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# Antiamoebic activity and cytotoxicity of ethanolic extract of *Cyperus rotundus* L.

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**ABSTRACT**

*Cyperus rotundus* L. (Family: Cyperaceae), has a number of pharmacological and biological activities including anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities. 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. The purpose of the paper was to investigate the *in-vitro* antiamoebic activity and cytotoxicity (MTT assay) of ethanol extract of *C. rotundus* L. (whole plant). The ethanol extract of *C. rotundus* (whole plant), with different concentration (500, 250 and 125 ppm) and metronidazole concentration (312.5 µg/ml) was investigated *in vitro* against *E. histolyica* trophozoites. The result was obtained from *C. rotundus* whole plant 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. In addition cytotoxicity (MTT assay) with different concentration (500, 250 and 125 ppm) in comparison to triton-x100 (the reference control) which verified the safety of the examined extract with an $IC_{50}$ less 100 µg/ml. In conclusion, these studies prove the potent activity of *C. rotundus* against *E. histolyica* trophozoites *in vitro* with verified safety evidence for use.

**Keywords:** *In vitro*, antiamoebic, *Entamoeba histolyica*, Metronidazole, cytotoxicity (MTT-assay), *C. rotundus* (whole plant).

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## 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 (Amaral et al., 2006; Koko et al., 2008).

*Cyperus rotundus* L; (Family: Cyperaceae) is the largest family in the monocotyledons consisting of 109 genera and approximately 5,500 species (Govaerts et al., 2007). Also known as purple nut, sedge or nut grass is a

common perennial weed with slender, scaly creeping rhizomes, bulbous at the base and arising singly from the tubers which are about 1 to 3 cm long. The tubers are externally blackish in colour and reddish white inside, with a characteristic odour. The stems grow to about 25 cm tall and the leaves are linear, dark green and grooved on the upper surface. Inflorescences are small, with 2 to 4 bracts, consisting of tiny flowers with a red-brown husk. The nut is three-angled, oblong-ovate, yellow in colour and black when ripe. *C. rotundus* is indigenous to India, but are now found in tropical, subtropical and temperate regions (Uddin et al., 2006).

In Asian countries, the rhizomes of *C. rotundus*, which are used as traditional folk medicines for the treatment of stomach and bowel disorders, and inflammatory diseases, have been widely, investigated (Dang et al., 2011; Gupta et al., 1971; Won-Gil et al., 2001). *C. rotundus* is a traditional herbal medicine (Weenen et al., 1999; Zhu et al., 1997). The tuber part of *C. rotundus* is one of the oldest known medicinal plants used for the treatment (Yu *et al*., 2004; Zeid *et al*., 2008). Infusion of this herb has been used (Umerie and Ezeuzo, 2000). It is a multipurpose plant, widely used in traditional medicine around the world as herbal remedies (Oliver-Bever, 1986; Puratuchikody et al., 2006; Joshi and Joshi, 2000; El-Kamali and El-Khalifa, 1999). A number of pharmacological and biological activities have been reported for this plant (Zhu et al., 1997; Durate et al., 2005; Sundaram et al., 2008; Raut et al., 2006; Kilani et al., 2005; Kilani et al., 2007; Kilani et al., 2008; Dhillon et al., 1993; Pal and Dutta, 2006; Neffatti et al., 2008).

*C. rotundus* has a broad spectrum of applications as herbal remedies in China, Africa, Latin America, India, Saudi Arabia and Sudan (Nandihalli and Bendixen, 1987). In Chinese pharmacopoeia, it was described as an agent to regulate circulation, normalize menstruation, and relieve pain (Huang, 1999).

In Sudan the tubers of *C. rotundus* L. are used in stomach disorders and bowels irritation. An infusion of the tubers is used in dyspepsia, diarrhea, dysentery, as cites, vomiting, cholera and fevers. The tubers are given in large doses as an anthelmintic. A poultice of the fresh tubers is used to cure wounds, ulcers and sores; it is also applied to the breast to promote the flow of milk. Paste is used in scorpion stings (El-Ghazali et al., 1994).

The methanolic extract of the tubers showed an anti-inflammatory effect for the treatment of inflammatory diseases mediated by over production of nitric oxide and superoxide (Seo et al., 2001). Moreover, it showed significant antidiarrhoeal activity in castor oil induced diarrhea in mice (Seo et al., 2001). The dried tubers are used to treat dysmenorrhea and other menstrual irregularities. The aqueous extract of the dried tubers has an inhibitory effect on the uterus, (uterine relaxation) in both pregnant and non-pregnant women, and relieving pain. The herb can stimulate gastric and salivary secretion. In addition, the aqueous extract of the dried tubers has antibacterial and anti-malarial effects (Huang, 1999).

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 (WHO, 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). 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. *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 socioeconomic status, poor sanitation and nutrition especially in the tropics (Ravdin and Stauffer, 2005).

Thus the majority of *E. histolytica* infections, morbidity and mortality occur in Africa, Central and South America and the Indian sub-continent (Haque et al., 2000). Metronidazole is the drug now widely used and recommended in the treatment of amoebiasis (Townson et al., 1994). But it is less effective in the tissue than in the gut lumen (Bhopale et al., 1995). In addition, it can eradicate only up to 50% of laminae infections (Tierney et al., 1998). Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis (Upcrof et al., 2006). Hence, the present study was conducted to investigate the antiamoebic activity and cytotoxicity of *C. rotundus* (whole plant) in Sudan.

## MATERIALS AND METHODS

### Plant materials

*C. rotundus* (whole plant) was collected from Central Sudan during the period of January to February 2014. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan.

### Preparation of crude extract

Extraction was carried out for the whole plant of *C. rotundus* by using overnight maceration techniques according to the method described by Harbone (1984). About 50 g of powdered material was macerated in 250 ml of ethanol for 3 h at room temperature. Occasional shaking for 24 h at room temperature was performed and, the supernatant was decanted. Thereafter, the supernatant was filtered under reduced pressure by rotary evaporator at 55°C. Each residue was weighed and the yield percentage was calculated and then stored at 4°C in tightly sealed glass vial ready for use. The remaining extracts which were not soluble were successively extracted using ethanol with the described technique. The extracts were kept in freeze dryer for 48 h, (Virtis, USA) until they were completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept and stored at 4°C until required.

### *In vitro* testing of extract for antiamoebic activity

#### *Parasite isolate*

*E. histolytica* used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All parasite 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 parasite 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. (2002), 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* (Arguello-Garcia et al., 2004). 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], 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:

$$\text{Mortality of parasites (\%)} = \frac{\text{Negative control} - \text{tested sample with extract}}{\text{Negative control}} \times 100\%$$

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 *C. rotundus*.

### 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 C. rotundus extract*

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 sub cultured 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:

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

**Table 1.** Preliminary quantitative data on yield percentage (%) of *C. rotundus* (whole plant) used for the antiamoebic activity and cytotoxicity study.

| Scientific name of plant | Family name | Part used | Yield (%) |
|---|---|---|---|
| *Cyperus rotundus* L. | Cyperaceae | Whole plant | 10.5 |

***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, that is, 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.

After four days, 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 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 ± S.D. Statistical analysis for all the assay results were done using Microsoft Excel program (2007).

## RESULTS AND DISCUSSION

The whole plant of *C. rotundus* family (Cyperaceae) was screened for antiamoebic activity against (*Entamoeba histolytica*) trophozoites *in vitro*, and tested for cytotoxicity using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line after the evaluation of the yield percentage of the plant which was found to be 10.5% (Table 1).

The antiamoebic potential of the ethanolic extract of *C. rotundus* (whole plant), with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with concentration (312.5 µg/ml) was investigated against *E. histolytica* trophozoites *in vitro*. However, the ethanol extract of *C. rotundus* (whole plant) showed 100% inhibition at a concentration 500 µg/ml after 96 h; which was compared with Metronidazole giving 96% inhibition at concentration 312.5 µg/ml at the same time against *E. histolytica* (Figure 1).

The whole plant of *C. rotundus* which was screened for antiamoebic activity against (*E. histolytica*) trophozoites *in vitro* showed antiamoebic activity with an inhibition concentrations (IC) more than 1.32 µg/ml and increasing successively during the days of the screening (Table 2).

Moreover, these results obtained in this study are similar to studies carried out for antimalaial activity by Thebtaranonth and Thebtaranont (1995) and the previous comprehensive screening of Sudanese medicinal plants for their antiprotozoal activity (Samia et al., 2004; Ali et al., 2002; Koko, 2005).

The promising activity of the plant in this study may be explained for the chemical constituents- Cyprotene, cypera-2, 4-diene, a-copaene, cyperene, aselinene, rotundene, valencene, ylanga-2, 4- diene, g-gurjunene, trans-calamenene, d-cadinene, g-calacorene, epi-a-selinene, a-muurolene, g-muurolene, cadalene, nootkatene which the plant proved to contain (Joulain and Konig, 1998), in addition to mustakone, cyperol (Nyasse et al., 1988), isocyperol (Hikino and Takemoto, 1967) cyperotundone (Hikino et al., 1966), and acyperone (Howe and Mc Quillin, 1955; Haaksma et al., 1992).

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the cytotoxicity effects of ethanolic extract of *C. rotundus* (whole plant) by using MTT-assay including (Vero cell line). Table 3 indicated the inhibition percentage (%) of Vero cell line growth *in vitro* by ethanolic extract of *C. rotundus* (whole plant) for different concentrations 125 to 500 µg/ml and showed an $IC_{50}$ **>** 100 (µg/ml) which is verifying the plant safety. This result was similar to that produced by Ahmed et al. (2012), who found that the plant extract gave similar result using the Brine Shrimp Bioassay.

## CONCLUSION

This result enhances the ethno botanical uses of *C. rotundus* (whole plant) as antiamoebic in cases associated with amoebiasis in Sudan. Further investigations regarding the mode of action and other

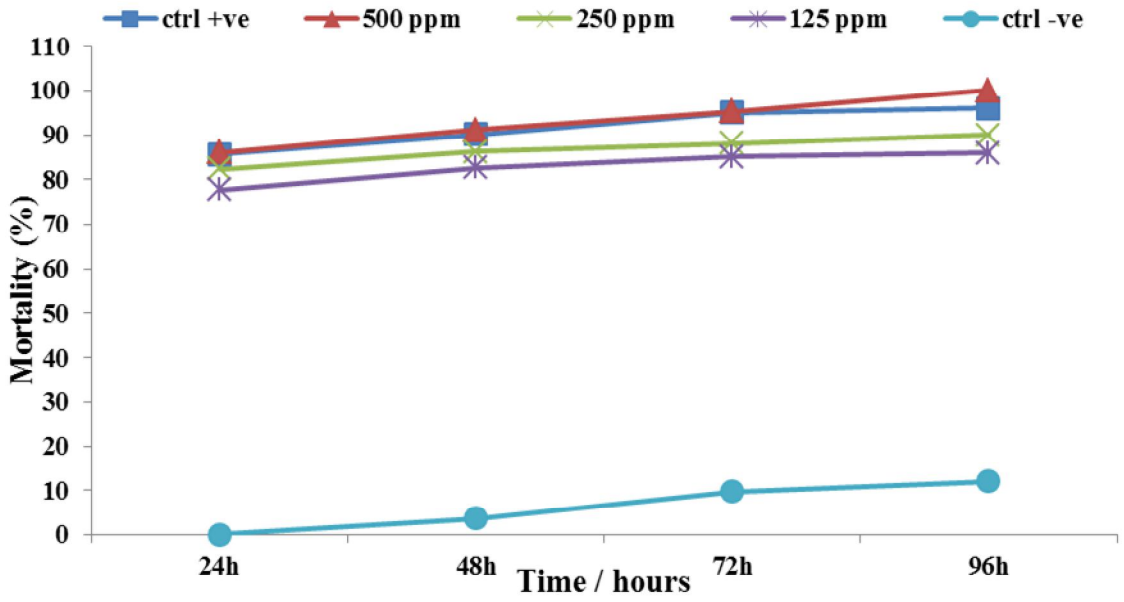


**Figure 1.** *In vitro* activity of *C. rotundus* ethanol extract against *E. histolytica.*

**Table 2.** Inhibition concentration (IC) *C. rotundus* (whole plant) ethanol extract against *E. histolytica*.

| IC (µg/ml) | $IC_{50}$ | $IC_{90}$ | $IC_{95}$ | $IC_{99}$ |
|---|---|---|---|---|
| After one day | 1.32 | 918.97 | 2081.79 | 4004.52 |
| After two days | 1.43 | 432.61 | 979.14 | 1882.12 |
| After three days | 1.58 | 266.59 | 506.19 | 845.45 |
| After four days | 4.97 | 207.44 | 330.76 | 480.40 |

Key: $IC_{50}$ Inhibition concentration 50%, $IC_{90}$ Inhibition concentration 90%, $IC_{95}$ Inhibition concentration 95%, $IC_{99}$ Inhibition concentration 99%.

**Table 3.** Cytotoxicity of *C. rotundus* extract on normal cell lines (Vero cell line) as measured by the MTT assay.

| No. | Name of sample | Concentration (µg/ml) | Absorbance | Inhibition (%) ± SD | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|---|
| | | 500 | 0.98 | 40.39 ± 0.07 | |
| 1 | *C. rotundus* (whole plant) | 250 | 1.04 | 30.80 ± 0.09 | > 100 |
| | | 125 | 1.34 | 16.84 ± 0.01 | |
| 2 | *Control | | 0.09 | 95.96 ± 0.01 | |

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

related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

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