentration within 24 hours. The cytotoxicity of methanol and petroleum ether extract had varying degrees of toxicity to vero cell lines with IC_{50} 731.79 µg/ml for the methanol extract and 11.22 µg/ml for the petroleum ether extract.

Keywords: *Prosopis Juliflora* (Leaves), antigiardial, amoebicidal, cytotoxicity.

INTRODUCTION

The history of the first introduction of *Prosopis juliflora* into India is about 130 years old. It domain of the species in arid and semi-arid tropical regions is mostly in plains and valleys but in many places it grows at altitudes of up to 1200 m above mean sea level (Khare, 2010; Singh and Pandey, 1998). *P. juliflora* is used to treat colds, diarrhea, dysentery, inflammation, itch, measles, sore throat, wounds and sexually transmitted diseases. The plant also possessed antiemetic, antibacterial, expectorant and antiseptic activities. Its juice is used in folk remedies for the cancerous condition (http://www.hort.purdue.edu accessed on 23/03/08). Pain and inflammation are common complaints in many patients suffering from acute conditions (Vikrant and Arya, 2001; Vogel, 2002). Anti-inflammatory agents inhibit the synthesis of prostaglandin synthesis which is one of the most important mediators of inflammation. Other mechanism of anti inflammatory activity the stabilization of lysosomal membrane in leucocytes (lysosomal enzymes destroy cartilage and other issues and perpetuate inflammation) and antagonism of certain actions of bradykinin (Tripathi, 1994). The infection of intestinal parasite is one of the most familiar in the outgrowth countries its negatively effects on the feed and healthy case of human (W.H.O., 1984). Some of the intestinal parasites cause sudden and acute diarrhea continues for many days as in cases of giardiasis and amoebiasis (FAbu-zeid, et al., 1989). The intestinal parasites may be caused anemia and a different grade of malnutrition (Aust et al., 1974).

Giardia lamblia is one of the most common intestinal pathogenic protozoan parasite (Newman et al., 2001). It

is becoming increasingly important among HIV/AIDS patients. There are reports that some cases of acute and chronic diarrhea in AIDS patients may be associated with giardial infection. However, Metronidazole, the common drug of choice, can cause mutagenicity in bacteria (Legator et al., 1975) and is carcinogenic in rodents (Rustia and Shubik, 1972). It also possesses undesirable side effects and treatment failures have been reported (Llibre et al., 1989).

Entamoeba histolytica is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually (World Health Organization, 1997; Ravdin and Stauffer, 2005). Intestinal amoebiasis due to the infection of *E. histolytica* is ranked third on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis (Farthing et al., 1996). Amoebiasis is the infection of human gastrointestinal tract by *E. histolytica*; a protozoan parasite capable of invading the intestinal mucosa and that may spread to other organs, mainly the liver which usually leads to amoebic liver abscess. This infection remains a significant cause of morbidity and mortality world-wide (Stanley Jr and Reed, 2001). Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers, immigrants, homosexuals and institutionalized persons.

In this study the plant *Prosopis juliflora* was selected to evaluate the activity of the petroleum ether and methanol crude leaves extracts against *Giardia lamblia* and *Entamoeba histolytica* trophozoites *in vitro.* Furthemore, the this study was carried to investigate the cytotoxicity of the same plant against vero cell line.

MATERIALS AND METHODS

Plant materials

The *Prosopis juliflora* (leave) were collected from central Sudan between January 2014 and February 2014. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI). All plant parts were air-dried, under the shadow with good ventilation and then ground finely in a mill until their uses for extracts preparation.

Preparation of crude extracts

Extraction was carried out according to the method described by Harbone, 1984. To prepare extracts for screening of antigiardial and amoebicidal activity, briefly 50 g were macerated in 250 ml of petroleum ether for 3 hours at room temperature with occasional shaking for 24 hours at room temperature, the supernatant was decanted and clarity field by filtration through a filter paper, after filtration, the solvent was then removed by rotary evaporator at 55 °C. Each residue was weighed and the yield percentage was determined (% of dry weigh) and stored at -20 °C for further analysis in tightly sealed glass vial. The remaining extracts which not soluble by petroleum ether successively extracted by methanol using the previous technique.

Parasite isolate

G. lamblia and *E. histolytica* used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All positive samples were examined by wet amount preparation. Then the positive sample was transported to the laboratory in RPMI1640 medium. Trophozoites of *G. lamblia* and *E. histolytica* were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ±1^{o}C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

In vitro susceptibility assays

In vitro susceptibility assays used the sub- culture method of Cedilla *et al*, which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *Entamoba histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis (Arguello-Garcia et al., 2004).*

Five mg from each extract was dissolved in 50 µl of dimethyl sulfoxide (DMSO) at Eppendorf tube containing 950 µl of distilled water in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20°C for further analysis.

Sterile 96-well microtite plate used for different plant extracts, positive control and negative control. Three out of 8 columns of microtitre plate wells (8 columns × 12 rows) were chosen for each extract, 40 µl (micro-liters) of an extract solution (5 mg/ml) were added to the first column wells C-1: On the other hand, twenty µl of complete RPMI medium were added to the other wells the second column and third column (C-2 and C-3). Serial dilutions of the extract were obtained by taking 20 µl of extract to the second column wells and taking 20 µl out of the complete solution in C-2 wells to C-3 wells and discarding 20 µl from the total solution of C-3 to the remaining 20 µl serial solutions in the successive columns. 80 µl of culture medium was complemented with parasite and added to all wells. The final volume in the wells was 100 µl. In each test metronidazole pure compound [(1-(2-hydroxyethl)-2-methyl-5 nitroimidazole], was used as positive control in concentration 312.5 ppm, whereas untreated cells were used as a negative controls (culture medium plus trophozoites).

For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was

determined with haemocytometer three times for counting after 0, 24, 48, and 72 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

Mortality of parasite (%) =

$$\frac{\text{(Control negative – tested sample with extract)}}{\text{Control negative}} \times 100\%$$

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the studied plants.

Microculture Tetrazolium (MTT) Assay

Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, blue colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Patel et al., 2009).

Preparation of Extracts, Solutions

Using a sensitive balance 5 mg of each extracts were weighed and put in eppendorf tubes. Fifty micro-liters of DMSO were added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

Cell Line and Culturing Medium

Vero (Normal, African green monkey kidney) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37^{o}C. The cells were subcultured twice a week.

Cell counting

Cell counts were done using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

$$\text{(Cells/ml) } N = \frac{\text{number of cells counted X dilution factor X } 10^4}{4}$$

Procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. One ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. Three duplicated concentrations for each extracts i.e. 6 wells for each extracts. All wells in rows A, B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control (Triton X). 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension was added to complete all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37^{o}C for 96 hours. On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37^{o}C. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 15 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

$$\% \text{ cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where, At = Absorbance value of test compound; Ac =Absorbance value of control.

Statistical analysis

All data were presented as means ± standard deviation (SD) Microsoft Excel program (2007) was used for analysis of all the assays results to determine significant

Table 1. Extract Yield % the plant *Prosopis juliflora* Leaves of methanol and petroleum ether extract

Scientific Name of Plant	Family name	Part Used	Yield%	
			Petroleum ether	Methanol
Prosopis juliflora	Fabaceae	Leaves	2.5	23.9

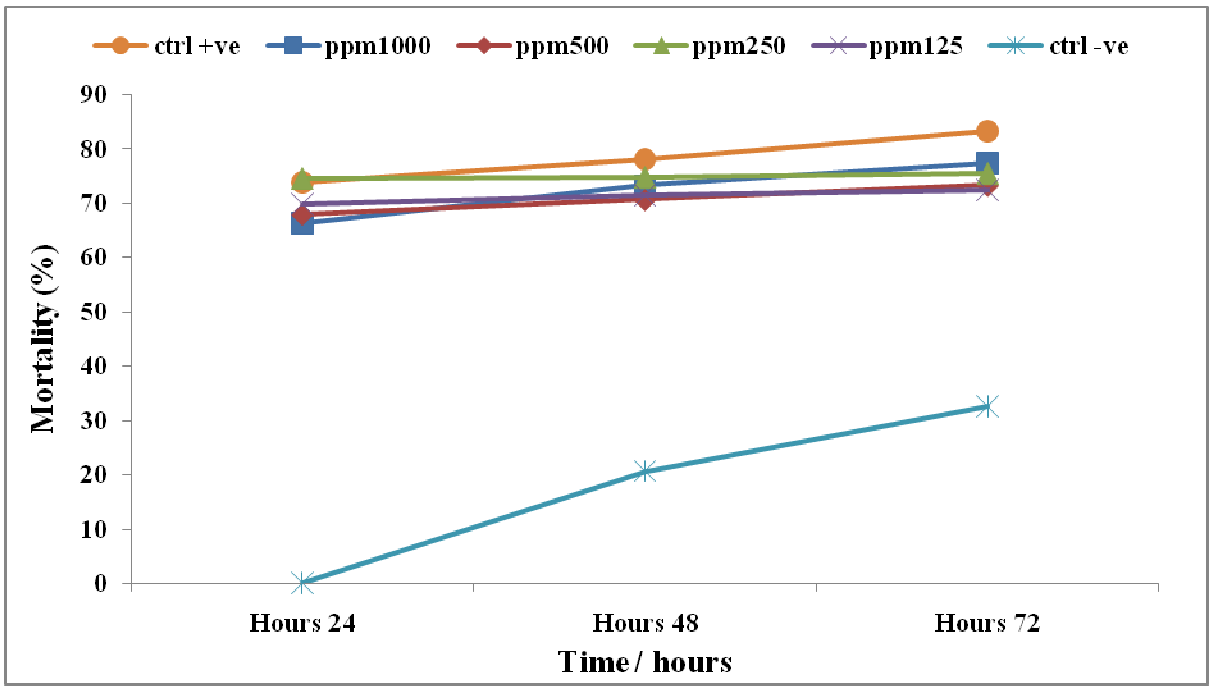

Figure 1. *In vitro* activity of *Prosopis juliflora* methanol extract against *G.lamblia.*

difference between control and plant extracts at level of P < 0.05.

RESULTS AND DISCUSSION

Giardiasis is the most common cause of parasitic gastro-intestinal disease and it is estimated that up to two hundred million people are chronically infected with *giardia lamblia* globally, and 500,000 new cases reported annually (World Health Organization, 1998). *Giardia lamblia* is a major cause of diarrhoea in humans (Lauwaet et al., 2010). *Giardia* is a flagellate protozoan with worldwide distribution that causes significant gastrointestinal diseases in a wide variety of vertebrates including cats and human .Giardiasis is one of the intestinal protozoa that cause public health problems in most developing countries as well as some developed countries. *Giardia lamblia* is considered to be one of the leading causative agents of diarrhoea in both children (Noor Azian et al., 2007; Dib et al., 2008; Addy et al., 2004) and adults (Ayeh-Kumi et al., 2009; Nyarango et al., 2008).

In this study the yield percentage (%) of *Prosopis Juliflora* leaves petroleum ether, methanol extract was 2.5, 23.9 respectively (Table.1) Moreover, the highest effective concentration of *Prosopis Juliflora* petroleum ether extract against *Giardia lamblia* was 1000 ppm with mortality of 78.91% after 72 hours. And the lowest antigiardial activity was 38.55 % mortality with 1000 ppm concentration in 24 hours in the same extract. While 312.5 ppm of metronidazole was gived 83.42% mortality after 72 hours explained in Figures (1, 2).

Among parasitic infections amoebiasis caused by *Entamoeba histolytica* ranks third worldwide in lethal infection, after malaria and schistosomiasis (Walsh, 1988; Petri Jr and Mann, 1993). Although it is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from the symptoms of amoebiasis such as hemorrhagic colitis and amoebic liver abscess. These infections result in 50000–100000 deaths annually (Ravdin, 1995). Data from some parts of Iran showed that 7.9% of the *E. histolytica/E. dispar* isolates were *E. histolytica* or mix infection and 92.15% were *E. dispar (Hooshyar et al., 2004).* In anther hand The highest effective concentration of *Prosopis Juliflora* methanol

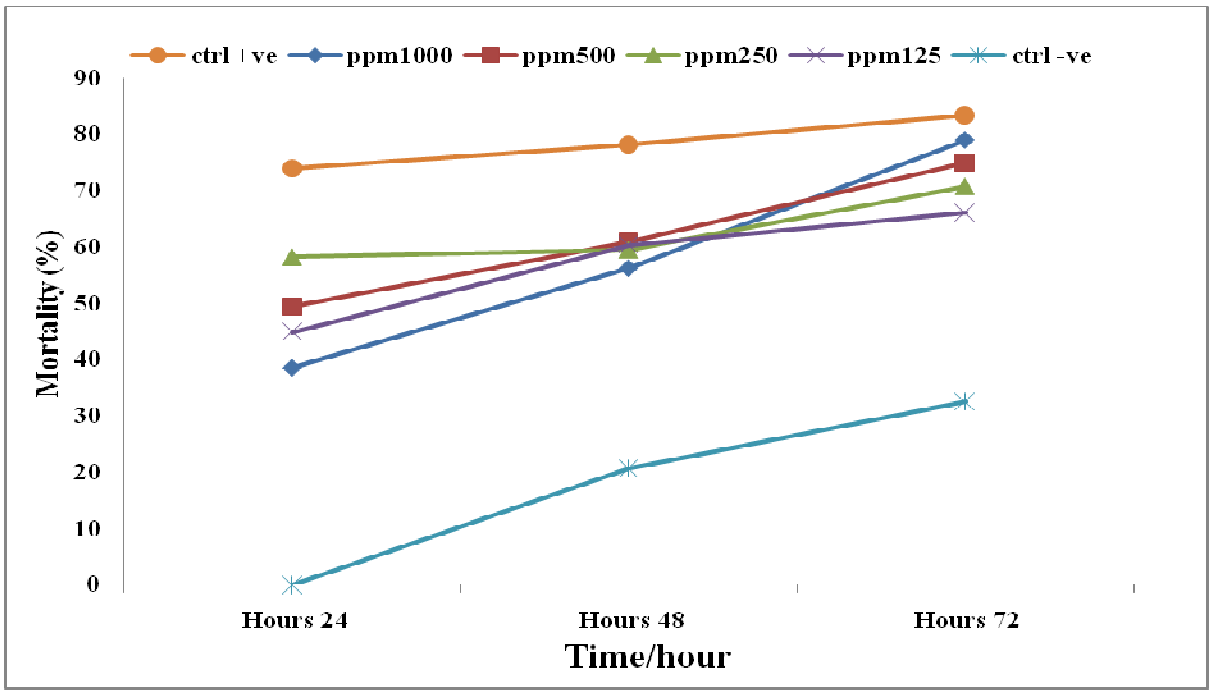

Figure 2. *In vitro* activity of *Prosopis juliflora* petroleum ether extract against *G.lambli.*

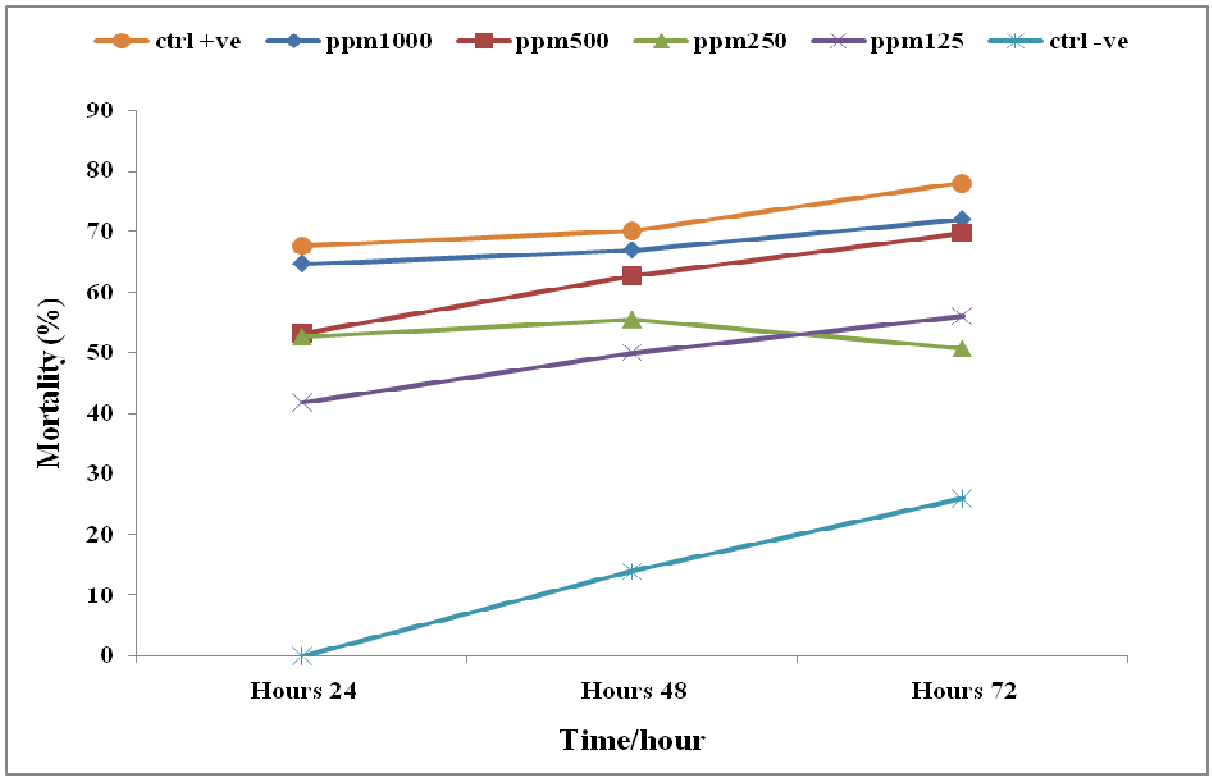

Figure 3. *In vitro* activity of *Prosopis juliflora* methanol extract against *E.histolytica.*

extract against *Entamoba histolytica* was1000 ppm with mortality of 71.97% after 72 hours. And the lowest antiamobic activity was 31.88 % mortality with 125 ppm concentration in 24 hours in petroleum ether extract. While 312.5 ppm of metronidazole gived 78.01% mortality at after 72 hours. Figures (3, 4).

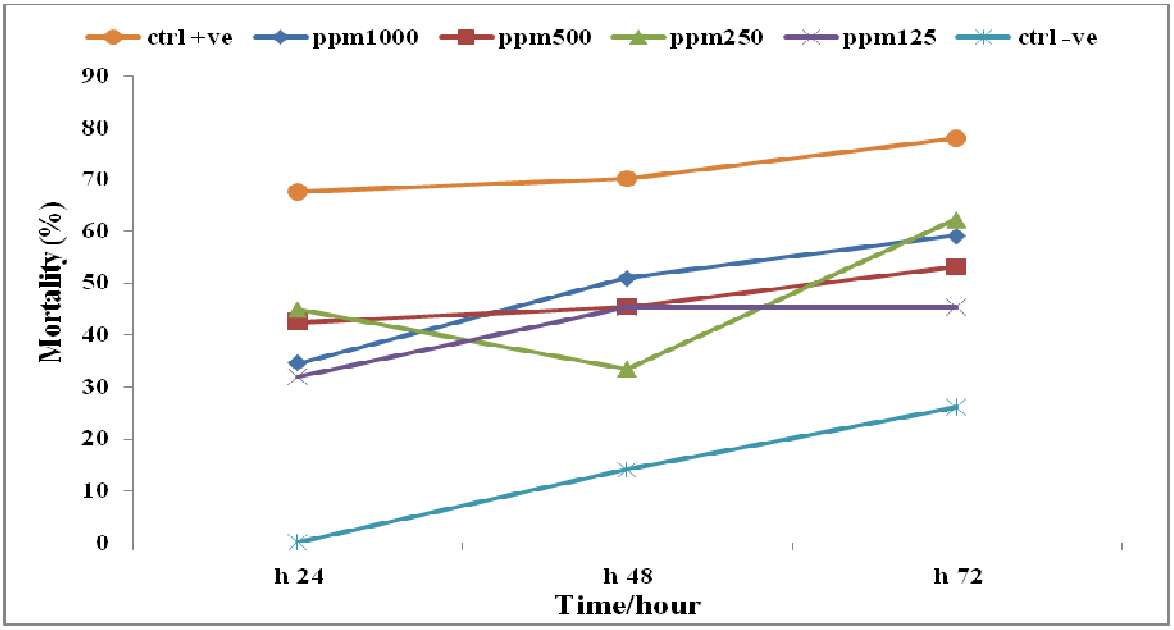

Figure 4. *In vitro* activity of *Prosopis juliflora* petroleum ether extract against *E.histolytica.*

Table 2. Inhibition percentage, Statistical Analysis and IC_{50} of *Prosopis juliflora* MTT assay against vero cell line

Name of plant (part)	Concentration (µg/ml)	pet.ether		Methanol	
		Inhibition (%) ± SD	IC_{50} (µg/ml)	Inhibition (%) ± SD	IC_{50} (µg/ml)
Prosopis juliflora (leaves)	500	39.44 ± 0.47	>100	88.52 ± 0.03	11.22
	250	35.82 ± 0.39		87.74 ± 0.01	
	125	18.18 ± 0.45		76.33 ± 0.02	
*Control	95.3 ± 0.01				

Key: *Control = Triton-x100 was used as control positive at 0.2 µg/ml.

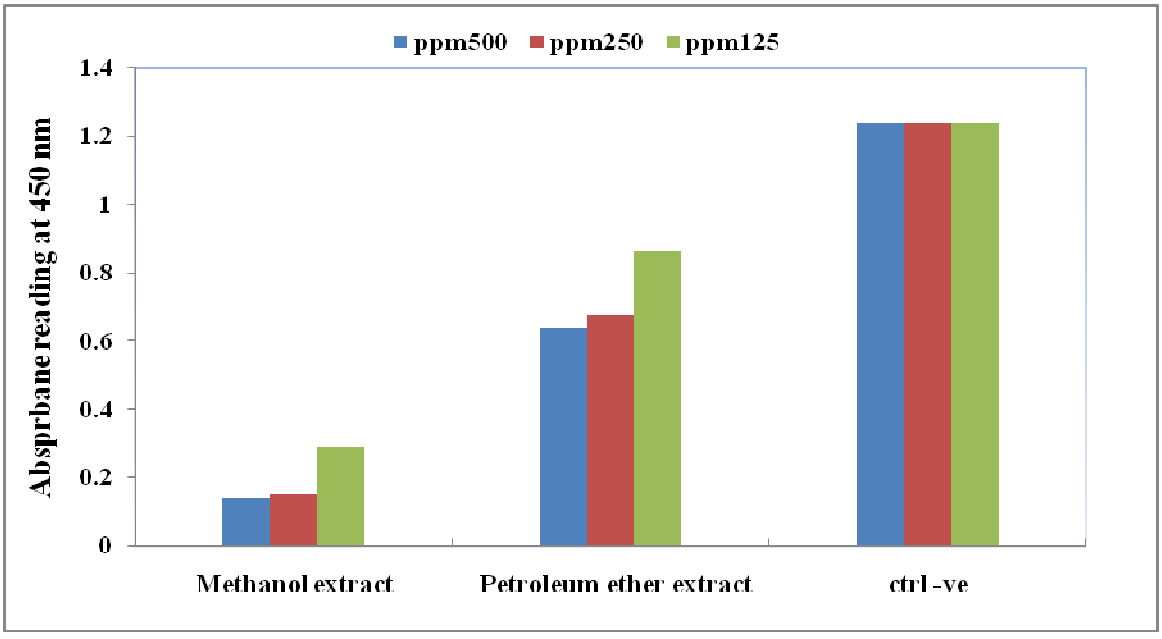

Figure 5. MTT reduction cytotoxic assay for evaluation of the plant extract.

The methanol extracts from *Prosopis Juliflora* giving a high cytotoxicity with 88.52% Inhibition and IC_{50} 11.22 µg/ml in the experiment for their cytotoxicity activity against vero cells by using MTT assay table (1). The results of cytotoxicity evaluation of extract was ranging from (500 to 125) ppm as shown in Figure (5) and table (2).

The maximum concentration used was 500 µg/ml. When this concentration produced less than 50% inhibition, the IC_{50} cannot be calculated.

This table indicates the inhibition percentage of vero cell line growth *in vitro* by methanolic and Petroleum ether extract of the *Prosopis juliflora* (leaves). MTT colorimetric assay was used. Reading in triplicate for different concentrations 500-125 µg/ml.

The result of MTT assay verified the safety of the examined extract of petroleum ether and toxic of extract of methanol.

CONCLUSION

It has been concluded that the leaves of the *Prosopis juliflora* petroleum ether extract can solved the problem of diarrhea that caused by *Giardia lamblia* and *Entamoeba histolytica* instead of Metronidazol which has been demonstrated to have side effects and they can be used traditionally or can be formulated.

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