ORIGINAL ARTICLE CODEN: AAJMBG

Antiamoebic and Cytotoxicity of Ethanolic leaves Extract of *Acacia nilotica* (L)

Ahmed Saeed Kabbashi[1*] and Mohammed Ismail Garbi[2]

[1]Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), P.O. Box 2404, National Center for Research, Khartoum, Sudan and [2]Department of Microbiology, Faculty of Medical Laboratory Sciences, International University of Africa. P.O. Box 2469 Khartoum, Sudan

Abstract: *Background: Acacia nilotica* (L) related to family Fabaceae-Mimosoideae. The division of *Acacia nilotica* is Magnolophyta and class is Magnolipsida. The genus is *Acacia* and species is *nilotica*. The ailments treated by this plant include colds, congestion, fever, gallbladder, hemorrhage, hemorrhoids, leucorrhoea, ophthalmic, sclerosis and small pox. Acacia bark is drunk for intestinal pains and used for treating acute diarrhea. Other preparations are used for gargle, toothache, ophthalmic and syphilitic ulcers. The roots of Acacia are used to treat Tuberculosis. The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually. 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. *Objectives:* This study was carried out to evaluate antiamoebic activites (*Entamoeba histolyica*) and cytotoxicity (MTT assay) of ethanol extract of *A. nilotica* (leaves). *Method:* The extract of *A. nilotica* (leaves), with different concentration (500 ppm, 250 ppm and 125 ppm) and metronidazole concentration (312.5 µg/ml) to be investigated *in vitro* against *Entamoeba histolyica* trophozoites. And cytotoxicity (MTT assay) with different concentration (500 ppm, 250 ppm and 125 ppm) and compare triton-100 (the reference control). *Result:* The result was obtained from *A. nilotica* leaves ethanol extract which exhibited 100% mortality within 96 h, at a concentration 500 ppm; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time. And MTT assay verified the safety of the examined extract. *Conclusion:* These studies conducted for both *A. nilotica* leaves was proved to have potent activities against *Entamoeba histolyica* trophozoites *in vitro*. And MTT assay verified the safety.

Keywords: In vitro, antiamoebic, Entamoeba histolyica, Metronidazole, cytotoxicity (MTT assay), Acacia nilotica (leaves).

Introduction

Medicinal plants are still invaluable source of safe, less toxic, lower price, available and reliable natural resources of drugs all over the world. People in Sudan and in other developing countries have relied on traditional herbal preparations to treat themselves. Therefore, it is useful to investigate the potential of local plants against these disabling diseases [1- 2].

Acacia nilotica (L) commonly called *Acacia* belongs to the (family: Mimosaceae). It is known as “Bagaruwa” among the “Hausa” speaking people of northern Nigeria. The plant is a tree with yellow mimosa-like flowers and long grey pods constricted between seeds. The bark and branches are dark with fissures. The branches bear spikes about 2 cm long. The leaves are five and densely hairy with 3 - 6 pairs of pinnae consisting of 10 - 20 pairs of leaflets that are narrow with parallel margins that are rounded at the apex and with a central midrib closely crowded. The inflorescence consists of bright yellow flowers in auxillary head on stalks that are half way up. The flowering period of the plant is between November and March. The powdered bark of the plant with little salt is used for treating acute diarrhea [3].

The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually [4-5]. Intestinal amoebiasis due to the infection of *E. histolytica* is ranked third

© 2015. Al Ameen Charitable Fund Trust, Bangalore

on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis [6].

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 [7]. Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers, immigrants, homosexuals and institutionalized persons. *E. histolytica*-associated dysentery is a common occurrence in the less developed and developing countries of the world, but is more common in areas of low socio-economic status, poor sanitation and nutrition especially in the tropics [5].

Thus the majority of *E. histolytica* infections, morbidity and mortality occur in Africa, Central and South America and the Indian sub-continent [8]. Metronidazole is the drug now widely used and recommended in the treatment of amoebiasis [9]. But it is less effective in the tissue than in the gut lumen [10]. In addition, it can eradicate only up to 50% of laminae infections [11]. Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis [12]. The present study was conducted to investigate the antiamoebic activity and cytotoxicity of *A. nilotica* (leaves) in Sudan.

Material and Methods

Plant materials: The *A. nilotica* (leaves) was collected from central Sudan between January 2008 and February 2008. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants Research Institute (MAPRI). Plant leaves was air-dried, under the shadow with good ventilation and then ground finely in a mill until their uses for extracts preparation. Table (1): showed the yield % of ethanolic *A. nilotica* (leaves) antiamoebic and cytotoxicity investigated in this study.

Table-1: ***Acacia nilotica*** **(leaves) investigated of antiamoebic and cytotoxicity**

Scientific Name of Plant	Family name	Part Used	Yield %	Traditional medicine
Acacia nilotica	Mimosaceae	leaves	7.50	antimalarial patients as a tonic, antimicrobial, antidiarrhoea and dysentery.

This table indicates the scientific names, families, parts used, yield% of ethanol extract and traditional uses of *Acacia nilotica* (leaves).

Preparation of crude extracts: Extraction was carried out for the leaves of *A. nilotica* plant by using overnight maceration techniques according to the method described by [13]. About 50 g was macerated in 250 ml of ethanol for 3 h at room temperature with occasional shaking for 24 h at room temperature, the supernatant was decanted and clarity field by filtration through a filter paper, after filtration, the solvent was then removed under reduced pressure by rotary evaporator at 55°C. Each residue was weighed and the yield percentage was calculated then stored at 4°C in tightly sealed glass vial ready for use. The remaining extracts which is not soluble by successively extracted by Ethanol using the previous technique. Extracts kept in deep freezer for 48 h, then induced in freeze dryer (Virtis, USA) until completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept in 4°C until the time of their use (Figure 1).

Figure-1: Leaves part of *A. nilotica*.

© 2015. Al Ameen Charitable Fund Trust, Bangalore

Antimoebic activity of A. nilotica (leaves) extract:

Parasite isolate: E. histolytica used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All taken samples were examined by wet amount preparation; the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of *E. histolytica* were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ± 1°C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

Inoculums: E. histolytica was inoculated in the RPMI 1640 medium and incubated at 37 ± 1°C for 48 h. parasites were counted under the microscope by haemocytometer chamber.

In vitro susceptibility assays: *In vitro* susceptibility assays used the sub-culture method Cedillo-Rivera *et al*. [14], which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* [15]. 5 mg from each extract and compound was dissolved in 50 µl of dimethyl sulfoxzide (DMSO) at Eppendorf tube containing 950 µl D.W 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 and negative control. Three columns of a microtitre plate wells [8 columns (C) × 12 rows (R)] 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 were added to the other wells of 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 (a trichomonocide) pure compound [(1-(2-hydroxyethl)-2-methyl-5 Nitroimidazole], a was used as positive control in concentration 312.5 µg/ml, 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 24, 48, 72 and 96 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

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

Only 100% inhibition of the parasite was considered, when there was no motile parasite observed.

Cytotoxicity Screening: Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the studied *A. nilotica* (leaves).

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 [16].

Preparation of Extracts, Solutions: 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.

© 2015. Al Ameen Charitable Fund Trust, Bangalore

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:

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

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. 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 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°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 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

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

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

Statistical analysis: All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program (2007).

Results

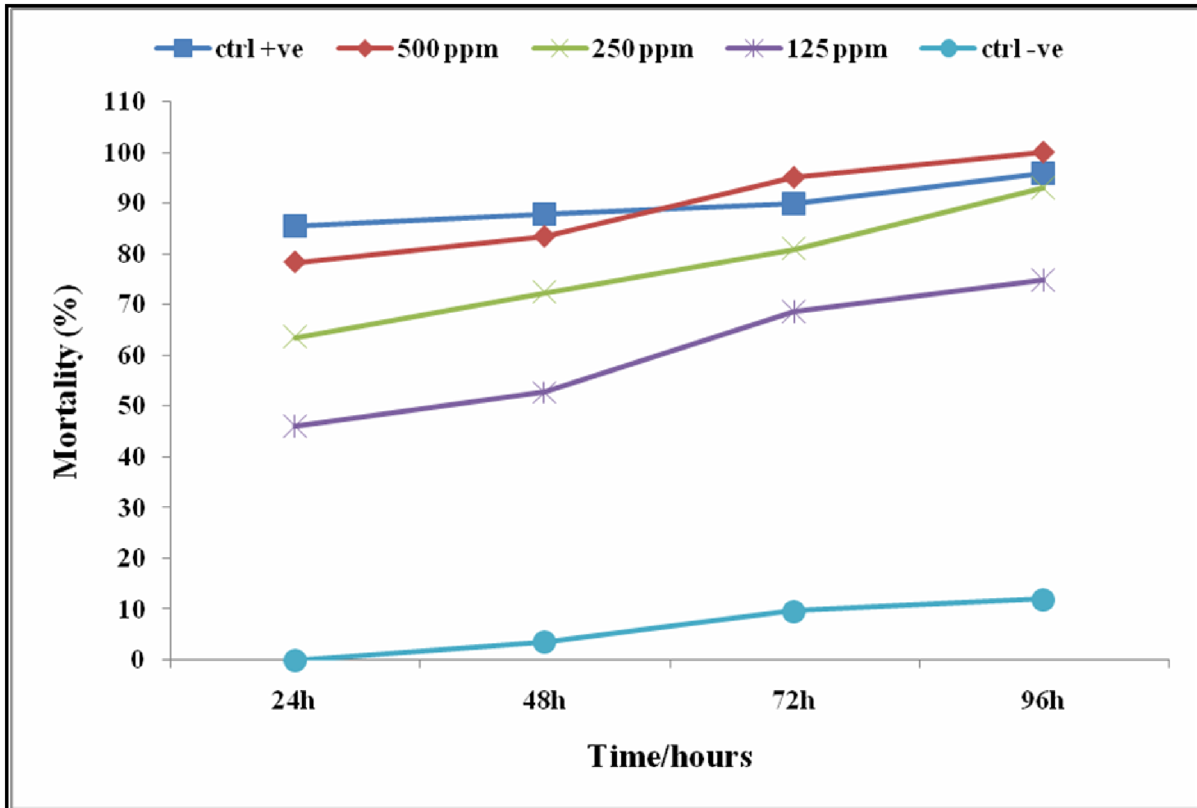
The leaves of *A. nilotica* family (Mimosoideae) was screened for antiamoebic activity against (*Entamoeba histolytica*) trophozoites *in vitro*. And cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line.

Antiamoebic activity of A. nilotica (leaves) extract: The antiamoebic potential of the ethanolic extract of *A. nilotica* (leaves) was extracted by ethanol, with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with

© 2015. Al Ameen Charitable Fund Trust, Bangalore

concentration (312.5 µg/ml) to be investigated against *E.* trophozoites *in vitro*. Ethanol extracts of *A. nilotica* (leaves). showed 100% inhibition at a concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time against *Entamoeba histolytica* (Figure 2).

Fig-1: *In vitro* activity of *A. nilotica* (leaves) ethanolic extract against *Entamoeba histolytica.*



Cytotoxicity assay of A. nilotica (leaves) extract:

Table-2: Cytotoxicity of *A. nilotica* extracts on normal cell lines (Vero cell line) as measured by the MTT assay:

No.	Name of plant (part)	Concentration (µg/ml)	Absorbance	Inhibition (%) ± SD	IC_{50} (µg/ml)
1	*A. nilotica* (leaves)	500	1.43	50.9 ± 0.05	> 100
		250	1.53	47.5 ± 0.03	
		125	2.07	29.1 ± 0.02	
2	*Control		0.14	95.3 ± 0.01	

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

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 of Vero cell line growth in vitro by ethanolic extract of *A. nilotica* (leaves). MTT colorimetric assay was used. Reading in triplicate for different concentrations 125-500 µg/mL.

© 2015. Al Ameen Charitable Fund Trust, Bangalore

Discussion

The leaves of *A. nilotica* family (Mimosoideae) was screened for antiamoebic activity against (*Entamoeba histolytica*) trophozoites *in vitro*. And cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line.

Antiamoebic activity of A. nilotica (leaves) extract: Amoebiasis is caused by *Entamoeba histolytica,* a protozoan parasite of humans and the causative agent of intestinal amoebiasis. This disease is a major health problem in developing countries [17]. Although it is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from symptoms associated with amoebiasis, such as hemorrhagic colitis and amoebic liver abscess [18]. Several means of transmitting *E. histolytica* are known, ingestion of the infective cyst occur in food and water and it may also be transferred via homosexual men [19]. Ethanol extracts of *A. nilotica* (leaves). Showed 100% inhibition at a concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time against *Entamoeba histolytica*.

Interestingly, the cytotoxicity assays was conducted in this study to evaluate the ethanolic extract of *A. nilotica* (leaves) their cytoxtoxicty effects by using MTT-assay include (Vero cell line). The result of MTT assay verified the safety of the examined extract.

Cytotoxicity assay of A. nilotica (leaves) extract: The cytotoxicity assays were conducted in this study to evaluate the ethanolic extract of *A. nilotica* (leaves) their cytoxtoxicty effects by using MTT-assay include (Vero cell line). The result of MTT assay verified the safety of the examined extract.

Conclusion

This result enhances the ethno botanical uses of *A. nilotica* (leaves) as antiamoebic in cases associated with amoebiasis in Sudan. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

Acknowledgements

The authors are grateful to Dr. Amel Mahmoud Abdrabo, Head department of Microbiology and Parsitology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) Khartoum, Sudan.

References

1. Amaral FMM, Ribeiro MNS, Barbosa-Filho JM, Reis AS, Nascimento FRF, Macedo RO. Plants and chemical constituents with giardicidal activity. *Braz J Pharmacogn,* 2006; 16(Supl):696-720.
2. Koko WS, Mesaik MA, Yousaf S, Galal M, Choudhary MI. *In vitro* immunomodulating properties of selected Sudanese medicinal plants. *J Ethnopharmacol,* 2008; 118:26-34.
3. Banso A. Phytochemical and antibacterial investigation of bark extracts of *Acacia nilotica, Journal of Medicinal Plants Research* 2009; 3(2):082-085.
4. World Health Organization. Weekly Epidemiological Records, *WHO* 1997; 72:97-100.
5. Ravdin JI, Stauffer MM. *Entamoeba histolytica* (Amoebiasis). In Mendell, G. L., Benneth, J. E. Dolin, R. (ed) Mendell, Doglas and Benneth) Principles and Practice of Infectious Diseases. 6th ed. Philadelphia, P. A. *Churchill Livingstone* 2005.
6. Farthing MS, Cavellos AM, Kelly P, Cook GC. Intestinal Protozoa; In Manson's Tropical Disease. 20th Edition, London W.B. *Saunder Company.* 1996; 1255-1267.
7. Stanley Jr SL, Reed SL. Microbes and microbial toxin: paradigms for microbial-mucosal. Interactions VI. *Entamoeba histolytica*: parasite-host interactions. *Am J Physiol Gastrointest Liver physiol,* 2001; 280(6):1049-1054.
8. Haque R, Mollah NU, Ali IK, Alam K, Eubanks A, Lyerly D, Petri Jr WA. Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. *J Clin Microbiol,* 2000; 38(9):3235-3239.
9. Townson SM, Boreham PF, Upcroft P, Upcroft JA. Resistance to the nitroheterocyclic drugs. *Acta Trop,* 1994; 56:173-194.
10. Bhopale K, Pradhan K, Masani K, Karl C. Additive effect of diloxanidefuorate and Metronidazole (Entamizole R) in mouse caecalamoebiasis. *Indian J Exp Biol,* 1995; 33:73-74.
11. Tierney LM, Mcphee JS, Papadaksis MA. Current medical diagnosis and treatment Appleton and Lange (Ed.), *Stanford, Conn* 1998; 344-353.
12. Upcrof JA, Dunn LA, Wrigh JM, Benakli K, Upcrof P, Vanelle P. 5-nitroimidazole drugs

© 2015. Al Ameen Charitable Fund Trust, Bangalore

effective against Metronidazole-resistant *Trichomonas vaginalis* and *Giardia dodenalis* spp. *Animicrob agent Chemother,* 2006; 50:344-347.

13. Harbone B. Phytochemical methods. 2nd. New York, *Champan Hall,* 1984; 4;4-7.
14. Cedillo-Rivera R, Chave B, Gonzalez-Robles A, Tapia-Contreras A, Yepez-Mulia L. In vitro effect of nitazoxanide against Entamoba histolytica, Gairdia lamblia and Trichomonas vaginalis trophozoites. *J Eukaryotic Microbiol,* 2002; 49:201-208.
15. Arguello-Garcia R, Cruz-suto M, Romero-Montoya L, Ortega-Pierres G. Variability and variation in drug susceptibility among *Giardia duodenalis* isolates and clones exposed to 5-nitromidazoles and benzimidazoles *in vitro*. *J Antimicrob Chemother,* 2004; 54:711-721.
16. Patel S, Gheewala N, Suthar A and Shah A. *In-Vitro* Cytotoxicity Activity of *Solanum Nigrum* Extract Against Hela Cell Line And Vero Cell Line. *International Journal of Pharmacy and Pharmaceutical Sciences,* 2009; 1(Suppl 1):38-46.
17. Stanley SL. Amoebiasis. Lancet 361:1025-1034. Tabanca NE, Bedir N, Kirmer KH, Baser SI, Khan MR, Jacob, Khan IA. Antimicrobial compounds from *Pimpinella* species growing in Turkey. *Planta Med.* 2003; 69:933-938.
18. Ravdin JI. State of the art clinical article. *Clin. Infect. Dis.* 1995; 20:1453-1466.
19. Tanyuksel M. & Petri WA. Laboratory Diagnosis of Amoebiasis. *Clin. Microbiol. Rev.* 2003; 16:713-729.

*All correspondences to: Dr. Ahmed Saeed Kabbashi, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), P.O. Box 2404, National Center for Research, Khartoum, Sudan. E-mail: ahmedsak88@gmail.com

© 2015. Al Ameen Charitable Fund Trust, Bangalore