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Mediterranean Journal of Chemistry **2015**, 4(4), 201-208

Phenolic composition and antioxidant, antimicrobial and cytochrome P450 inhibition activities of *Cyperus rotundus* tubers

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Abstract: The chemical composition of a *Cyperus rotundus* methanol extract was investigated. The phenol content determined by the Folin-Ciocalteu method was 83.6 ± 5.42 mEGA.g^{-1} and the flavonoid content assayed by the $AlCl_3$ method was 32.65 ± 3.5 mEQ.g^{-1}. The phenolic composition determined by HPLC-DAD showed the presence of phenolic acids (chlorogenic, caffeic, ferulic, syringic acids) and flavonoids (luteolin, quercetin, apigenin, myricetin). The antioxidant activity of the extract was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\cdot}$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS$^{\cdot+}$) radical scavenging assays and the β-carotene bleaching test. The EC_{50} values were 5.76 ± 0.83 µg/mL and 18.08 ± 0.6µg/mL for DPPH$^{\cdot}$ and ABTS$^{\cdot+}$, respectively. The antioxidant activity coefficient (AAC) was 670.8 ± 4.2. The antibacterial activity determined by the disc diffusion and submerged culture methods against four Gram negative and two Gram positive bacteria showed effects against all strains. Cytochrome P450 (CYPs) inhibitory activity was evaluated against CYP1A2, CYP3A4 and CYP2D6, which was the most inhibited (IC_{50} =11.13± 0.04µg/mL).

Keywords: *Cyperus rotundus*, chemical composition, antioxidant activity, antimicrobial activity, cytochrome inhibition

Introduction

For many years, plants have been used as therapeutic resources either as herbal teas or other homemade remedies, as crude extracts, or as standard enriched fractions in pharmaceutical preparations such as tinctures, fluid extracts, powders, pills, and capsules[1,2]. Plants are rich sources of bioactive molecules, most of which probably evolved as chemical defenses against infections[3]. Increasingly, natural products have been studied for their antibacterial antioxidant and anticancer activities[4,5]. Recently, the ability of numerous herbs to act as substrates or inhibitors for different cytochrome P450 (CYPs) has been reported[6]. The relationship between cytochrome P450 and chemical carcinogenesis has been extensively studied. CYP activation of certain anticancer drugs has long been known and the importance of this process as a way of targeting novel anticancer therapy is being explored.

However, certain CYPs involved in hormone and vitamin metabolism and in the metabolic activation of genotoxic substances have been reported to play important roles in tumor formation and development[7].

Cyperus rotundus L., (family Cyperaceae), also known as purple nutsedge or nutgrass, is a common perennial weed with slender, scaly creeping rhizomes, bulbous at the base and arising singly from tubers which are about 1-3 cm long[8]. *C. rotundus* is widely distributed in the Mediterranean basin areas[9]. In Tunisia, it is widespread in the northeast, center and south of the country[10]. *C. rotundus* is a traditional herbal medicine; the tuber part is one of the oldest known medicinal elements used for the treatment of dysmenorrhea and menstrual irregularities. Infusions of this herb have been used for pain, fever, diarrhea[11].

A number of pharmacological and biological activities including anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic antimicrobial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities have been reported for this plant[12,13].

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DOI: http://dx.doi.org/

Therefore, the objectives of the present study were to characterize the chemical composition of *C.rotundus*, to assess the biological activities of a methanol extract as an antioxidant and as an antimicrobial agent against standard microorganisms, and to investigate for the first time its inhibitory effect on three CYPs isoforms, namely CYP1A2, CYP3A4 and CYP2D6.

Experimental Section

Plant material and reagents

Cyperus rotundus tubers were collected from Kebili in the southwestern part of Tunisia. Samples were authenticated by the National Institute of Agronomic Research of Tunisia (INRAT).Vivid CYP blue screening kits were purchased from Invitrogen Corporation (Carlsbad, CA, USA). All the other reagents were of the highest purity available and were purchased from the Sigma-Aldrich Chemical Company.

Preparation of methanol extract

Fifty grams of *C. rotundus* tubers were crushed and extracted with 80% aqueous methanol (3 x 300 mL) by agitated maceration at room temperature for 72 h. The extracts obtained from three extractions were combined, filtered through a Whatman No.4 filter paper and concentrated under reduced pressure.

Total phenolic and flavonoid contents

The total phenolic content was assayed using the Folin-Ciocalteu reagent according to the method modified by Turkoglu et al. (2006)[14]. An aliquot (0.1mL) of a suitable diluted extract was added to 0.5 mL of the Folin-Ciocalteu reagent and 1mL of deionized water. The mixture was shaken and allowed to stand for 1 min, before adding 1.5 mL of 20% Na_2CO_3 solution. The absorbance at 760 nm was recorded after two hours.

The total flavonoid content was measured according to Al-Dabbas et al. (2006)[15], in which 1.5 mL of the extract was mixed with 1.5 mL of 2% $AlCl_3$ methanol solution. After 10 min, the absorbance was read at 367.5 nm.

Total phenolic and flavonoid contents are expressed in mg gallic acid equivalents per gram of extract (mg GAE/g) and in mg quercetin equivalents per gram of extract (mg QE/g), respectively. Analyses were performed in triplicate.

HPLC-DAD analysis

Chromatographic separation was carried out on a HPLC-DAD Agilent 1200 using a C18 column kept at 30°C. A gradient solvent comprised of 0.5% acetic acid in water (A) and methanol (B) was applied for a total running time of 40 min. The following proportions of solvent B were used for elution: 0.5-20 min, 0-85%; 20-30 min, 85%; and 30-40 min, 85-0%. The flow rate was 0.5 mL/min, and 5 µL of each solution at 100 mg/mL was injected and detected at two wavelengths (254 and 330 nm).

Antioxidant activity

DPPH radical scavenging assay: *C. rotundus* methanol extract was tested for the scavenging effect on DPPH radicals according to the method of Al-Dabbas et al. (2006)[15] , in which 2 mL of extract solution in methanol was added to 2 mL of a 10^{-4}mol/L DPPH methanolic solution. Solutions were then kept in the dark at room temperature for 30 minutes. The scavenging activity on the DPPH was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a UV-vis spectrophotometer. As a positive control, synthetic antioxidant BHT was used. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using Equation 1:

Equation 1. % inhibition = [1- A_1/A_0] x 100

where A_1 and A_0 are the absorbance of the tested sample and the blank after incubation, respectively.

ABTS radical cation decolorization assay : Spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was done according to a previously described method[16]. ABTS radical cations were produced by reacting 2 mM ABTS in distilled water with 70 mM potassium persulfate ($K_2S_2O_8$) stored in the dark at room temperature for 24 h. Then, 1 mL of the ABTS radical cation solution was added to 1 mL of *C. rotundus* extract at
different concentrations. The absorbance was measured at 734 nm, 30 min after mixing the prepared solution. The percentage of radical scavenging was calculated for each concentration relative to a blank
containing no scavenger using Equation2:

Equation 2. Inhibition (%) = [(A_0−A_1)/A_0] × 100

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The sample concentration providing 50% of the free radical inhibition (EC_{50}) was calculated by plotting inhibition percentage against sample concentration. EC_{50} was expressed as Equivalents Trolox (TEAC)

β-Carotene bleaching test: The antioxidant activity of *C. rotundus* extract was determined according to a slightly modified version of the β-carotene bleaching method described by Suja et al. (2005)[17]. Two milligrams of β-carotene were dissolved in 5 mL of chloroform and 0.5 mL β-carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween-40 emulsifier in a round bottom flask. Then chloroform was removed in a rotary vacuum evaporator. The resulting mixture was diluted with 50 mL of oxygenated distilled water. To 4 mL of this emulsion, 0.2 mL of the test sample in ethanol (0.2 mg/mL) was added. BHT was

used for comparative purposes. A solution with 0.2 mL of ethanol and 4 mL of the above emulsion was used as control. A mixture prepared as above without β-carotene served as the blank, and was used to zero the spectrophotometer. The tubes were covered with aluminum foil and maintained at 50°C in a water bath. Absorbance of the emulsion at 470 nm was taken at baseline (t = 0 min) and after every 15 min. Measurement of absorbance continued until the color of β-carotene disappeared in the control reaction (t = 120 min). All determinations were performed in triplicate. Antioxidant activity coefficient (AAC) was calculated according to the following Equation 3:

Equation 3. AAC = [$A_{A\,(120)}$-$A_{C\,(120)}$ / $A_{C\,(0)}$-$A_{C\,(120)}$] × 1000

where $A_{A\,(120)}$ is the absorbance of antioxidant at 120 min, $A_{c\,(120)}$ is the absorbance of the control at 120 min and $A_{C\,(0)}$ is the absorbance of the control at 0 min.

Antibacterial activity

Disc diffusion method: The *in vitro* antibacterial activity of the tested sample was carried out by the disc diffusion method against two Gram positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Streptococcus A*ATCC 11 700) and four Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 14 028, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13833). In this test, nutrient agar (NA) was used as the culture medium[18].The NA plates were prepared by pouring 15 mL of molten medium into sterile Petriplates. The plates were allowed to solidify for 5 min and 100µL of the inoculum suspension was swabbed uniformly; the inoculum was allowed to dry for 5 min. Different concentrations of extract dissolved in dimethyl sulfoxide (DMSO) (20, 50, 75 and 100 mg/L) were loaded on 6 mm sterile individual discs. The loaded discs were placed on the surface of the medium and the compound was allowed to diffuse for 5 min. The plates were then incubated at 37°C for 24 h. The negative control was prepared using the respective solvent. At the end of incubation, inhibition zones that had formed around the discs were measured with a transparent ruler in millimeters. The test was performed in triplicate.

Submerged culture method: The MIC and MBC were determined using the submerged culture method[19] with some modifications. The activities of six strains of microorganism were determined as follows: equal volumes of each bacterial strain culture, containing approximately 1 10^6CFU/mL, were applied onto flasks containing 20 mL of nutrient broth and the methanol extract at concentrations ranging from 20 to 5000 mg/L. These serially diluted cultures were then incubated at 37°C for 18 h, then 1 ml of the culture medium was removed from each broth assay flask and sub-cultured in fresh nutrient agar. After incubation at 37°C for 24 h, the MIC was determined as the lowest concentration at which the microorganism did not demonstrate visible growth, and the least concentration showing no visible growth on sub-culture was taken as the MBC.

Cytochrome P450 enzyme inhibition

The inhibitory effect of the methanol extract was determined using 96-well microtiter plates (Sumilon 96F)[20], based on reading the fluorescence of 3-cyano-7-hydroxycoumarin produced by CYP hydroxylase activities at an excitation wavelength of 460 nm and an emission wavelength of 409 nm. CYP inhibitory activity was assayed using the vivid CYP Blue Substrate. 10µM 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) was the substrate used for CYP3A4, and 7-ethyloxymethyloxy-3-cyanocoumarin (EOMCC) was the substrate used for CYP1A2 (3µM) and CYP2D6 (10µM). The positive control was erythromycin for CYP3A4, safrole for CYP1A2 and cimetidine for CYP2D6. In the first step, serial dilutions of the methanol extract were performed by distilled water. In the second step, 40 µL of each sample was added to 50 µL of a CYP/NADPH-CYP reductase mixture in a 96-well plate (5nM of CYP1A2, 10nM of CYP2D6 and 5nM of CYP3A4). The obtained solution was then pre-incubated for 20 min at room temperature. The reaction was then initiated by adding 10 µL of the substrate/NADP+ mixture. The plate was incubated for 15 min. Finally, the reaction was ended by the addition of 10 µL of each stop solution (10 µM of ketoconazole for CYP3A4, 30 µM α-naphthoflavone for CYP1A2 and 15 µM quinidine for CYP2D6). Activity was measured as the rate of fluorescent metabolite production over the course of the reaction. The IC_{50} values were calculated by linear interpolation. The test was performed in triplicate.

Statistical analysis

Results are expressed as mean ± SD of three independent experiments. Statistical significant differences were considered at $p < 0.05$ using one-way analysis of variance (ANOVA) with Statgraphics Centurion.

Results and Discussion

Chemical composition

The total phenol content of *C. rotundus* extract, reported as mg gallic acid equivalents/g dried extract, was estimated to be equivalent to 83.6 ± 5.42 mg EGA/g; this value is comparable to that found by Ardestani and Yazdanparast, (2007)[9] which was 78.55 mg EGA/g in a dried extract. The flavonoid content was equal to 32.65 ± 3.5 mg EQ/g. The analysis of methanol extract composition by HPLC-DAD allowed us to characterize its phenolic composition.

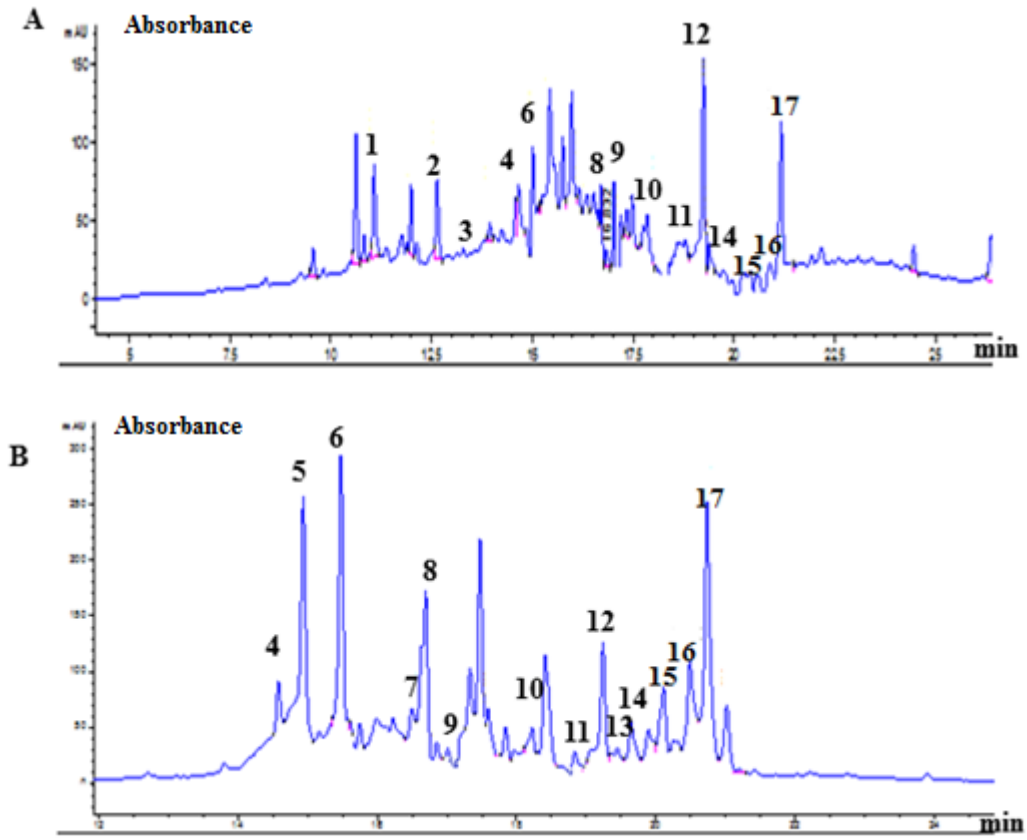

Figure 1. HPLC-DAD chromatograms of *Cyperus rotundus* methanol extract at 254 nm (A) and 330 nm (B)

The chemical composition of *Cyperus* extract (Fig.1) was characterized by the presence of phenolic acids (chlorogenic (1), caffeic (2), ferulic (3), *p*-coumaric (4), syringic (5), cinapic (6), salicylic (7), ellagic (8) and trans-cinnamic (9)) and flavonoids (myricetin (10), quercetin (11), luteolin (12), hesperetin (13), genistein (14), kaempferol (15), apigenin (16) and rhamnetin (17)) which can be classified into several groups (hydroxycinnamic acids, hydroxbenzoic acids, flavanols, flavanones and flavones).

Previous RP-HPLC analysis of *C. rotundus* revealed the presence of phenolic compounds such as gallic acid, caffeic acid, salicylic acid,*p*-coumaric acid, luteolin, quercetin, kaempferol and epicatechin[21,22].

The crude extract of *C.rotundus* was used to assess the antioxidant and antimicrobial activities and the inhibitory effect against cytochrome P450. The HPLC/DAD analysis of the plant material allowed us to identify the individual phenolic compounds present in the studied material that could participate in the biological activities of the extract. However, the use of the crude extract to assess the different biological activities could be more informative than using individual phenolic compounds. The extract may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in an extract[23].

Antioxidant activity

The antioxidant activity of the *C.rotundus* methanol extract was assessed by radical scavenging assays using DPPH and ABTS radicals and the β-carotene bleaching method (Figure 2).

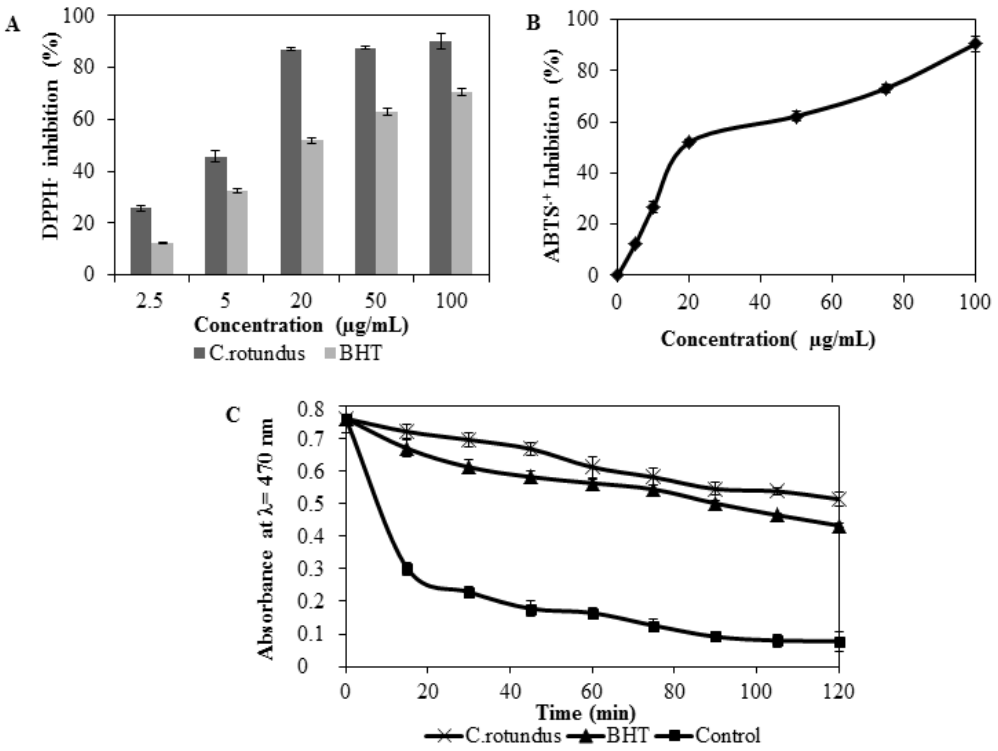

Figure 2. Evaluation of antioxidantactivity of *Cyperusrotundus*methanolextract by DPPH· test (A), ABTS·+ test (B) and β-carotenebleachingmethod (C)

Free radical scavenging tests

As shown in Fig.2A and Fig.2B, the *C. rotundus* extract (CRE) exhibited potent radical scavenging ability in a dose-dependent manner, pointing to its antioxidant activity. The antioxidant ability of CRE to scavenge purple colored DPPH· And blue-green colored $ABTS^{\bullet+}$radicals was compared to that of BHT and Trolox, respectively. CRE showed free radical scavenging activity with high potency. The DPPH radical scavenging activity provided an EC_{50} value of 5.76 ± 0.83 µg/mL (lower than that of BHT, 18.4± 1.3µg/mL).The EC_{50} value for the $ABTS^{\bullet+}$ radical was 18.8 ± 0.6µg/mL which represented 12072.87 µM ETrolox/g of the extract as the TEAC value.

β-carotene bleaching test

As shown in Figure 2C, there was a considerable decrease in absorbance in the control sample. This reduction was due to the accumulation of malondialdehyde compounds from linoleic acid oxidation, which is not stable. In the presence of CRE and BHT, the absorbance is more stable, which can be explained by their antioxidant activity based on their capacity to reduce malondialdehyde formation. Further oxidation causes malondialdehyde to be converted to secondary products such as alcohols and acids that cannot be detected. The antioxidant activity of CRE was found to be higher than that of BHT with an AAC of 670± 4.2.

The present results are in agreement with those from previous research by Kilani et al. (2011)[13], Nagulendran et al. (2007)[21] and Singh et al. (2012)[24] who demonstrated the antioxidant activity of *Cyperus* extracts. The antioxidant activity of CRE could be related to its phenolic composition. There seems to be a good correlation between its phenolic content and the antioxidant activity.

The presence of some phenolic acids such as chlorogenic and caffeic acids as well as the flavonoids quercetin and luteolin have been shown to have important antioxidant activity[25].

Antibacterial activity

The crude extract was screened for its antibacterial activity against *S. aureus*, *Strept. A*, *E. coli*, *S. enteritidis*, *P. aeruginosa*, and *K. pneumoniae* using the disc diffusion and submerged culture methods. The choice of these microorganisms was made due to the fact that some of them are causative agents of human intestinal infection.

Table 1. Inhibition diameters of *Cyperus rotundus* methanol extract against six bacterial strains

Bacterial strain	Inhibition Diameter (mm)				
	Antibiotic	Extract Concentration mg/L			
	50µg/mL	20	50	75	100
Gram+ bacteria					
Staphylococcus aureus ATCC 25923	15.7±1.2	6.0±0.5	7.0±0.4	11.0±1.0	13.0±1.2
*Streptococcus A*ATCC 11 700	14.2±0.8	4.0±0.2	5.0±0.7	9.0±1.1	11.0±0.9
Gram- bacteria					
Pseudomonas aeruginosa ATCC 9027	2.1±0.5	0	0	3.0±0.8	5.0±0.3
Salmonella enteritidis ATCC 14 028	14.7±1.4	3.0±0.1	5.0±0.3	7.0±0.7	9.0±1.2
Esherichia coli ATCC 25922	12.3±0.5	2.0±0.5	3.0±0.2	6.0±0.4	8.0±0.0
Klebsiella pneumoniae ATCC 13 833	12.6±1.5	0	2.4±0.9	5.0±0.6	7.0±0.3

Means ± SD (n = 3), Internal diameter = 6mm, Inhibition diameter = external diameter-internal diameter, significant difference ($p<0.05$)

The inhibition diameters (mm) of the extract on the selected microorganisms are shown in Table 1. The results indicated greater inhibition at the highest concentration. This is in agreement with a previous report[26] that the mode of action of plant extracts is concentration-dependent. It was found that Gram positive bacteria were more sensitive than Gram negative bacteria. *S.aureus* presented the highest inhibition zone of (13 mm) at 100 mg/L while *P.aeruginosa* was the most resistant bacterium with only a 5 mm inhibition zone at the same concentration.

Several studies have demonstrated the higher sensitivity of Gram positive bacteria compared to Gram negative which can be attributed to the difference in the outer layers of Gram negative and Gram positive bacteria. Gram negative bacteria, regardless of the cell membrane, have an additional layer of the outer membrane, which consists of phospholipids, lipopolysaccharide and proteins; the membrane is impermeable to most molecules. Nevertheless, the presence of pores in this layer will allow the free diffusion of molecules with a molecular weight below 600 Da[27].

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Cyperus rotundus* methanol extract

Strains	MIC (mg/L)	MBC (mg/L)
***Staphylococcus aureus* ATCC 25923**	20	200
***Streptococcus A* ATCC 11 700**	50	250
***Pseudomonas aeruginosa* ATCC 9027**	250	>5000
***Salmonella enteritidis* ATCC 14 028**	75	1500
***Esherichia coli* ATCC 25922**	150	2000
***Klebsiella pneumoniae* ATCC 13 833**	200	3000

This result was confirmed by the submerged culture method as shown in Table 2. The MIC and the MBC showed that *S. aureus* was the most sensitive bacterium with MIC and MBC values of 20 and 200 mg/L respectively. The antimicrobial activity of several *C. rotundus* extracts was previously investigated on Gram positive and Gram negative bacteria; the ethyl acetate and methanol extracts showed significant inhibitory activity against *S. enteritidis*, *S. aureus* and *E. Faecalis*[13,24]. The antimicrobial capacity of CRE is attributed to its phenolic compounds which are well-known for their antimicrobial activity[28].

The antimicrobial properties of phenolic compounds have been reported many times. Cowan (1999)[29] showed that quercetin is one of the most active principal antimicrobial agents in plant extracts. Caffeic acid, chlorogenic acid and gallic acid have been shown to exhibit some antimicrobial properties[29,30].

Cytochrome P450 enzyme inhibition

The inhibitory effect of CRE was investigated against three CYP enzymes .The results show that CYP inhibition was dose-dependent (Data not shown). The inhibition percentages of CYP1A2, CYP3A4 and CYP2D6 activities reached 85.36% for 0.2 mg/mL, 83.25% for 0.05 mg/mL and 67.28% for 0.025 mg/mL of the extract, respectively.

The EC_{50} values are presented in Table 3. The EC_{50} value is negatively related to inhibitory CYP activity. A lower EC_{50} value indicates greater inhibition of CYP activity by the tested sample. The lowest EC_{50} value of the *C.rotundus* methanol extract (11.13µg/mL) was obtained with CYP2D6, which was the most inhibited enzyme, followed by CYP3A4 and CYP1A2 with EC_{50} values of 13.44and 59.48µg/mL, respectively. The EC_{50} values of CRE were higher than those of the reference compounds (significant difference, $p<0.05$).

Table 3. IC_{50} Values of *Cyperus rotundus* methanol extract for cytochrome P450 enzyme CYP1A2, CYP3A4 and CYP2D6 inhibition activity

Samples	IC_{50} (µg/mL)		
	CYP		
	1A2	2D6	3A4
***C.rotundus* extract**	59,48± 0,02	11,13± 0,04	13,44± 0,01
Safrole[a]	0,64 ± 0,10	-	-
Cimetidin[b]	-	5,19 ± 1,74	-
Erythromycin[c]	-	-	0,62 ± 0,10

[a]positive contol of CYP1A2
[b]positive contol of CYP2D6
[c]positive contol of CYP3A4

Our results are comparable to those of Usia et al. (2006)[31] who demonstrated the inhibitory capacities of cytochrome P450 CYP3A4 and CYP2D6 by several medicinal plant extracts.

The CYP inhibition activity observed for CRE could be attributed to the presence of phenolic compounds such as phenolic acids and flavonoids (flavones, flavanones and flavonols). Previous research has indicated the strong inhibitory activity of the latter compounds on cytochrome P450[32]. Flavonols such as quercetin and kaempferol that possess a 3-OH group have inhibitory activity against CYP2D6, CYP1A2 and CYP3A4. Flavonoids with more phenolic hydroxyl (-OH) groups strongly inhibit CYP3A4 activity[33].

It is important to note that the additive and synergistic effects of phytochemicals in plants are responsible for their potent bioactive properties[34]. This, by implication, means that both minor and major phenolic compounds can contribute to the antioxidant, antimicrobial and the cytochrome P450 enzyme inhibition activities exhibited by the studied plant material.

Conclusion

The results of the present investigation reveal that the use of *C.rotundus* extract as an antioxidant nutraceutical may reduce the oxidation and microbial infections. It may also be an effective regulator of cytochrome P450 enzymes, partly due to the protective action provided by its phenolic compounds.

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