Medicine and Medical Sciences (LRJMSS) ISSN: 2354-323X Vol. 3 issue 1 pp. 012-018, January, 2016
Available online http://www.landmarkresearchjournals.org/lrjmms/home
INDEXING: ISI Impact Factor (IF)=1.264; Scopus; Index Copernicus.

Full Length Research Paper

Antigiardial and Cytotoxicity of Ethanolic Extract of Cyperus rotundus L

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Accepted 12 January, 2015

The purpose of the paper was to investigate the in-vitro antigiardial activities (Giardia lamblia) and cytotoxicity (MTT assay) of ethanol extract of Cyperus rotundus L (whole plant). The ethanol extract of C. rotundus were screened for their antigiardialal activities (Giardia lamblia) and screened for their cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), obtained from C. rotundus (whole plant) ethanol extract which exhibited 100% inhibition at a concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 95% inhibition at concentration 312.5 µg/ml at the same time against Giardia lamblia. MTT assay verified the safety of the examined extract. In conclusion: These studies conducted for C. rotundus (whole plant) were proved to have potent activities against Giardia lamblia trophozoites in vitro.
Index Terms- In vitro, antigiardial, Giardia lamblia, Metronidazole, cytotoxicity (MTT-assay), Cyperus rotundus L, (whole plant).

Keywords: Sudan, Medical Plant, Cytotoxicity, Antigirdial.

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 at., 2006; Koko et al., 2008).

Cyperus rotundus L; (Family-Cyperaceae), also known as purple nutsedge or nutgrass, is a common perennial

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Figure 1 Laboratory Sample of *Cyperus rotundus* L (whole plant).

weed with slender, scaly creeping rhizomes, bulbous at the base and arising singly from the tubers which are about 1-3 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 2-4 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 used widely as analgesic, sedative, antispasmodic, antimalarial, stomach disorders and to relieve diarrhoea (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 of dysmenorrheal and menstrual irregularities (Yu et al.,2004; Zeid et al.,2008). Infusion of this herb has been used in pain, fever, diarrhoea, dysentery, an emmenagogue and other intestinal problems (Umerie and Ezeuzo 2000). It is a multipurpose plant, widely used in traditional medicine around the world to treat stomach ailments, wounds, boils and blisters (Oliver-Bever 1986; Puratuchikody et al.,2006; Joshi and Joshi 2000; El-Kamali and El-Khalifa 1999). A number of pharmacological and biological activities including anti-*Candida*, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antibacterial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities have been reported for this plant (Durate et al.,2005; Sundaram et al.,2008; Raut and Gaikwad 2006; Kilani et al.,2005; Zhu et al.,1997; Kilani et al.,2007; Kilani et al.,2008; Dhillon et al.,1993; Pal and Dutta 2006; Neffatti et al.,2008).

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 diarrhea in humans (Lauwaet and Andersen 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 et al 2007; Dib and Wen 2008; Addy et al., 2004). and adults (Ayeh-Kumi et al.,2009; Nyarango et al.,2008). The present study was conducted to investigate the antigiardial activity and cytotoxicity of *C. rotundus* (whole plant) in Sudan.

MATERIALS AND METHODS

Plant Materials

The *C. rotundus* (whole plant) were collected from central Sudan between January 2008 and February 2008. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan.

The *C. rotundus* (whole plant) were air-dried, under the shadow with good ventilation and then ground finely until their uses for extracts preparation (Fig. 1).

Preparation of Crude Extracts

Extraction was carried out for the fruits of C*. rotundus* (whole plant) plant by using overnight maceration techniques according to the method described in Harbone (Harbone 1984). About 50 g round 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 evaporatorion 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 not soluble was successively extracted using ethanol with the described technique. The extracts were kept in deep freezer 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.

Table 1 below indicates the scientific name, family name, part used, yield% of ethanol extract and traditional uses of Cyperus rotundus L whole plant.

Parasite Isolate

G. lamblia used in all experiments was taken from patients of Ibrahim Malik Hospital (Khartoum). All taken samples were examined by wet mount preparation; the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of *G. lamblia* 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

G. lamblia 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*. (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], 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:

Mortality of cells (%) = (Control negative – tested sample with extract) × 100% Control negative

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 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).

Table 1 Preliminary quantitative data on the amount of *C. rotundus* whole plant used in the antigiardial activity and cytotoxicity study

Scientific Name of Plant	Family name	Part Used	Yield %	Traditional medicine
Cyperus rotundus L.	Cyperaceae	Whole plants	10.5	anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antibacterial, antioxidant, cytotoxic, apoptotic, anti-pyretic and analgesic.

Preparation of C. Rotundus 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.

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:

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

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:

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

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 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 $P < 0.05$.

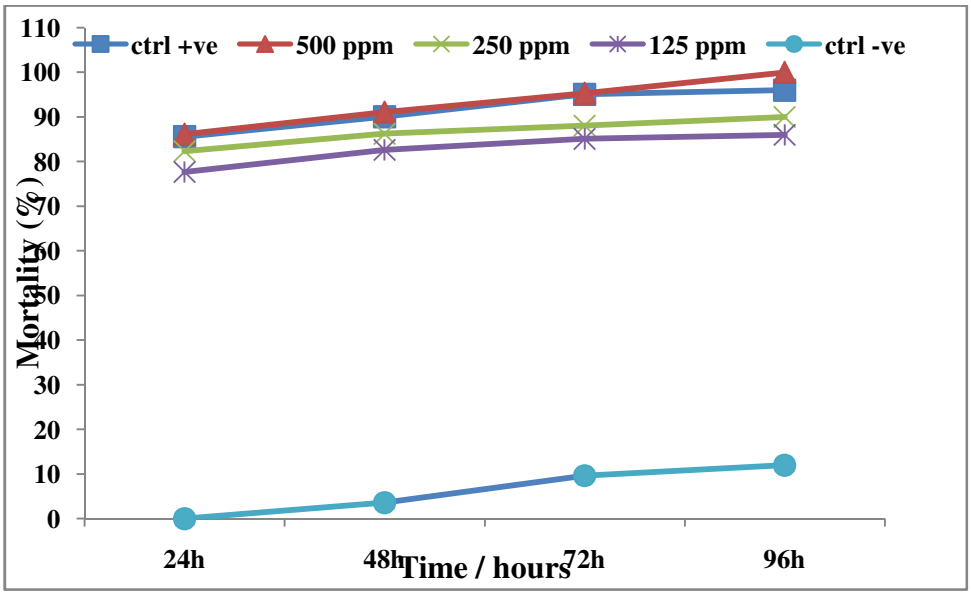

Figure 2 In vitro activity of C. rotundus ethanol extract against G.lamblia.

Table 2 Cytotoxicity of *C. rotundus* 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	***C. rotundus*** **(whole plants)**	**500**	0.98	40.39 ± 0.07	**> 100**
		250	1.04	30.80 ± 0.09	
		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.

RESULTS AND DISCUSSION

The whole plant of *C. rotundus* family (Cyperaceae) were screened for antigiardial activity against (*giardia lamblia*) trophozoites *in vitro*, and screened for cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line.

Antigiardial Activity of C. Rotundus (whole plant) Extract.

Giardia lamblia is an important cause of acute and chronic gastrointestinal disease throughout the world and has been identified as the etiologic agent in numerous waterborne outbreaks of diarrheal disease. Although *G. lamblia* is among the most prevalent enteric protozoal infections in humans, it is relatively recently that improvements in t he *in vitro* cultivation of this organism have allowed reliable, reproducible tests to assess the *in vitro* activity of therapeutic agents against *G. lamblia* (Boreham et al.,1984). Despite the previous comprehensive screening of Sudanese medicinal plants for their antiprotozoal activity (Samia et al.,2004; Hiba et al.,2002; Koko et al., 2008).

The antigiardial potential of the ethanolic extract of the *C. rotundus* (whole plant) were extracted by ethanol, with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with concentration (312.5 µg/ml) to be investigated against *giardia lamblia* trophozoites *invitro*. Ethanolic extract of *Cyperus rotundus* (whole plant) inhibited 100% inhibition concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 95% inhibition at concentration 312.5 µg/ml at the same time against *giardia lamblia* (Fig. 2).

Cytotoxicity Essay of C. Rotundus (whole plant) Extract

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 *C. rotundus* (whole

plant). MTT colorimetric assay was used. Reading in triplicate for different concentrations 125-500 µg/mL.

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the ethanolic extract of *C. rotundus* (whole plant) 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 *C. rotundus* (whole plant) as antidiarrheal in cases associated with Giardiasis 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.

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