

## Full Length Research Paper

## ***In vitro* susceptibilities of the clinical isolate of *Entamoeba histolytica* to *Euphorbia hirta* (Euphorbiaceae) aqueous extract and fractions**

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*Euphorbia hirta* (Euphorbiaceae) has been used widely in traditional medicine as a treatment against infectious pathogens. This medicinal plant is also well known for its diverse biological activities. The present study aimed to evaluate the susceptibilities of the clinical isolates of *Entamoeba histolytica* to *E. hirta* aqueous extract, methanol fraction, methylene chloride fraction, and hexane fraction. The clinical isolates of *E. histolytica* grown on polyxenic medium were treated with *E. hirta* aqueous extract (AE), methanol fraction (MF), methylene chloride fraction (CH<sub>2</sub>Cl<sub>2</sub> F), and hexane fraction (HF). Metronidazole (MTZ) was used as the reference drug. Furthermore, the effects of the extract as well as the fractions on the activity of *E. histolytica* ribonuclease (RNase), aldolase, acid and alkaline phosphatases (ACP and ALP) were evaluated. The methanol fraction of *E. hirta* inhibits significantly the clinical isolate of *E. histolytica* growth with the IC<sub>50</sub> of 67.18 ± 7.40 µg/ml after 72 h of incubation but remains lower compared to metronidazole (IC<sub>50</sub><10 µg/ml). The aqueous extract and methylene chloride showed moderate activities, whereas no amoebicidal activity was found associated to the hexane fraction. The enzymes activity assay showed that the inhibitory effect of the methanol fraction against *E. histolytica* RNase, aldolase acid and alkaline phosphatases activities were comparable to that of metronidazole and significantly higher than those of aqueous extract and methylene chloride fraction. According to the above mentioned results, the methanol fraction of *E. hirta* exhibits anti-amoebic activity and inhibition of enzymes involved in the metabolism or survival of *E. histolytica*.

**Key words:** *Entamoeba histolytica*; *Euphorbia hirta*; Anti-amoebic activity.

### INTRODUCTION

Amoebiasis is caused by a protozoan parasite, *Entamoeba histolytica* with or without clinical symptoms and it is the third leading cause of death from parasitic diseases after malaria and schistosomiasis (WHO, 1997). This infection

remains a major health problem in developing countries and its prevalence varies between countries and between regions with different socio-economic conditions (Jackson, 2000). Sometimes, it may reach 50% of the

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population in regions with poor sanitary conditions (Caballero et al., 1994). The most effective and commonly used drug for treatment of this intestinal protozoan infection is metronidazole (MTZ). However, this drug has been reported to have unpleasant side effects such as metallic taste, headache, dry mouth, and to a lesser extent nausea, glossitis, urticaria, pruritus, and dark colored urine. Carcinogenic, teratogenic, and embryogenic effects have also been documented (Upcroft et al., 1999; Upcroft and Upcroft, 2001) in addition to the fact that it may lower both cell mediated and humoral immune responses in drugs recipients (Saxena et al., 1985). Therefore, immunocompromised recipients of MTZ including those with AIDS (full form) would constitute a high risk. According to these observations above, the search of alternative anti-amoebic compounds with high activity, and low toxicity is still necessary. Segment of the world's population relies on traditional remedies to treat a plethora of diseases. Medicinal herbs constitute an indispensable part of traditional medicine practised all over the world due to the cost, easy access, and ancestral experiences (Martini-Bertolo, 1980). *Euphorbia hirta* belongs to the genus *Euphorbia* and the family of Euphorbiaceae. It is a common herb in the pan-tropic and partly subtropic areas worldwide, including China, India, Philippines, Australia, Africa, Malaysia, and so on (Huang et al., 2012). *E. hirta* is an important medicinal herb with various pharmacological behaviours. The flavonol glycosides afzelin, quercetin and myricitrin, isolated from *E. hirta* showed inhibition of the proliferation of *Plasmodium falcifarum* at different concentrations (Jackson, 2000). The leaves, flowers, stems, and root extract of the plant exhibited antimicrobial activity against *E. coli*, *C. albicans*, *S. aureus*, and *P. mirabilis* (Mohammad et al., 2010). The anti-diarrheal effect of the *E. hirta* herb decoction was studied in mice. It demonstrated an activity in models of diarrhea induced by castor oil, arachidonic acid, and prostaglandin E 2. Quercitrin, a flavonoid isolated from this crude drug contributed to the anti-diarrheal activity at a dose of 50 mg/kg, against castor oil and prostaglandin E2-induced diarrhea in mice (Galvez et al., 1993). The *in vitro* and *in vivo* immunomodulatory properties of *E. hirta* is reported elsewhere (Ramesh and Vijaya, 2010). The finding has been proven through macrophage activity testing, carbon clearance test, and mast cell de-granulation assay. The aqueous extract of the leaves of *E. hirta* Linn could serve as an immunostimulant on the experiment of the pathogen-infected *Cyprinus carpio* Linn. (Cyprinidae). The anti-inflammatory activity of the chemicals in *E. hirta* showed that the flavonoids quercitrin (converted to quercetin in the alimentary canal) and myricitrin, as well as the sterols 24-methylenecycloartenol and sitosterol, exert noteworthy and dose dependent anti-inflammatory activity. Triterpene beta-amyrin also seems to exert a similar anti-inflammatory activity (Ekpo and Pretorius, 2007). The crude and polyphenolic extracts of *E. hirta* exhibited anti-amoebic potential (Tona et al., 2000).

However, the activity of the fractions obtained from this plant have not yet been tested against *E. histolytica*. Therefore, the present study was planned to investigate the *in vitro* susceptibilities of the clinical isolates of *E. histolytica* to *E. hirta* (euphorbiaceae) aqueous extract and fractions.

## MATERIALS AND METHODS

### Biological materials

#### Plant material

The aerial part of *E. hirta* (Euphorbiaceae) collected in Yaounde (Cameroon) on April 2011 during morning time was used in the present study.

#### Microorganisms

Clinical isolates of *E. histolytica* trophozoites from stool sample of Indian patients suffering from amoebiasis collected at the Department of Medical Parasitology of the Postgraduate Institute of Medical Education and Research (PGIMER) of Chandigarh, India were used for polyxenic cultivation.

#### *Euphorbia hirta* extract and fractions preparation

The aerial (leaves and stems) part of *E. hirta* was harvested, washed in chlorinated water and dried at room temperature (Moundipa et al., 2005). Dried materials were reduced in powder form and the extract was obtained by decoction of 200 g of the powder in 1000 ml of distilled water for 3 h. Decoction obtained was concentrated in the oven at 50°C for 72 h and the concentrated product constituted the aqueous extract. The extraction yield was calculated according to the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{mass of extract obtained}}{\text{mass of powder introduced}} \times 100$$

Fractionation of the aqueous extract was performed by using different polarity based solvent from hexane (nonpolar) to methanol (polar) (Zubair et al., 2011). To obtain the hexane fraction (HF), 200 g of the above aqueous extract was macerated in 1000 ml of pure hexane until exhaustion of the solvent. Resulting solution was concentrated using a Büchi Rotavapor R-210/R-215 with the temperature of 40°C. The solid material obtained after a total evaporation of the solvent was conserved and constituted our hexane fraction. Then, methylenechloride fraction (CH<sub>2</sub>Cl<sub>2</sub> F) was obtained by subjecting the residue to a second maceration in the same volume of pure methylene chloride followed by the concentration as described previously. The residue obtained from the second step was extracted with 1000 ml of pure methanol as described above to obtain the methanol fraction (MF). Crude aqueous extract and fractions obtained were subjected to *in vitro* assays for the determination of their anti-amoebic potential.

#### *E. histolytica* cultivation

##### Polyxenic cultivation

Biphasic medium of Boeck and Drbohlav (Parija and Rao, 1995) that involves solid phase (ringer's solution + egg) and liquid phase (lock's solution containing nutrients) was used for *E. histolytica* polyxenic cultivation. Before inoculation, complete media were pre-

incubated at 37°C for 30 min to 1 h and 1000 µl of diarrheal stool sample containing viable trophozoites of *E. histolytica* were introduced in each tube. The tubes were incubated at 37°C and the *E. histolytica* growth verified after every 48 or 72 h. Then, the tubes were removed from the incubator and shaken to detach parasites from the solid phase and left for 5 min then the supernatant was decanted to obtain the subculture. The pellet containing the parasites was introduced in a tube containing pre incubated new medium as previously described (Moundipa et al., 2005).

#### Amoebicidal effect of the *E. hirta* extract and fractions assays

*E. hirta* aqueous extract (AE) and its fractions (AE, MF, CH<sub>2</sub>Cl<sub>2</sub> F) were prepared using sterile DMSO (Sigma-Aldrich, and culture medium leading to concentrations of 200, 20, 2 and 0.2 mg/ml respectively). Each mixture was filtered with sterile syringe filters (Ø 22 µm) and aliquots were prepared from these stock solutions. Parasites grown were harvested at midlog phase at the concentration of 10<sup>7</sup> cells/ml of culture by counting using the haemocytometer (Neubauer, Hausser Scientific) and inoculated in tubes containing new 5 ml media in which 25 µl of plant materials were added. MTZ was used as a standard drug and was tested at 0.1, 1, 5 and 10 µg/ml. AE was tested at the concentration of 50, 100, 200 and 400 µg/ml; whereas all the different fractions were tested at 25, 50, 100 and 200 µg/ml. One control tube was used in which parasites were incubated on culture medium containing 0.5% DMSO without any drug. Each testing concentration was made in triplicate and the experiment was repeated three times for each compound. All the tested tubes were incubated at 37°C as described by Chitravanshi et al., 1992 and the viability was evaluated by trypan blue method after 24, 48 and 72 h.

Amoebicidal activity was evaluated using the method described by Bansal (1987). In 1.5 ml micro centrifuge tube, 25 µl of parasite suspension and 225 µl of 0.4% trypan blue solution prepared in 0.9% NaCl was introduced. The mixture was homogenized and 10 µl of this mixture was used for cells counting. The chamber was covered with cover slip and the viable (bright) cells as well as the dead (blue) cells were counted at 40X on a light microscope. The concentration of the cell has been calculated using the following formula:

$$N = (n \times d) / v$$

Where, N= concentration of viable cells/ml; n= number of the viable cells counted in the chamber, d= dilution factor and v= the volume of the chamber (0.1 µl)

The percentages of inhibition were calculated also using the formula below and IC<sub>50</sub> were determined using the software Graphpad Prism 3.0

$$\text{Percentage inhibition (\%)} = (N_C - N_T) / N_C \times 100$$

N<sub>C</sub> = Number of viable cells in the control tube and N<sub>T</sub> = Number of viable cells in testing tube.

#### Effect of *E. hirta* extract and fractions on some enzymes of *E. histolytica*

The effect of extract, fractions and metronidazole on some enzyme activities of polyxenically cultivated clinical isolate of *E. histolytica* was studied as described (Sohni et al., 1995).

Amoeba from different tubes of each type of culture were pooled and washed five times with cold phosphate-buffered saline then centrifuged at 1/min for 10 min. The sediment was suspended in cold phosphate-buffered saline to yield a concentration of 10<sup>6</sup> cells/ml

following which the suspension was homogenized in cold 0.25 M sucrose solution for 3 h at 4°C. The homogenate was then centrifuged in cold and opalescent supernatant was used as the crude enzyme extract. The amount of total protein in the crude enzyme extract was determined according to the protein colorimetric method assay described by Bradford (1976). All the assays compounds (extract, fractions and metronidazole) were tested at the concentration of 100, 200, 400, and 800 µg/ml. Each concentration was made in triplicate and the experiments were repeated three times.

#### RNase activity assay

A micro centrifuge tube with a capacity of 1.5 ml was used for preparing the reaction mixture of 1.25 ml containing 150 µM of phosphate buffer (pH 7.6), 1.25 mg of yeast RNA, 0.5 ml of enzyme extract and the testing drug (Sparh and Hollingworth, 1961). The mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of 0.25 ml of uranyl acetate reagent. The suspension was mixed and chilled for 30 min to precipitate undigested RNA. The precipitate was centrifuged and the absorbance was read at 260 nm against blanks. An enzyme unit was defined as the amount of enzyme which gives an increase in absorbance of 0.1.

#### Aldolase activity assay

Aldolase activity was assayed according to the method describe by Sibley and Lehninger (1949) with some modifications. In a 1.5 ml micro centrifuge tube, a reaction mixture of 1.25 ml containing 0.5 ml of Tris buffer (pH 8.6), 0.125 ml of 0.05 M fructose-1,6-diphosphate, 0.125 ml of 0.0035 M hydrazine sulphate solution in 100 µM EDTA (pH 7.5) 0.25 ml of enzyme extract and the testing drugs was introduced. The mixture was incubated at 37°C for 30 min. The reaction was stopped by addition of 10% trichloroacetic acid (TCA) and the absorbance of supernatant was read after at 240 nm against blanks. An enzyme unit was defined as the amount of enzyme which gives an increase in absorbance of 1.00.

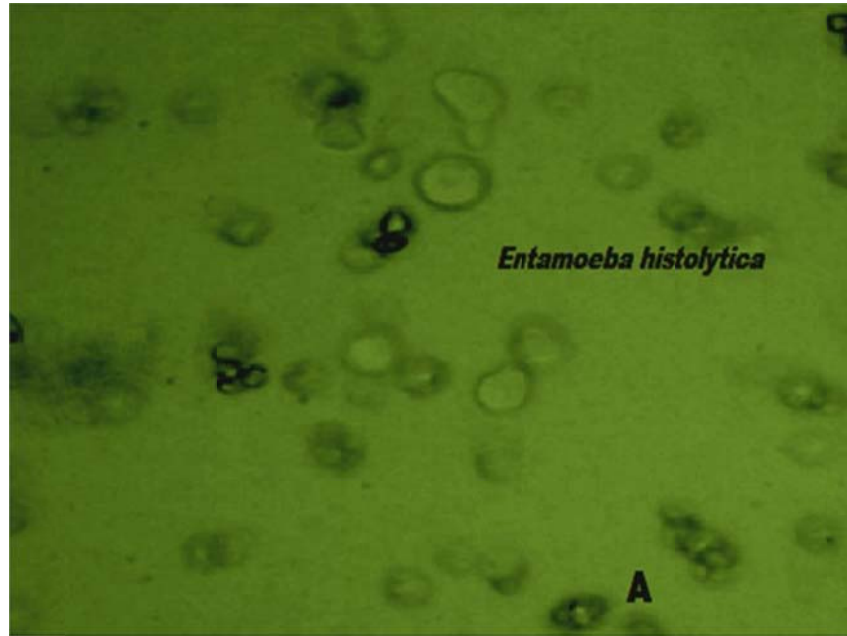
#### Acid and alkaline phosphatase (ACP and ALP) activity assay

Aldolase activity was assayed according to the method described by Ashrafi et al. (1969). For the ALP analysis, a reaction mixture containing 50 µl of enzyme extract, 100 µl of substrate p-nitrophenylphosphate (PNP) 1% in 0.1 M glycine/NaOH buffer pH 9, as well as the tested compounds were prepared in a micro plate of 96 wells and the final volumes were adjusted to 250 µl. The reaction mixtures for the ACP activity assay contained 50 µl of enzyme extract, 100 µl of 1% substrate PNP in citrate buffer pH 4, as well as the tested compounds were prepared in a micro plate of 96 wells and the final volumes were adjusted to 250 µl. Both ACP and ALP mixtures were incubated at 37°C for 30 min. The reaction was stopped by addition of 100 µl 0.1 N NaOH and the absorbance was read at 405 nm against blanks. An enzyme unit was defined as the amount of enzyme which catalyses the formation of 1 µmol of p-nitrophenolate ion.

The specific activities were calculated for all the enzymes assayed according and the IC<sub>50</sub> of the inhibition were determined using the software Graphpad Prism 5.0.

#### Statistical analysis

The tests were performed in triplicate and all data are presented as mean ± SD (standard deviation) values. Statistical analysis was performed using GraphPadPrism and student's t-test was used to determine P-values for the differences observed between test compounds and control. Results were considered significantly different when P ≤ 0.05.



**Figure 1.** *Entamoeba histolytica* growing on polyxenic Boeck and Drbohlav medium (Pechangou January 30<sup>th</sup> 2013).

## RESULTS

### Amoebicidal effect of the *E. hirta* aqueous extract and fractions against polyxenic culture of clinical isolates of *E. histolytica*

The clinical isolates of *E. histolytica* grown maintained on biphasic medium of Boeck and Drbohlav (Figure 1) were incubated with different plant extract and fractions. MTZ and *E. hirta* aqueous extract, methanol fraction, methylene chloride fraction exhibited amoebicidal effects that were concentration dependant (Figure 2). In contrast no amoebicidal activity was found to be associated with HF. The IC<sub>50</sub> values of MTZ and *E. hirta* AE, MF, and CH<sub>2</sub>Cl<sub>2</sub> F were respectively about 4.30, 145.95, 67.18, and 194.04 µg/ml after 72 h of incubation. *E. hirta* MF exhibited higher amoebicidal effect than AE and CH<sub>2</sub>Cl<sub>2</sub> F but, remained lower as compared to reference drug MTZ activity (Table 1).

### Effect of *E. hirta* extract and fractions on some enzymes of *E. histolytica*

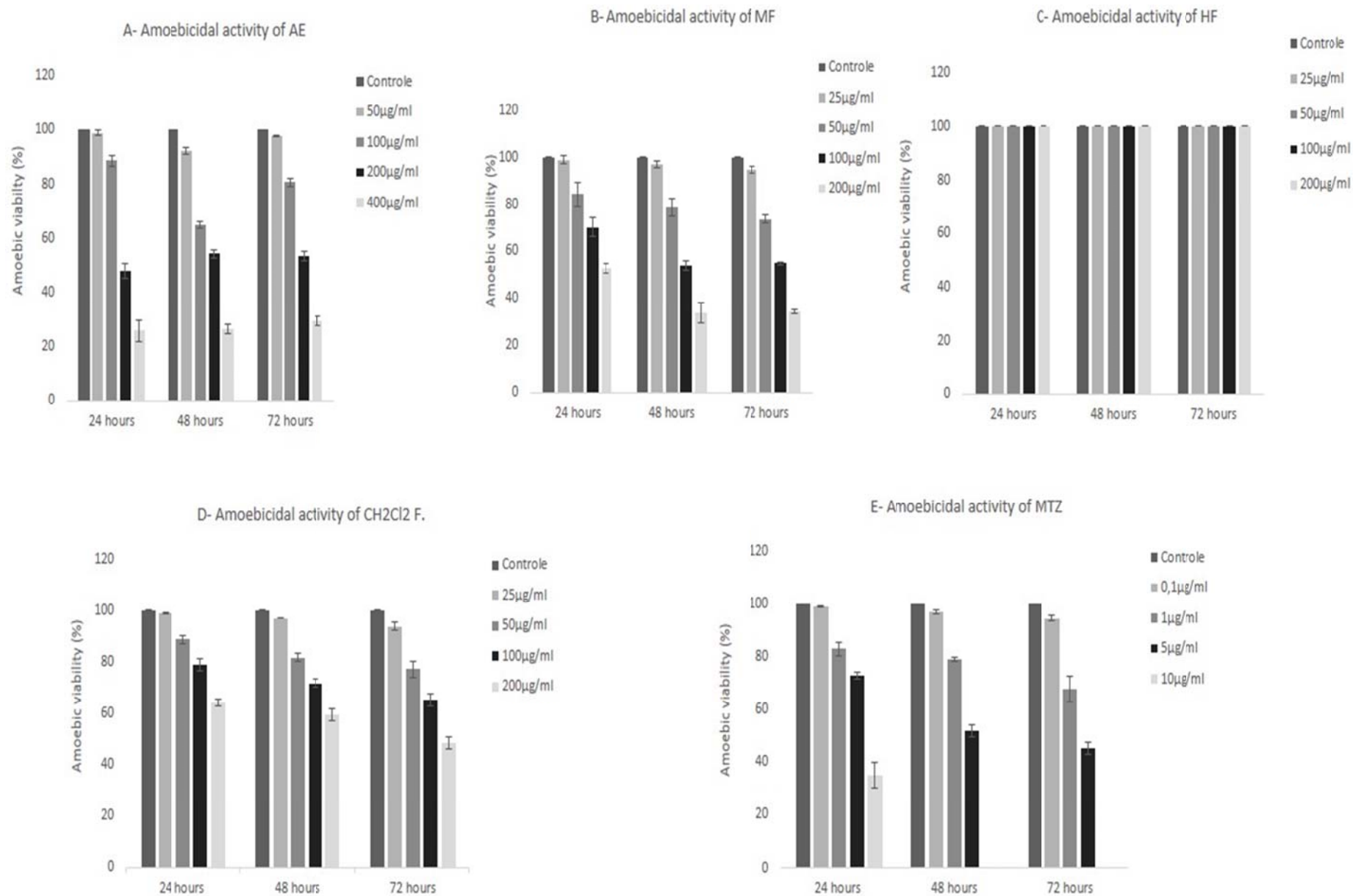
The results of the experiments on the effect of metronidazole and *E. hirta* AE, MF, and CH<sub>2</sub>Cl<sub>2</sub> F on enzymes of *E. histolytica* by mean of specific activities is presented in Figure 3. Specific activity was defined as the enzyme unit per milligram of protein. The specific activity units of compound containing reactions were converted into a percentage basis by correlating with those of

controls, the inhibitory effect of MF is significantly higher than those of AE and CH<sub>2</sub>Cl<sub>2</sub> F, and comparable to that of metronidazole for all the enzymes assayed. The higher inhibition percentage observed with MF was on ACP activity (73.0435 ± 2.30%) at the concentration of 800 µg/ml. The lower percentage observed with the same fraction was on ALP activity (52.2 ± 1.42 %) (Table 2).

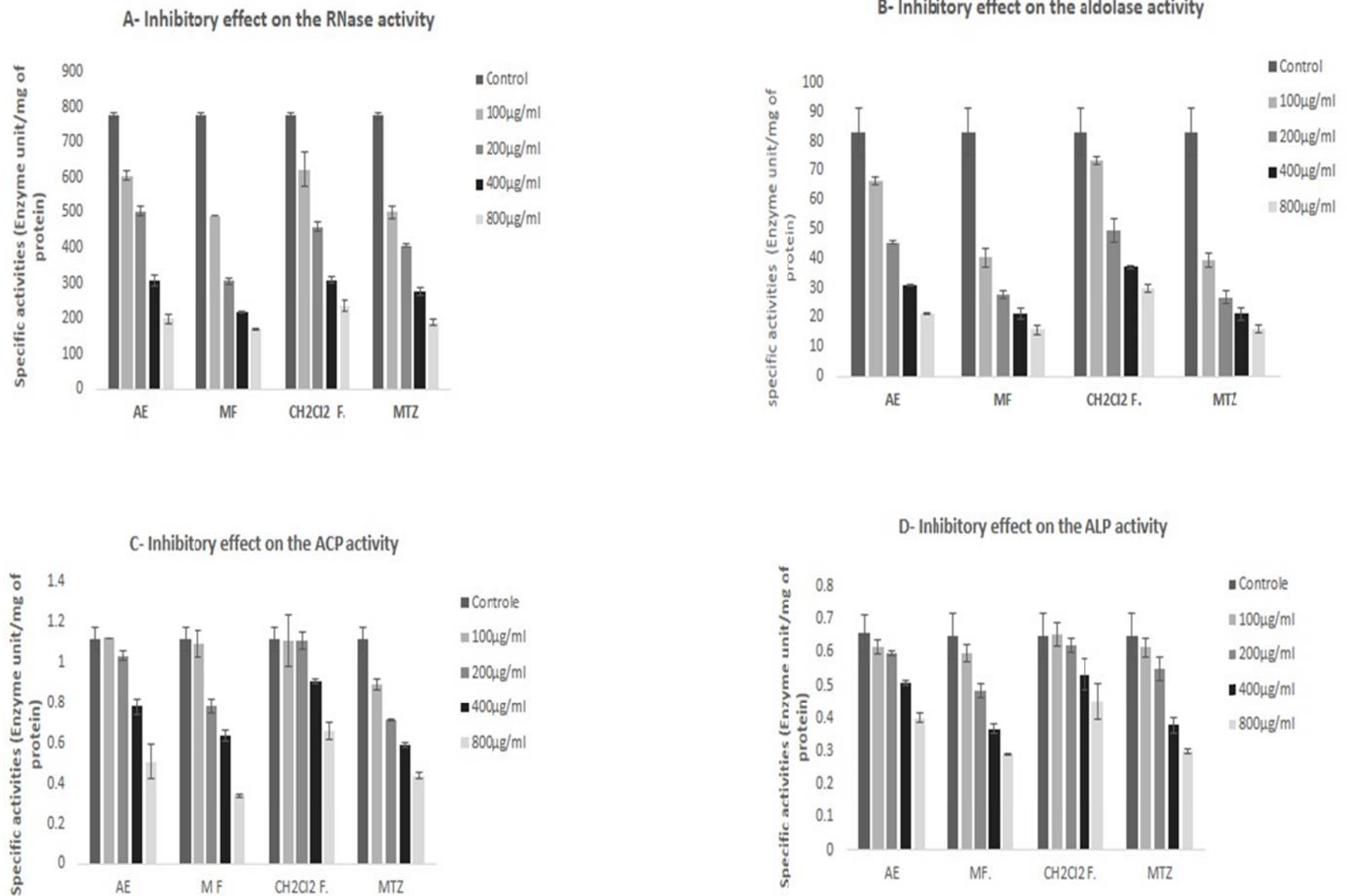
## DISCUSSION

Clinical isolates of *E. histolytica* were collected from patients with intestinal amoebiasis and cultivated on polyxenic Boeck and Drbohlav medium. Figure 1 shows the presence of *E. histolytica* strain in culture. For the antiamoebic assays HM1:IMSS strain cultivated in axenic culture is mainly used. However in this study, clinical isolates cultivated in polyxenic culture are used as it is well documented that *E. histolytica* is more virulent in association with suitable bacterial cells (Bracha and Mirelman, 1984; Wittner and Rosenbaum, 1970).

It is apparent from the data presented in Figures 2 and 3 that MF of *E. hirta* is more efficient than CH<sub>2</sub>Cl<sub>2</sub> F and AE. After 48 h of incubation, the amoebicidal effect of the AE decreased. This may be attributed to the resistance of *E. histolytica* against the extract. The same observation was made by Moundipa et al. (2005) with the same extract. The antiamoebic effect of polyphenolic extract of *E. hirta* has been elucidated by Tona et al. (2000). The polyphenols are the major compounds found in the aerial part of *E. hirta* including phenols, flavonoids and alkaloids (Huang et al., 2012) which are



**Figure 2.** *In vitro* Amoebicidal effect of (A) aqueous extract, (B) methanol fraction, (C) Hexane fraction, (D) methylene chloride fraction of *E. hirta* and (E) metronidazol after 24, 48 and 72 h of incubation. These results are the average of three experiments by mean of standard deviation, repeated three times and compared to the controle (DMSO).



**Figure 3.** *In vitro* inhibitory effect of *E. hirta* extract/fractions and metronidazole on the activity of (A) RNase, (B) aldolase, (C) acid phosphatase (ACP), and (D) alkaline phosphatase (ALP). These results are the average of three experiments by mean of standard deviation, repeated three times and compared to the control.

**Table 1.** IC<sub>50</sub> of the Amoebicidal effect of the aqueous extract, methanol fraction, methylene chloride fraction of *E. hirta* and metronidazole after 24, 48 and 72 h of incubation.

Compounds	IC <sub>50</sub> (mean ± SD) (µg/ml)		
	24 h	48 h	72 h
AE	170.45 ± 15.91	103.9 ± 3.3	145.95 ± 8.98
MF	93.13 ± 9.06 <sup>a</sup>	83.85 ± 4.37 <sup>a</sup>	67.18 ± 7.40 <sup>a</sup>
CH <sub>2</sub> Cl <sub>2</sub> F	262.07 ± 9.01	238.22 ± 13.38	194.04 ± 7.81
HF	N	N	N
MTZ	8.33 ± 0.43 <sup>b</sup>	5.12 ± 0.13 <sup>b</sup>	4.30 ± 0.48 <sup>b</sup>

a= significant difference between methanol fraction and other extract/fractions; b= significant difference between extract or fraction compared to metronidazole (n=3; p≤0.05). N= mean test non performed.

**Table 2.** IC<sub>50</sub> values of the metronidazole and *E. hirta* aqueous extract and fractions on *E. histolytica* enzymes activity.

Compounds	IC <sub>50</sub> (mean ± SD) (µg/ml)			
	Aldolase	RNase	ACP	ALP
MTZ	463.71 ± 42.84 <sup>a</sup>	658.32 ± 29.6 <sup>a</sup>	391.29 ± 17.83 <sup>a</sup>	652.52 ± 11.51 <sup>a</sup>
AE	569.50 ± 13.02	829.93 ± 10.04	783.58 ± 10.57	1081.01 ± 50.42
MF	400.19 ± 14.24 <sup>b</sup>	639.93 ± 30.25 <sup>b</sup>	394.42 ± 1.59 <sup>b</sup>	707.76 ± 14;79 <sup>b</sup>
CH <sub>2</sub> Cl <sub>2</sub>	816.54 ± 36.85	848.84 ± 37.18	922.49 ± 22.25	1300.77 ± 35.7

a= significant difference between extract or fraction compared to metronidazole; b= significant difference between methanol fraction and other extract/fractions (n=3; p≤0.05).

particularly polar compounds, thus found in greater amount in MF than CH<sub>2</sub>Cl<sub>2</sub> F. It can also be noted that MTZ exhibited a significantly greater amoebicidal effect as compared to extract and fractions. This can be attributed to the crude nature of the extract and fractions. The anti-amoebic activities of *E. hirta* studied previously (Moundipa et al., 2005, Tona et al., 2000) were focussed only on the amoebicidal effect based on the viability evaluation of amoeba in culture. However, in this study we also investigated the effect of *E. hirta* extracts on important bio-molecules of *E. histolytica* which are essential for its survival.

Several mechanisms for the pathogenesis of *E. histolytica* by which the parasite can engage in tissue damage are available. These mechanisms include secretion of enzymes and cell free cytotoxins, contact dependent cytolysis and phagocytosis (Sohni, et al., 1995). Some of the secreted enzymes which have been investigated in the present study are believed to play an important role in the virulence and survival of the parasite. The nucleopolymerases play an important role in the metabolism of all living cells. Amoebas which were fed with cholesterol have been shown to increase in lysosomal level of DNase and RNase activities (Narain, 1979). It is also well documented previously that cholesterol increases the virulence of *E. histolytica* (Meerovitch and Ghadirian, 1978).

There is significantly higher level of ACP than ALP in trophozoites of *E. histolytica* and axenically growth amoeba

exhibit increased level of ACP activity (Sohni et al., 1995). ACP may play an important role in the utilization of phagocytised food materials. It is also demonstrated that ACP gene expression increases during invasion and cells lesions by *E. histolytica* suggesting that this enzyme plays an important role during tissue invasion by the pathogenic amoeba (Fernandes et al., 2014).

In the present study, clinical isolates of amoeba grown in poly-xenic medium exhibit also a higher level of ACP than ALP. It is apparent from the Figure 3 that MF of *E. hirta* has inhibitory effect non significantly different from that of MTZ for all the enzyme activities studied. CH<sub>2</sub>Cl<sub>2</sub> F and AE have inhibitory effects lower than those of MF.

However further studies of MF of the aerial part of *E. hirta* are suggested, mainly to confirm the finding in axenic culture of *E. histolytica* and its effect on the activity and expression of the cysteine proteinase which is the main compound involved in virulence of *E. histolytica*. In future *in vivo* studies can be carried out to confirm the anti-amoebic activity of *E. hirta* extracts, so that these can be used for therapeutics.

## Conclusion

Methanol fraction of the aerial part of *E. hirta* exhibited higher anti-amoebic activity than methylene chloride fractions and aqueous extract against clinical isolate of *E. histolytica*. The extract and fractions also had varying

degree of inhibition on enzymes of *E. histolytica* which are thought to play a role in its survival and virulence. The present finding justifies the use of *E. hirta* aerial part in the traditional medicine for the treatment of dysentery.

### Conflict of Interests

The authors have not declared any conflict of interests.

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