

Research article

Antimicrobial activity and Cytotoxicity of Ethanolic Extract of *Cyperus rotundus* L.

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Abstract :

The purpose of the paper was to investigate the *in-vitro* antimicrobial activity and cytotoxicity (MTT assay) of ethanol extract of *Cyperus rotundus* L (whole plant). The ethanol extract of *C. rotundus* was tested against four standard bacteria i.e.: two Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*), two Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and against two standard fungi species i.e. *Aspergillus niger* and *Candida albicans* using the agar plate diffusion method. The cytotoxicity was tested against Vero cell line using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). The ethanol extract of *C. rotundus* (whole plant) exhibited inhibitory effects against most of the tested organisms with the zone of inhibition ranging from 19 to 31 mm in length. The largest inhibition zone in case of bacteria was obtained for the Gram-positive against bacteria *S. aureus* (31 mm) and *B. subtilis* (30 mm) while in case of fungi highest inhibition was observed against *C. albicans* (26 mm). MTT assay verified the safety of the examined extract.

In conclusion: This study conducted for *C. rotundus* (whole plant) proved to have potent activities against antibacterial as well as antifungal activity *in vitro*. **Copyright © AJPPS, all rights reserved.**

Keywords: *In vitro*, antimicrobial, Cytotoxicity (MTT-assay), *Cyperus rotundus* L, (whole plant).

I. 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] .

Cyperus rotundus L; (Family-Cyperaceae), 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 1-3 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 [3]. 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 [4, 5, 6]. *C. rotundus* is a traditional herbal medicine used widely as analgesic, sedative, antispasmodic, antimalarial, stomach disorders and to relieve diarrhoea [7, 8]. The tuber part of *C. rotundus* is one of the oldest known medicinal plants used for the treatment of dysmenorrheal and menstrual irregularities [9, 10]. Infusion of this herb has been used in pain, fever, diarrhoea, dysentery, an emmenagogue and other intestinal problems [11]. It is a multipurpose plant, widely used in traditional medicine around the world to treat stomach ailments, wounds, boils and blisters [12, 13, 14, 15]. 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 [16, 17, 18, 19, 8, 20, 21, 22, 23, 24].

Cyperus rotundus has a broad spectrum of applications as herbal remedies in China, Africa, Latin America, India, Saudi Arabia and Sudan [25] .

In Asian countries, *Cyperus rotundus* the rhizomes are used as traditional folk medicines for the treatment of spasms, stomach disorders, bowel disorders and inflammatory diseases [26]. In Chinese pharmacopoeia, it was described as an agent to regulate circulation, normalize menstruation, and relieve pain [27].

In Sudan the tubers of *Cyperus rotundus* L. are used in stomach disorders and bowels irritation. An infusion of the tubers is used in dyspepsia, diarrhea, dysentery, ascites, 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 [28].

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 [26]. Moreover, it showed significant antidiarrhoeal activity in castor oil induced diarrhea in mice [26]. 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 [27].

The growth and acid production of *Streptococcus mutans* were reduced by the tuber extract of *C. rotundus* - *S. mutans* is known as the causative bacteria in the formation of dental plaque and dental caries - Moreover, the same tuber extract inhibited the adherence of *S. mutans* to saliva-coated hydroxyapatite beads. Glucosyltransferase enzyme, which synthesizes water-insoluble glucan from sucrose, was also inhibited by the tuber extract. So, these results suggested that *C. rotundus* may inhibit cariogenic properties of *S. mutans* [29]. *n*-Hexane extract of the tubers of *C. rotundus* proved to be a new herbal supplement for controlling body weight because it induced a significant reduction in weight gain without affecting food consumption or inducing toxicity [30]. Also, *C. rotundus* extract has antihyperglycemic and antioxidant activities [31, 32].

In the study acetone and ethanol extracts showed significant broad spectrum antibacterial activity in disc diffusion method [10]. Antimicrobial activity tests were carried out on human pathogens bacteria (Gram negative and Gram positive) and fungi viz. *C. albicans* and *A. niger*. The highest percentage of inhibition was observed against *K. pneumoniae* (133.33%). Amoxicillin 20µg/ml and ethanol (as fungicide) 70% were used as positive control. Moderate inhibition was observed in case of *A. niger* and *S. aureus* (90 and 70% respectively). No zone of inhibition was observed in *Acinetobacter* and *Candida*. The oil of *C. rotundus* showed a remarkable activity against Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis* [33, 34]. Another study stated that a marked inhibitory effect of *C. rotundus* was observed against *Salmonella enteritidis*, *Staphylococcus aureus* and *Enterococcus faecalis* with total oligomers flavonoids (TOFs) and ethyl acetate extracts [35, 36]. Essential oils and alcoholic extracts from the leaves and/or roots of 35 medicinal plants commonly used in Brazil were screened for anti *Candida albicans* activity. Essential oils from 13 plants showed anti *Candida* activity, including *Aloysia triphylla*, *Anthemis nobilis*, *Cymbopogon martini*, *Cymbopogon winterianus*, *Cyperus articulatus*, *Cyperus rotundus* L., *Lippia alba*, *Mentha arvensis*, *Mikania glomerata*, *Mentha piperita*, *Mentha sp.*, *Stachys byzantina*, and *Solidago chilensis*. The ethanol extract was not effective at any of the concentrations tested. Chemical analyses showed the presence of compounds with known antimicrobial activity, including 1,8-cineole, geranial, germacrene-D, limonene, linalool, and menthol [37]. The present study was conducted to investigate the antimicrobial activity and cytotoxicity of *C. rotundus* (whole plant) in Sudan.

II. MATERIALS AND METHODS

Plant materials

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

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



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

Preparation of crude extracts

Extraction was carried out for the whole plant of *C. rotundus* plant by using overnight maceration techniques according to the method described in Harbone [38]. 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 were not soluble were successively extracted using ethanol with the described technique. The extracts were kept in freez 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.

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

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

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

Test microorganisms

The ethanolic extract of *C. rotundus* whole plant were tested against four bacterial species: two Gram-positive bacteria viz., *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923), two Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and two fungal strains viz, *Aspergillus niger* (ATCC 9763) and *Candida albicans* (ATCC 7596). The bacterial and fungal strains used in the study were obtained from the Department of Microbiology, of the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) and National Health Laboratory of Khartoum in Sudan.

The bacterial cultures were maintained on nutrient agar and incubated at 37°C for 18 h and then used for the antimicrobial test.

***In vitro* testing of extracts for antimicrobial activity**

The cup-plate agar diffusion method described in Kavanagh, [39] was used adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. One ml of the standardized bacterial stock suspension (between 10^8 and 10^9 CFU/ml) was thoroughly mixed with 100 ml of molten sterile nutrient agar which was maintained at 45°C. 20 ml aliquots of the inoculated nutrient agar were distributed into sterile Petri-dish plates. The agar was left to set and in all of these plates 4 cups (10 mm in diameter) were cut using a sterile cork borer (No. 4) and agar discs were removed. Each cups were filled with 0.1 ml sample of the ethanolic extracts using an automatic microlitre pipette, and thereafter the extracts were allowed to diffuse at room temperature for two hours. The plates were then incubated in an upright position at 37°C for 18 h. Two replicates were carried out for each extract against each of the test organisms. After incubation the diameters of the resultant growth inhibition zones were measured and averaged. The mean values were tabulated.

Antifungal testing: The same method used for the antibacterial test was employed. However, the growth media used in case of fungi, was Sabouraud dextrose agar instead of nutrient agar. The inoculated medium was incubated at 25°C for two days for *Candida albicans* and three days for *Aspergillus niger*.

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 [40].

Preparation of *C. rotundus* extracts

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:

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

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:

$$\% \text{ Cell inhibition} = 100 - \left\{ \frac{\text{Ac}-\text{At}}{\text{Ac}} \right\} \times 100$$

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.

III. RESULTS AND DISCUSSION

The whole plant of *C. rotundus* family (Cyperaceae) was screened for antimicrobial activity against two Gram positive bacteria (*B. subtilis*, *S. aureus*), two Gram negative bacteria (*E. coli*, *P. aeruginosa*) as well as two fungi namely (*A. niger* and *C. albicans*) using the cup plate agar diffusion method, and screened for cytotoxicity using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line.

Antimicrobial activity of *C. rotundus* (whole plant) extract

The whole plant of *C. rotundus* family (Cyperaceae) was screened for antimicrobial activity against two Gram positive bacteria (*B. subtilis*, *S. aureus*), two Gram negative bacteria (*E. coli*, *P. aeruginosa*) as well as two fungi namely (*A. niger* and *C. albicans*) using the cup plate agar diffusion method.

The extracts whole plant of *C. rotundus* dissolved in DMSO (1:10) showed high activity (31 & 30 mm) against Gram positive bacteria (*S. aureus* & *B. subtilis*) and (20 & 26 mm) against (*A. niger* & *C. albicans*). It also showed (19 & 20) against Gram negative bacteria (*E. coli* & *P. aeruginosa*). Therefore this result showed that the extracts tested inhibited the growth of all microorganisms though the sensitivities of microorganisms varied.

Therefore this result showed that the Gram positive organisms are more active than the Gram negative organisms and the two fungi tested.

This result was similar to that produced by Aeganathan *et al.* [41] who found that the plant extract showed high activity against *B. subtilis* and *S. aureus* and fungi *A. niger* and *C. albicans* used different solvent: (methanol, chloroform and ethyl acetate). Muthu *et al.* [42] found also that chloroform, ethyl acetate and methanol fractionated compounds flower extracts were active against *B. subtilis*, *S. aureus*, and *P. aeruginosa*. Saadabi and Moglad [43], found that the chloroform and methanol extracts were active against

B. subtilis, *S. aureus*, and *P. aeruginosa*. Eltayeib and Ismaeel [44] found that the Rhizomes oil, was active against *B. subtilis*, *S. aureus*, and *P. aeruginosa*, *A. niger* and *C. albicans*.

The result of minimum inhibition concentration from **Table (3)** showed that 12.5 µg/ml was the lowest concentration at which all the tested microorganisms were inhibited. A comparison of observation given in **Tables (2 and 4)**, showed that the whole plant of *C. rotundus* dissolved in dimethyl sulphoxide inhibited all bacteria higher than 40 µg/ml Ampicillin and higher than 10 µg/ml Gentamicin. The whole plant extract of *C. rotundus* inhibited *A. niger* with a higher than 10 µg/ml of Clotrimazole, and inhibited *C. albicans* at more than 50 µg/ml of Nystatin.

Table (2): Antimicrobial activity of whole plant of *C. rotundus* against the standard bacteria and fungi :

Standard microorganisms	Concentration (100 mg/ml)	
	Mean Diameter of Growth Inhibition Zone (mm)	
Tested bacteria used		
<i>Bacillus subtilis</i>	30	
<i>Staphyococcus aureus</i>	31	
<i>Escherichia coli</i>	19	
<i>Pseudomonas aeruginosa</i>	20	
Tested fungi used		
<i>Apergillus niger</i>	20	
<i>Candida albicans</i>	26	

Key: Interpretation of results: MDIZ (mm) : >18 mm: Sensitive, 14 to 18 mm: Intermediate: <14 mm: Resistant. (-): No inhibition; Concentration used 100 mg/ml at 0.1ml/cup.

Table (3): The antimicrobial activity whole plant of *C. rotundus* against the standard bacteria and fungi :

Standard microorganisms	Concentrations (mg/ml)			
	100	50	25	12.5
	Mean Diameter of Growth Inhibition Zone (mm)			
Tested bacteria used				
<i>Bacillus subtilis</i>	30	25	21	18
<i>Staphyococcus aureus</i>	31	26	22	19
<i>Escherichia coli</i>	19	18	16	15
<i>Pseudomonas aeruginosa</i>	20	19	18	16
Tested fungi used				
<i>Apergillus niger</i>	20	19	18	16
<i>Candida albicans</i>	26	22	20	18

Table (4): Antibacterial and antifungal activity of reference antibiotics against standard microorganisms.

Drugs	Concentrations (µg/ml)	Standard microorganisms used			
		Gram positive		Gram negative	
		Tested bacteria used			
		Mean Diameter of Growth Inhibition Zone (mm)			
		<i>Bacillus subtilis</i>	<i>Staphyococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Ampicillin	40	15	25	-	16
	20	14	20	-	13
	10	13	18	-	12
	5	12	15	-	-
Gentamicin	40	29	35	32	23
	20	22	33	30	22
	10	20	30	17	21
	5	17	28	-	19
Tested fungi used (M.D.I.Z. mm)					
		<i>Apergillus niger</i>		<i>Candida albicans</i>	
Clotrimazole	40	30		42	
	20	22		40	
	10	19		33	
	5	16		30	
Nystatin	50	28		17	
	25	26		14	
	12.5	23		-	

Cytotoxicity assay of *C. rotundus* (whole plant) extract

Table (5): 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	<i>C. rotundus</i> (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
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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 IC₅₀ 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 cytotoxicity effects of ethanolic extract of *C. rotundus* (whole plant) by using MTT-assay including (Vero cell line). The result of MTT assay verified the safety of the examined extract. This result was similar to that produced by Ahmed *et al.* [45] who found that the plant extract gave similar result using the Brine Shrimp Bioassay.

V. CONCLUSION

The whole plant extract of *C. rotundus* showed the various degree of inhibitory activity against the microorganisms tested. The obtained results may justify the use of the Sudanese whole plant of *C. rotundus* as antimicrobial therapy in traditional medicine in Sudan and the neighboring countries. 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.

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