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Antigiardial, antiamoebic and cytotoxic activity of the leaves extracts of *Vitex trifolia*

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ABSTRACT

The World Health Organization ranks diarrheal disease as the second (after acute respiratory infections) most common cause of morbidity and mortality in children in the developing world. *Entamoeba histolytica* and *Giardia lamblia* are two of the most important and most widespread diarrhea-related parasitic protozoa in the world. Metronidazole is used as a drug of choice against amoebiasis and giardiasis. However, like a lot of other chemical agents, this drug has its own side effects. The present study was carried out to evaluate anti-giardial, anti-amoebic activity and explore the cytotoxicity of *Vitex trifolia* (leaves) variety supreme court leaves petroleum ether and methanolic extracts *in vitro*. The highest activity against *Giardia lamblia*, with respect to time, was obtained from petroleum ether extract which exhibited 75.25% mortality within 72 h with a concentration of 1000 ppm followed by the same extract which exhibited 72.07% mortality within 72 h with a concentration of 500 ppm. On the other hand, the lowest anti-giardial activity was recorded by petroleum ether extract 58.92% mortality with 125 ppm concentration in 72 h. The highest activity against *Entamoeba histolytica*, with respect to time, was obtained from methanolic extract which exhibited 61.64% mortality within 72 h with a concentration of 1000 ppm. On the other hand, the lowest anti-amoebic activity was recorded by petroleum ether extract 53.62% mortality with 125 ppm concentration within 72 h. The cytotoxicity of methanol and petroleum ether extract had varying degrees of toxicity to Vero cell lines with IC_{50} 349.07 μ g/ml for the methanol extract and 369.77 μ g/ml for the petroleum ether extract.

Keywords: *Vitex doniana* (Leaves), anti-giardial, anti-amoebic, cytotoxicity, Sudan.

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INTRODUCTION

Vitex trifolia is basically a sea side shrub from the family Lamiaceae or Verbenaceae. The *Vitex* genus family is comprised of about 250 species of shrubs and trees; it is widely cultivated in warm temperate and subtropical regions (El-Kousy et al., 2012). *V. trifolia* L. is a shrub or shrubby tree that may grow up to 6 m. Its origin is unknown and several varieties have been described in distant countries as India and Mexico and Northern Sudan (McMillan, 1976). Several *Vitex* species are used

as folk remedies in Mexico. *Vitex mollis* is reported as a remedy to alleviate dysentery, as well as an analgesic and anti-inflammatory medicine; other folk uses include the treatment of scorpion stings, diarrhea and stomach ache (Argueta et al., 1994). Antimalarial, antimicrobial, and antifungal activity has been reported for *V. gaumeri*, *V. agnus-cas-tus* and *V. negundo*, respectively; *V. negundo* is also used as an anti-inflammatory agent, *Vitex negundo* (Family: Verbenaceae), is an important

medicinal plant found throughout India. *V. trifolia* extracts from leaves and roots are the most important in the field of medicine and drug. Its leaves (Dharmasiri et al., 2003) and seeds (Chawla et al., 1992) are widely used externally for rheumatism and inflammations of joints and are also reported to have insecticidal properties. Internally, decoction of its leaves is taken as diuretic, expectorant, vermifuge, tonic and febrifuge. The chemical components of the essential oil of leaf isolated from *V. negundo* and other species while *V. gaumeri* is used to treat colds and coughing spells (Ekundayo et al., 1990; Damayanti et al., 1996). It is well known that a considerable number of plant species, besides their popular use as medicine in many countries, in India some species are present *Vitex glabrata*, *Vitex leucoxydon*, *Vitex penduncularis*, *Vitex pinnata*, and *Vitex trifolia* (Wealth of India, 1976) possess insecticidal activities. The genus *Vitex* sp. is not an exception. *V. negundo* has larvicidal activity against mosquito species *Culex quinquefasciatus* and *Anopheles stephensi* (Pushpalatha and Muthukrishnan, 1995), and acts as a deterrent to the mosquito *Aedes aegypti* (Hebbalkar et al., 1992). *Vitex rotundifolia* also shows better scientific word properties towards *A. aegypti*. Several other *Vitex* species are currently being investigated in specific programs of pest control (Rahman and Bhattacharya, 1982; Epila and Ruyooka, 1988; Sudarsanam et al., 1995). The infection of intestinal parasite is common in developing countries and has negative effects on the feed and human health (W.H.O., 1984). Some of the intestinal parasites cause sudden and acute diarrhea which continues for many days as in the cases of giardiasis and amoebiasis (Abu-zeid et al., 1989). The intestinal parasites may cause anemia and malnutrition (Aust et al., 1974).

Giardia lamblia is one of the most common intestinal pathogenic protozoan parasites (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). *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 caused by *E. histolytica* is ranked third after malaria and schistosomiasis on the list of parasitic protozoan infections leading to death (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 and Reed, 2001). Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers,

immigrants, homosexuals and institutionalized persons.

Vitex trifolia was selected to evaluate the activity of petroleum ether and methanol crude extracts against *Giardia lamblia* and *E. histolytica* trophozoites and also the cytotoxicity against Vero cell line was evaluated.

MATERIALS AND METHODS

Plant materials

V. trifolia study was collected from, Northern Sudan, collected on February 2012. The taxonomic identification of the plant was carried out at Medicinal and Aromatic Plants Research Institute, National Center for Research by W.E.A. Alla. A voucher specimen was deposited at the herbarium of the institute. The leaves were air-dried at room temperature (28 to 30°C) for three weeks and coarsely ground to powder by a mechanical grinder. Table 1 indicates the scientific names, families, parts used, yield percentage based on the dried weight of methanol and petroleum ether extracts and traditional uses of *Vitex trifolia*.

Preparation of crude extracts

Thirty grams of the coarsely ground material of the leaf were successively extracted for by soxhlet apparatus using petroleum ether, and methanol. The extracts were then filtered and evaporated under reduced pressure using rotatory evaporator apparatus.

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 mount 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 $37 \pm 1^\circ\text{C}$. 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 Cedillo-Rivera et al. (2002), which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica*, *Giardia intestinalis* and *Trichomonas vaginalis* (Arguello-Garcia et al., 2004).

5 mg from each extract was dissolved in 50 μl of dimethyl sulfoxide (DMSO) at Eppendorf tube containing 950 μl 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 microtitre plate was used for different plant extracts, positive control (metronidazole) and negative control (culture medium plus trophozoites).

Three out of 8 columns of microtitre plate wells (8 columns \times 12 rows) were chosen for each extract, 40 μl of an extract solution (5 mg/ml) were added to the first column wells C-1: On the other hand, 20 μl of complete RPMI medium was 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.

Table 1. *Vitex trifolia* selected to be investigated for their anti-giardial, anti-amoebic and cytotoxicity.

Scientific name	Family name	Part used	Yield percentage		Traditional medicine
			Petroleum ether	Methanol	
<i>Vitex trifolia</i>	Verbenaceae	Leaves	3.8	23.9	Used to treat colds, diarrhea, dysentery, inflammation, itch, measles, sore throat, wounds and sexually transmitted diseases, antimalarial, antimicrobial.

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 (a trichomonocide) pure compound [(1-(2-hydroxyethyl)-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:

$$\text{Mortality of parasite (\%)} = \frac{\text{Control negative} - \text{tested sample with extract}}{\text{Control negative}} \times 100\%$$

Cytotoxicity screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of *Vitex trifolia*.

Microculture tetrazolium (MTT) assay

Principle of MTT assay

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 *Vitex trifolia* extracts for MTT assay

Using a sensitive balance 5 mg of each extracts were weighed and put in eppendorf tubes. 50 µl 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°C. The cells were subcultured twice a week.

Cell line used

Vero cells (Normal, African green monkey kidney).

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:

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

MTT procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 min separating the cells from the supernatant that flicked out. 1 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. 3 duplicated concentrations for each extracts i.e. 6 wells for each of 8 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 were added completing 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°C for 96 h.

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 h at 37°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 10 min then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the following formula:

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

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

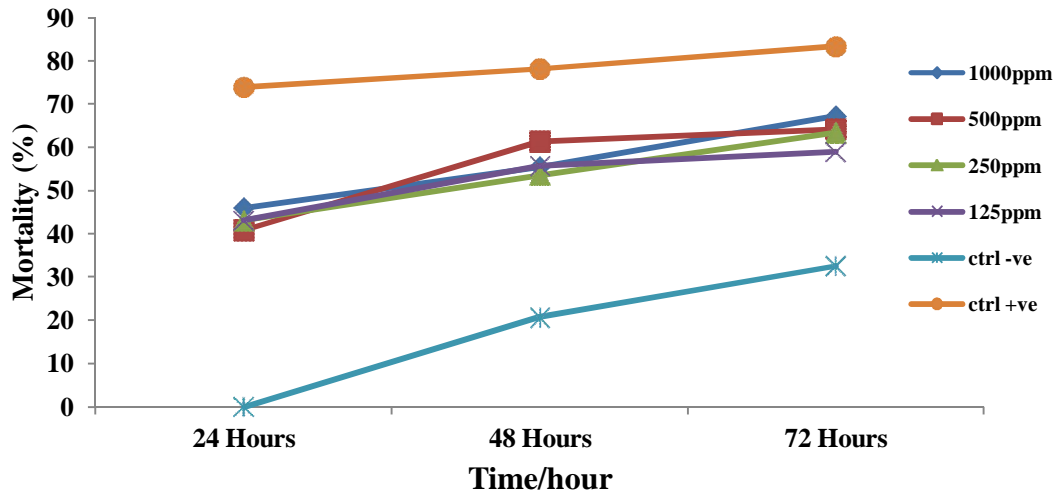


Figure 1. *In vitro* activity of *Vitex trifolia* methanol extract against *G. lamblia*.

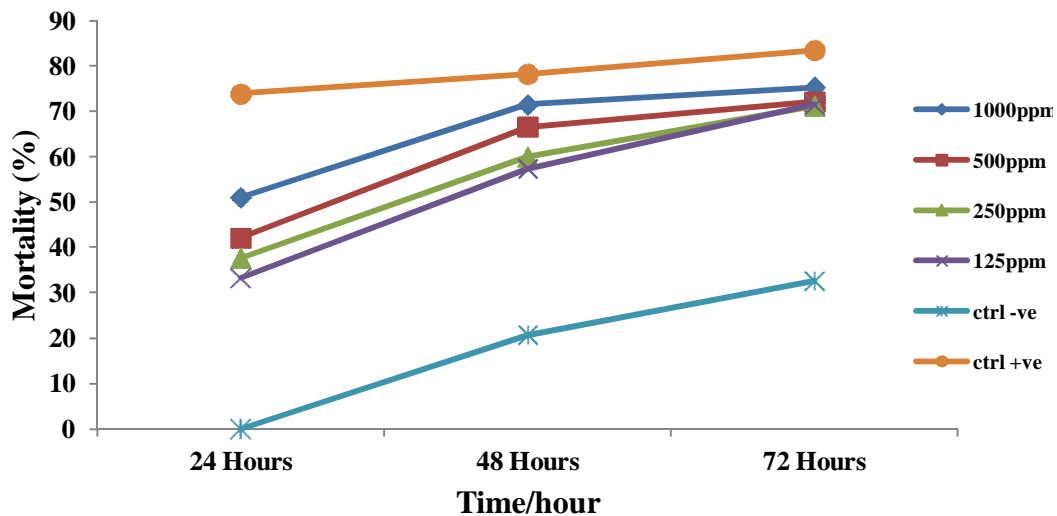


Figure 2. *In vitro* activity of *Vitex trifolia* petroleum ether extract against *G. lamblia*.

Statistical analysis

All data were presented as means \pm S.D. Statistical analysis for all the assays results were done using Microsoft Excel program Student t test was used to determine significant difference between control and plant extracts at level of $P < 0.05$.

RESULTS

The yield % of *V. trifolia* leaves petroleum ether and methanol extract was 3.8 and 23.9, respectively. The highest effective concentration of *V. trifolia* petroleum ether extract against *G. lamblia* was 1000 ppm with mortality of 75.25% after 72 h. The lowest anti-giardial activity was 33.33% mortality with 125 ppm concentration in 24 h in the same extract. 312.5 ppm of metronidazole

gave 83.42% mortality after 72 h (Figures 1 and 2).

On the other hand, the highest effective concentration of *V. trifolia* methanol extract against *E. histolytica* was 1000 ppm with mortality of 61.64% after 72 h. And the lowest antiamebic activity was 28.06% mortality with 125 ppm concentration in 24 h in petroleum ether extract; while 312.5 ppm of metronidazole was gave 78.01% mortality at after 72 h (Figures 3 and 4).

The methanol extracts from *V. trifolia* had moderate cytotoxicity with 59.20% Inhibition in 500 ppm and $IC_{50} > 100 \mu\text{g/ml}$ 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 $\mu\text{g/ml}$. When this concentration produced less than 50%

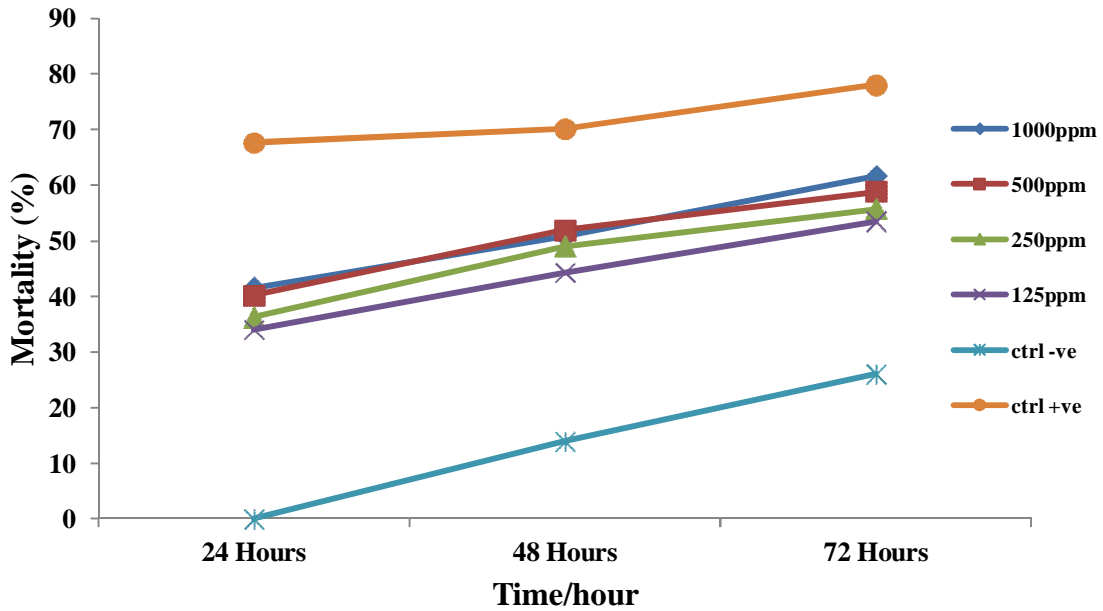


Figure 3. *In vitro* activity of *Vitex trifolia* methanol extract against *E. histolytica*.

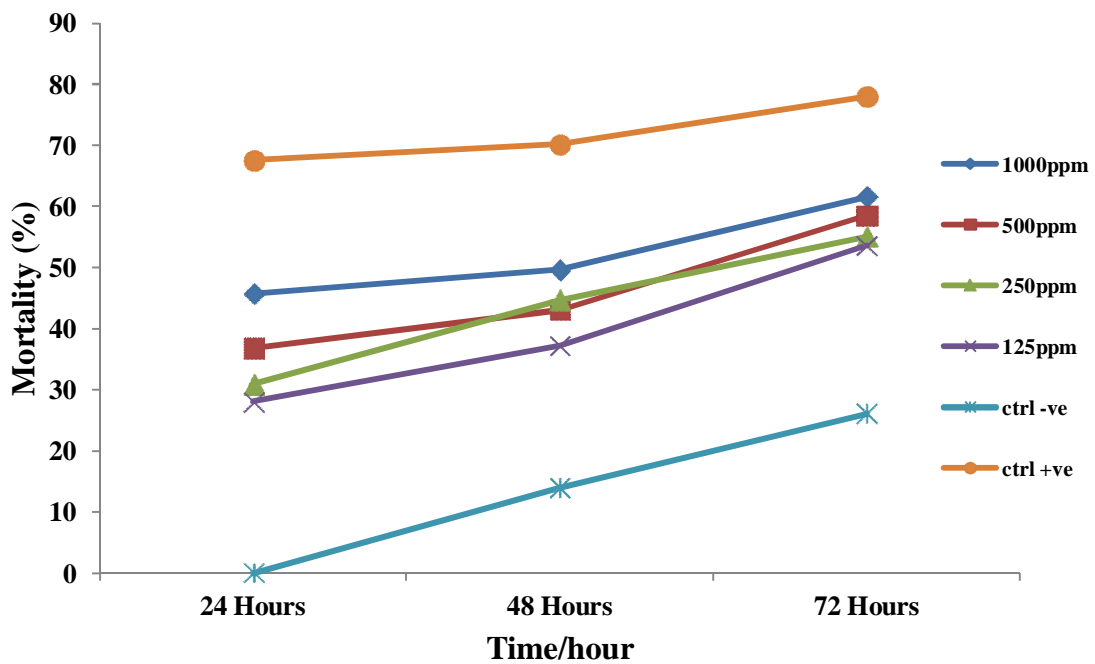


Figure 4. *In vitro* activity of *Vitex trifolia* petroleum ether extract against *E. histolytica*.

inhibition, the IC₅₀ cannot be calculated.

Table 2 indicates the % inhibition of Vero cell line growth *in vitro* by methanolic extract and Petroleum ether extract of the *Vitex trifolia* (leaves). MTT colorimetric assay was used.

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

DISCUSSION

Infectious diarrheal diseases caused by protozoa *E. histolytica* and *G. lamblia* are responsible for considerable morbidity and mortality worldwide mainly in developing countries. The problems of treating of gastrointestinal diseases by chemotherapy are well known. Therefore, new, safer and more effective drugs

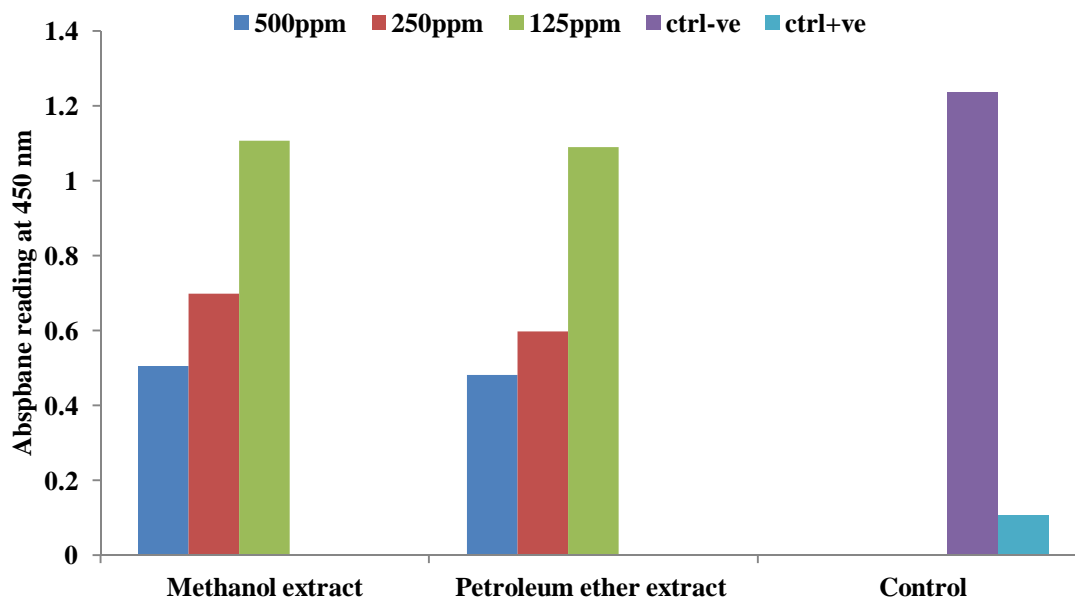


Figure 5. MTT reduction cytotoxic assay for evaluation of *Vitex trifolia* extracts.

Table 2. Inhibition percentage, statistical analysis and IC₅₀ of *Vitex trifolia* MTT assay against Vero cell line.

Name of plant (part)	Concentration (µg/ml)	Petroleum ether		Methanol	
		Inhibition (%) ± SD	IC ₅₀ (µg/ml)	Inhibition (%) ± SD	IC ₅₀ (µg/ml)
<i>Vitex trifolia</i> (leaves)	500	54.55 ± 0.21		59.20 ± 0.31	
	250	43.52 ± 0.65	>100	43.61 ± 0.15	>100
	125	-3.04 ± 0.16		10.48 ± 0.12	
*Control		95.3 ± 0.01		95.3 ± 0.01	

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

are necessary. In this context medicinal plants have made and are continuing to make important contributions to this area of therapeutics. This is the first report of anti-giardial and anti-amoebic activities of the *V. trifolia* extracts was investigated using *in vitro* bioassays that included the standard drug, metronidazole as positive control. The *V. trifolia* extracts against *E. histolytica* and *G. lamblia* are given in Figures 1 to 4. As indicated in the figures, the extract of methanol showed moderate anti-giardial activities, meanwhile the petroleum ether showed high effect against *G. lamblia*. Both methanol and petroleum ether extracts of *V. trifolia* were equally effective against *E. histolytica* and were less efficient than metronidazole. In other studies the petroleum ether and ethanol extracts of *V. trifolia* leaves was tested for gram-positive and gram-negative bacteria and showed moderate inhibiting activity (Rahman et al., 2001). This result also similarity with Thenmozhi et al. (2013) who find certain degree of anthelmintic activity of *V. trifolia* extracts. Other study on hexanic extract from leaves completely inhibited the growth of the fungal plant

pathogen *Fusarium* sp. (Hernandez, 1999).

Apparently, the introduction of the *V. trifolia* extracts showed effect against the aforementioned parasites compared with the standard drug metronidazole. In addition, the *V. trifolia* extracts were non-toxic to the Vero cell lines this result agree with Zullies Ikawati who studied the acute toxicity of the extract combination of *V. trifolia* leaves in rats and the LD₅₀ value showed that the highest dose can be administered without lethal effect, indicating that the extract has become safe (Kulkari, 2011).

Conclusion

Our results revealed a moderate pharmacological activity against *G. lamblia* and *E. histolytica* we suggested that the extracts have the potential of being used in parasitic infection. The results presented here providing motivation for further exploration of isolation active compounds, particularly as anti-giardial and anti-amoebic agents from *V. trifolia* extracts with important advantages for the

development of new anti-parasitic agents.

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