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Phytochemical, antimicrobial, antioxidant and antigenotoxic potentials of *Cyperus rotundus* extracts

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Abstract

The aqueous, ethyl acetate, methanolic and Total Oligomer Flavonoids (TOF) enriched extracts, obtained from the aerial parts of *Cyperus rotundus*, were investigated for their contents in phenolic compounds. Antioxidative activity using the NBT/riboflavin assay system, antimicrobial activity against Gram positive and Gram negative bacterial reference strains as well as antigenotoxic activity tested with the SOS chromotest assay were also studied. Significant antibacterial activity against reference strains; *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella enteritidis* and *Salmonella typhimurium*, was detected in the presence of ethyl acetate and TOF enriched extracts. In addition to their antimicrobial activity, the same extracts showed a significant ability to inhibit nitroblue tetrazolium reduction by the superoxide radical in a non enzymatic $O_2^{\cdot-}$ generating system, and were also able to reduce significantly the genotoxicity induced by nifuroxazide and Aflatoxin B1. The antioxidant, antimicrobial and antigenotoxic activities exhibited by *C. rotundus* depend on the chemical composition of the tested extracts.
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Keywords: Antimicrobial effect; Antigenotoxic activity; Antioxidant activity; *Cyperus rotundus*; Phenolic compounds; SOS chromotest

1. Introduction

Natural crude drug extracts and biologically active compounds isolated from plant species used in traditional medicine can be prolific resources for such new drugs. Microorganisms have been developing resistance to many antibiotics, due to the indiscriminate use of antimicrobial drugs inducing thus an increase of problems in clinical treatment of infectious diseases. In addition, antibiotics are sometimes associated with adverse effects in the host which include hypersensitivity, depletion of gut and mucosal microorganisms, immunosuppression and allergic reactions. Therefore, there is a need for alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen local medicinal plants for possible antimicrobial properties. Medicinal herbs represent a rich source from which novel antibacterial and antifungal chemotherapeutic agents may be obtained (Rates, 2001).

Oxidative DNA damage is generally regarded as carcinogenic and actively participates in many pathological processes, including cancer and aging. It can be caused by environmental factors such as ionizing radiation, UV light, a variety of chemical and genotoxic agents as well as by normal metabolism in which reactive oxygen species (ROS) are formed as by products (Bringmann et al., 2001). At present, there are several antigenotoxicity assays available, which include the micronucleus test, somatic mutation and recombination test (SMART), sister chromatid exchange (SCE) assay and the single cell gel electrophoresis (SCGE) or comet assay. The above-mentioned assays may involve a longer analysis time, a high cost, and specialized skill or may require addition of expensive reagents. Therefore, short-term bacterial assays, such as the Ames test

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and the SOS chromotest assay, are useful and give an estimation of the genotoxic/antigenotoxic potential of substances (Vahl et al., 1997). On the other hand, antioxidant enzymes can, however, minimize DNA damage. Superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as smaller molecules, such as Vitamin E, are mainly responsible for the primary defense against oxidative damage (Prabhakar et al., 2007). Although synthetic antioxidants have often been used to protect against free radicals by scavenging reactive oxygen or ending radical chain reactions, recent health concerns draw much attention to the use of natural antioxidative compounds (Lu and Foo, 2000; Rice-Evans and Burdon, 1993). In fact, much interest has been focused on compounds occurring in plants and herbs for their medicinal and biological activities (Bhatia et al., 2001; Zhao et al., 1999). Polyphenols, particularly flavonoids, which are widely distributed in plants and are present in considerable amounts in fruits, vegetables, spices, medicinal herbs and beverages, have been used to treat many human diseases, such as diabetes, cancers and coronary heart diseases (Broadhurst et al., 2000). Moreover, flavonoids have been shown to exhibit antiviral, antimicrobial, antiplatelet and antitoxic activities (Middleton and Kandaswami, 1994), as well as antioxidative and antigenotoxic activities (Bhouri et al., 2010). These activities are believed to be the result of their redox properties. In fact, polyphenols can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygens, or decomposing peroxides (Al-Dabbas et al., 2006).

Cyperus rotundus Linn, a sedge of the family of Cyperaceae, order Cyperales, is widely distributed in the Mediterranean basin areas. This plant, which grows naturally in tropical, sub tropical and temperate regions, is widespread in the northeast, center and south of Tunisia (Cuénod, 1954). *C. rotundus* is a traditional medicinal plant appearing among Indian, Chinese and Japanese natural drugs used against spasms, stomach disorders, and inflammatory bowel diseases. It has been widely investigated by several authors (Gupta et al., 1971; Singh and Singh, 1980; Thebtaranonth et al., 1995).

This paper reports the evaluation of the antioxidant, antimicrobial and antimutagenic activities of the aerial part extracts from *C. rotundus*. The total polyphenol, flavonoid and tannin contents of the extracts were determined. We report our findings and relate them to the phytochemical studies of the plant as well as its medicinal uses.

2. Materials and methods

2.1. Chemicals

Quercetin, nifuroxazide, Aflatoxin B1 (AFB1) and Nitroblue tetrazolium (NBT) were obtained from Sigma Aldrich Co (St. Louis, MO, USA). *O*-Nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenylphosphate (PNPP) and Riboflavin were purchased from Merck (Dramstadt, Germany). Mueller–Hinton agar was purchased from Fluka Biochemika (Spain). Tannic acid was procured from Fluka (Steinheim). All organic solvents were obtained from Normapur (UK). Digitonin, Folin-Denis reagent, and nitrite sodium ($NaNO_2$) were procured from Prolabo. Folin-Ciocalteau phenol reagent was obtained from BDH Laboratory (England). Aroclor 1254 from Supelco (Bellafonte, PA, USA).

2.2. Plant material

Cyperus rotundus aerial parts were collected in the region of Monastir in the center of Tunisia in October 2004. Botanical identification was carried out by Pr Chaieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia), according to the flora of Tunisia (Cuénod, 1954). A voucher specimen (Cp.10-04) has been kept in the laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, for future reference.

2.3. Preparation of plant extracts

The fresh aerial parts of *Cyperus rotundus*, were dried at room temperature and reduced to coarse powder. The powdered leaves were extracted by boiling water for 15 to 20 min. The crude extract obtained was filtered and lyophilized (aqueous extract). The residue was dissolved in water. In order to obtain an extract enriched with Total Oligomers Flavonoids (TOF), powder was macerated in water/acetone mixture (1v/2v), for 24 h with continuous stirring. The extract was filtered and the acetone was evaporated under low pressure in order to obtain an aqueous phase. Tannins in the aqueous phase, were precipitated with an excess of NaCl for 24 h at 5 °C. The mixture was then filtered and the filtrate solution was recovered. This latter was extracted with ethyl acetate, concentrated and precipitated with an excess of chloroform. The precipitate was separated and yielded TOF extract which was dissolved in water.

Ethyl acetate and methanol extracts were obtained by soxhlet extraction (6 h). The two types of extract, with different polarities were concentrated to dryness and the residue was kept at 4 °C. These two extracts were resuspended in DMSO. In the present study, four extracts were investigated.

2.4. Preliminary phytochemical analysis

Plant materials were screened for the presence of tannins, flavonoids, coumarins and sterols by using the methods previously described by Tona et al. (1998) and Kilani et al. (2005). Two milligrams of each extract were separately dissolved in 2 ml of the adequate solvent. The detection of major chemical groups was carried out by thin-layer chromatography (TLC) on silica gel 60 F_{254} from Merck (Dramstadt, Germany) (layer thickness, 0.25) as follows: for flavonoids, TLC was developed in n-Butanol/acetic acid/water 4:1:5 (toplayer), then spots were visualized with 1% $AlCl_3$ solution in methanol under ultraviolet light (UV) at $\lambda = 366$ nm. Coumarins were detected under UV (366 nm) thanks to their blue fluorescence, which becomes intense after spraying 10% potassium hydroxide solution in ethanol. Steroids were detected with Libermann–Burchard as a reagent using n-hexane/CH_2Cl_2 1:9 as a mobile phase. A range of colors is produced after

heating sprayed plates for 10 min at 100 °C. Tannin test was carried out with $FeCl_3$. Each class of tannins gave a specific coloration.

2.5. Quantitative analysis of extracts

2.5.1. Determination of total polyphenol and flavonoid contents

The polyphenol content of *C. rotundus* was quantified by the Folin–Ciocalteau reagent (Kumar and Chattopadhyay, 2007; Yuan et al., 2005). Aliquots of test samples (100 µl) were mixed with 2.0 ml of 2% Na_2CO_3 and incubated at room temperature for 2 min. After addition of 100 µl 50% Folin–Ciocalteau phenol reagent, the reaction tube was further incubated for 30 min at room temperature, and finally absorbance was read at 720 nm. Gallic acid was used as a standard.

A known volume of each extract was placed in a 10 ml volumetric flask to estimate flavonoid content according to the modified method of Zhishen et al. (1999). After addition of 75 µl of $NaNO_2$ (5%), 150 µl of freshly prepared $AlCl_3$ (10%), and 500 µl of NaOH (1 N) solutions, the volume was adjusted with distilled water until 2.5 ml. After 5 min incubation, the total absorbance was measured at 510 nm. Quercetin was used as a standard for constructing a calibration curve.

2.5.2. Determination of total sterol content

Twenty milligrams of each extract dissolved in 500 µl of acetone was mixed with 250 µl of a digitonin solution (2% in the alcohol) and heated to 60 °C for reaching half of volume. After cooling to room temperature for 15 min, the precipitate was separated on a weighed filter (M_0). Then, the filter was washed 10 times with water, 10 times with alcohol, once with acetone, once with alcohol, and finally once with anhydrous ether, the filter was dried for 3 h at 80 °C. After cooling, the filter was weighed (M_f). Sterol content was expressed according to the following formula:

$$\% \text{ Sterols} = (\text{Psteroids} / \text{Pextract}) \times 100, \text{where P steroids} = (M_f - M_0) \times 0.25.$$

2.5.3. Determination of tannin content

The method described by Pearson (1976) was used for the determination of tannin content of samples. Five grams of sample were dissolved in 50 ml of distilled water, and the mixture allowed to stand for 30 min with shaking at 10-min intervals. The solution was centrifuged at 5000 × *g* and the supernatant recovered (tannin extract). The extract was diluted in 100 ml of distilled water. Five milliliters of the diluted extract and 5 ml of standard tannic acid (0.1 g/L) were distributed into two different 50-ml volumetric flasks. One milliliter of Folin–Denis reagent was added to each flask followed by 2.5 ml of saturated sodium carbonate solution. The solutions were made up to the 50-ml mark with distilled water and incubated at room temperature for 90 min (Nwabueze, 2007). The absorption of these solutions was measured against that of the reagent blank (containing 5 ml distilled water in the place of extract or standard tannic acid solution) in a Beckmann spectrophotometer at 760-nm wavelength. Tannin content (mg/100 g) was calculated as: [(Sample reading - blank) / (Standard reading - blank)].

2.6. In vitro antimicrobial assay

Antimicrobial activity of *C. rotundus* extracts was tested on the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, and the Gram-negative bacteria *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076 and *Salmonella typhimurium* NRRLB 4420, using the micro dilution method (Eloff, 1998; Hayder et al., 2005; Roussis et al., 1998). Overnight grown microbial suspensions were standardized to approximately 10^5 cells/ml (Cremieux, 1991). The agar dilution method was used to determine the minimum inhibitory concentrations (MICs) of *C. rotundus* extracts: 100 µl of microbial suspension containing approximately 1×10^5 cells/ml, were added to 100 µl of the test extract dilution (concentrations ranging from 0.25 to 5 mg/ml in tubes). A set of tubes containing only microbial suspension served as the negative control. These serially diluted cultures were then incubated at 37 °C for 24 h. Subsequently, 10 µl of each culture were plated on substance-free Mueller–Hinton agar plates and further incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of plant extract that completely suppressed colony growth. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of the tested extract that kills 99.99% of the tested bacteria. The standard antibiotic ampicillin was used as positive control to check the sensitivity of the test organisms.

2.7. Determination of the scavenging of superoxide radicals

The inhibition of Nitroblue tetrazolium (NBT) reduction by photochemical generated O_2^-, was used to determine the superoxide anion scavenging activity of the extracts. The reaction was carried out at room temperature under fluorescent lighting (20 W, 20 cm). The standard incubation mixture contained 6.5 mM N,N,N′,N′-tetramethylethylenediamine (TMEDA), 4 µM riboflavin, 96 µM NBT, and 51.5 mM potassium phosphate buffer (pH 7.4). Superoxide generation was measured by increasing the amount of absorbance at 546 nm after 25 min incubation from beginning of illumination. The photochemically reduced riboflavins, generated O_2^-, which reduced NBT to form blue formazan. The sample extracts were added to the reaction mixture, in which O_2^- radicals are scavenged, thereby inhibiting the NBT reduction (Siddhurrajir et al., 2002).

Quercetin was used as a positive control. The degree of the scavenging was calculated by the following equation:

$$\text{Scavenging } (\%) = [\text{ODcontrol} - \text{ODsample} / \text{ODcontrol}] \times 100.$$

IC_{50} is defined as the concentration (µg/ml) of substrate that causes 50% loss of superoxide radicals (color) and was calculated by linear regression analysis.

2.8. SOS chromotest assay

The SOS chromotest employs the error-prone DNA repair pathway of *E. Coli* PQ37, also known as the SOS response, a complex regulatory network that is induced by DNA-damaging substances (Walker, 1987). The test involves incubation of the bacteria with the sample under investigation and subsequent determination of β-galactosidase (β-gal) activity (i.e. the level of SOS induction). Alkaline phosphatase (Ap) activity is also measured, as a toxicity control.

2.8.1. Bacterial tester strain

Escherichia coli PQ37 strain was kindly provided by Pr. I. Felzen Swalb (Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, RJ, Brazil) and has the genotype *F−thr leu his−4 pyr D thr galE galK lacU169 sRP300::Tn::10 rpoB rpsL uvrA rfa trp::Muc+sfiA::Mud* (*Apy lac*). The construction details of this strain were described by Quillardet and Hofnung (1985). Frozen permanent copies of the tester strain were prepared and stored at −80 °C.

2.8.2. Metabolic activation

The S9 microsome fraction is prepared from the livers of rats treated with Aroclor 1254 (Maron and Ames, 1983). The composition of the activation mixture for the SOS chromotest assay was the following per 10 ml of S9 mix: salt solution (1.65 M potassium chloride (KCl), 0.4 M magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)), 0.2 ml; 1 M glucose-6-phosphate (G6P), 0.05 ml; 0.1 M nicotinamide adenine dinucleotide phosphate (NADP), 0.15 ml; 0.4 M pH 7.4 Tris-buffer, 2.5 ml; Luria broth medium, 6.1 ml; S9 fraction, 1 ml. S9 fraction was used at a concentration of 1230 μg protein/ml of mix. The S9 fraction was stored at −80 °C.

2.8.3. Genotoxic and antigenotoxic assay

The SOS chromotest was employed to determine the effect of the *C. rotundus* extracts on aflatoxin B1 (AFB1: indirect acting mutagen) and nifuroxazide (direct acting mutagen) induced genotoxicity. Genotoxicity and antigenotoxicity assays were performed according to the procedure outlined by Quillardet and Hofnung (1985).

Exponential-phase culture of *E. coli* PQ37 was grown at 37 °C in Luria broth medium (1% bactotryptone, 0.5% yeast extract and 1% NaCl) supplemented with 20 μg/ml ampicillin, then diluted 1:9 into fresh medium. 100 μl aliquots were distributed into glass test tubes containing various doses of *C. rotundus* extracts in a 0.6 ml final volume. The extracts were dissolved in DMSO or distilled water and tested in triplicate, with or without exogenous metabolic activation.

A positive control was prepared by exposure of the bacteria to either nifuroxazide or AFB1. After 2 h of incubation at 37 °C, with shaking, 300 μl samples were used for assaying β-galactosidase (β-gal) and alkaline phosphatase (Ap) activities. In this assay, the β-galactosidase synthesis (*lacZ* gene) is dependent on *sfiA* activation and is used as a measure of SOS repair system induction. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. These enzyme activities were assayed in a Helios Alpha, SpectronicUnicam (Cambridge, England) spectrophotometer.

The SOS induction factor (IF) was calculated as the ratio of R_c/R_0, where R_0 and R_c are equal to (β-gal) activity/alkaline phosphatase (Ap) activity determined respectively in the absence and in the presence of the test compound at a concentration *c*. The IF in treated cells was obtained by comparing β-galactosidase and alkaline phosphatase activities in treated and untreated cells. The result was considered positive when the IF was >2.

In order to evaluate the effect of *C. rotundus* extracts on the SOS response induced by nifuroxazide (in the absence of the S9 activation mixture) and AFB1 (in the presence of the S9 activation mixture), 10 μl of nifuroxazide solution (10 μg/assay) or AFB1 solution (10 μg/assay) were added into tubes containing 10 μl of the extract tested concentration. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity induced by either nifuroxazide or AFB1 according to the formula:

$$\text{Inhibition } (\%) = 100 - (((IF_1 - IF_0) / (IF_2 - IF_0)) \times 100)$$

where IF_1 is the induction factor in the presence of both test compound and mutagen, IF_2 the induction factor in the absence of the test compound and in the presence of mutagen and IF_0 the induction factor of the untreated cells.

2.9. Statistical analysis

Data were collected and expressed as the mean±standard deviation of three independent experiments and IC_{50} values, from the *in vitro* data, were calculated by regression analysis.

3. Results and discussion

This study is designed to evaluate the antioxidant, antigenotoxic and antimicrobial activities of *C. rotundus* extracts against respectively superoxide radical, Aflatoxin B1 and nifuroxazide induced genotoxicity, and five Gram-positive and Gram-negative human pathogenic bacterial strains. The metabolite contents of the extracts as well as their preliminary phytochemical study were also determined.

3.1. Phytochemical study and metabolite content

The phytochemical study of *C. rotundus* extracts showed the presence of various quantities of flavonoids, coumarins and tannins in the aqueous, methanol and TOF enriched extracts. Ethyl acetate extract showed the presence of sterols and flavonoids (Kilani et al., 2005). The metabolite contents of the tested extracts are presented in Table 1.

TOF enriched extract exhibited the most important quantity of flavonoids and total polyphenolic compounds, followed by ethyl acetate, aqueous and methanolic extracts. In fact, 1 mg of TOF enriched extract was equivalent to 670 μg of gallic acid and 340 μg of quercetin. However, 1 mg each of ethyl acetate, methanolic and aqueous extracts were respectively equivalent to 440, 330 and 260 μg of gallic acid and 320, 290 and 200 μg of quercetin. The highest content of tannin was recorded in the

Table 1
Quantitative phytochemical Sceening (%) of extracts from *Cyperus rotundus*.

Metabolites	Extracts			
	Aqueous extract	TOF extract	Ethyl acetate extract	Methanol extract
Polyphenols (gallic acid equivalents)	260±11	670±20	440±12	330±11
Flavonoids (quercetin equivalents)	200±14.5	340±23	320±15	290±13
Tannins (mg/100 g)	59.61±8.5	229±13.25	117.1±8	68.7±6.25
Sterols (%)	–	–	2.75±0.25	–

– Not detectable.

TOF enriched extract with 229 mg/100 g and ethyl acetate extract with 117.1 mg/100 g. In comparison, tannin contents in the methanolic and aqueous extracts were respectively 68.7 and 59.61 mg/100 g. Only ethyl acetate extract contained sterols (2.75%).

Previous phytochemical screening of *C. rotundus* evidenced the presence of flavonoids and tannins (Harborne, 1980; Kilani et al., 2005; Sayed et al., 2001).

3.2. Antimicrobial activity

The antimicrobial activity of the tested *C. rotundus* extracts was evaluated against five reference bacteria. It appears that extracts from aerial parts of *C. rotundus* exhibited various levels of antibacterial effect against the tested bacterial strains. MIC values varied from 0.25 mg/ml to over 5 mg/ml, and MBC values varied from 0.5 to over 5 mg/ml (Tables 2 and 3).

TOF enriched extract displayed strong activity against both Gram-negative and Gram-positive bacteria. *Enterococcus faecalis* was the most susceptible bacterial species to TOF enriched extract, followed by *S. aureus*, *S. typhimurium* and finally *S. enteritidis* and *E. coli,* with MICs of respectively 0.25, 0.5, 0.5, 1 mg/ml and up to 5 mg/ml. In comparison ethyl acetate extract exhibits significant inhibitory effect against *S. aureus, S. enteritidis, E. faecalis* and *S. typhimurium* with the same MIC of 2.5 mg/ml.

In conclusion, ethyl acetate and TOF enriched extracts showed significant antimicrobial activities, whereas no inhibitory effect was exerted by both aqueous and methanolic extracts. This result is not surprising because the active extracts contain high amounts of flavonoids and other polyphenolic compounds. These families of compounds are reported to play a role in the prevention of colonization by parasites, bacteria and fungi (Chiang et al., 2003).

Preliminary screening for antibacterial activity of *C. rotundus* extracts against the tested bacteria revealed such activity with ethyl acetate and TOF enriched extracts. This suggests that compounds such as phenols or flavonoids (phenolic compound contents in active extracts is higher than in the other extracts) may act as antimicrobials (Rodriguez Vaquero et al., 2007; Sakanaka et al., 2000), and indicates that intermediate polarity compounds are active in the antibacterials.

Ethyl acetate and TOF enriched extracts contain higher quantities of flavonoids, tannins and polyphenols than aqueous and methanolic extracts (Table 1). However, their antibacterial activity is somewhat different from that observed with aqueous and methanolic extracts. We deduce that polyphenolic composition of TOF and ethyl acetate extracts is different from that of methanolic extract since they were extracted with solvents having different polarities. Also, the heating and crushing of plant material during the preparation of extracts or decoction may play a role in liberating active compounds from storage sites in plant tissues. Polyphenols such as tannins, flavonoids and phenolics, were the most prominent components of the TOF and ethyl acetate extracts investigated and could contribute to the antimicrobial activity of the *C. rotundus* extracts. In fact, it is known that polyphenols can form heavy soluble complexes with proteins, they can bind to bacterial adhesions, and by doing so disturb the availability of receptors on the cell surface (Haslam, 1996). The literature demonstrates that antibacterial activity can also be due to tannins, which are active compounds of several medicinal plants (Haslam, 1996). Tannins have been shown to form irreversible complexes with proline-rich proteins (Hagerman and Butler, 1981), resulting in the inhibition of cell wall protein synthesis. This property could explain the antibacterial mechanisms of plant extracts. Reports of several *in vitro* assays demonstrate potentially significant interactions with biological systems, such as viruses, bacteria and molluscs, as well as enzyme inhibiting, antioxidant, and radical-scavenging properties (De Bruyne et al., 1999). Their tendency to interfere with biological systems is, at least in part, due to a characteristic ability to form complexes with macromolecules, combined with a polyphenolic nature.

TOF enriched extract showed stronger antibacterial activity than ethyl acetate extract. Our previous studies revealed the presence of coumarins, in TOF enriched extract of *C. rotundus* (Kilani et al., 2005). Thus, it can be deduced that the moderate to good antibacterial activity observed with TOF enriched

Table 2
Antimicrobial activity of *Cyperus rotundus* extracts, expressed as MIC[a].

Extracts	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhimurium*	*Salmonella enteritidis*	*Enterococcus faecalis*
TOF extract	0.5	>5	0.5	1	0.25
Aqueous extract	>5	>5	>5	>5	>5
Ethyl acetate extract	2.5	5	2.5	2.5	2.5
Methanolic extract	5	>5	>5	>5	>5
Ampicillin[b]	0.0015	0.006	0.0039	0.0019	0.0025

[a] Values in mg/ml, means of three experiments.
[b] Positive control.

Table 3
Antimicrobial activity of *Cyperus rotundus* extracts, expressed as MBC[a].

Extracts	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhimurium*	*Salmonella enteritidis*	*Enterococcus faecalis*
TOF extract	1	>5	1	1.25	0.5
Aqueous extract	>5	>5	>5	>5	>5
Ethyl acetate extract	5	>5	5	5	5
Methanolic extract	5	>5	>5	>5	>5
Ampicillin[b]	0.225	0.275	0.26	0.085	0.125

[a] Values in mg/ml, means of three experiments.
[b] Positive control.

extract may be attributed at least in part to coumarins. In fact, the antimicrobial properties of coumarins from other plant species such as *Daphne gnidium* have been reported (Cottiglia et al., 2001).

We conclude that the antibacterial effect of ethyl acetate and TOF enriched extracts corresponds to a synergic effect of different compounds implied in the antibacterial activity in each extract.

In our study, extracts showed no selective activity towards one group of bacteria. It is interesting to note that *C. rotundus* extracts exhibited antimicrobial activity, particularly towards organisms of interest to the medical field such as *Staphylococci*, *Enterococci* and *Salmonella*. Finally, the use of natural products as antibacterials (Conner, 1993; Dorman and Deans, 2000) seems to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food.

3.3. Superoxide radical-scavenging activity

The assay was based on the capacity of different extracts from *C. rotundus* aerial parts to enhance the formation of formazan in comparison to the NBT/riboflavin reference signal. The increase of purple color, typical to formazan was followed spectrophotometrically at 560 nm. Data are collected in Table 4.

TOF, ethyl acetate and methanolic extracts were very potent radical scavengers. These extracts decreased respectively, by 72.1%, 74.34% and 66.5%, NBT photoreduction at a concentration of 10 mg/ml and gave IC_{50} values of 60, 50 and 90 µg/ml, respectively. These extracts were more active than the positive control, quercetin, used in the assay. It seems that this activity is mostly related to the presence of the phenolic compounds such as flavonoids, tannins and coumarins in these extracts.

The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Duenas et al., 2006). Moreover, radical-scavenging activity is one of various mechanisms to contribute overall activity, thereby creating a synergistic effect.

The significant antioxidant effect of TOF, ethyl acetate and methanolic extracts may be ascribed to the presence of high quantities of flavonoids compared to the aqueous extract. However, we cannot, exclude the possibility that other compounds having antioxidant properties participate in the inhibitory effects of the *C. rotundus* extracts. Moreover, the present results support the notion that the protective effect of whole plant extracts is due to the combined and synergistic effects of a complex mixture of phytochemicals, the total activity of which may result in health benefits (Liu, 2004).

Although aqueous extracts contain almost the same quantity of flavonoids as the methanolic extracts, they showed less significant antioxidant effect. We suppose that flavonoid components of aqueous extracts are different from those detected in methanolic extracts. On the other hand, aqueous extracts showed the lowest antioxidant activity compared to the other tested extracts. This may be due to variation in the quality and quantity of different compounds present in the aqueous extracts.

In our study, we have shown that the tested extracts contained total phenolic compounds (as gallic acid equivalents) in the following order: TOF > ethyl acetate > methanolic > aqueous extracts, and total flavonoids (as quercetin equivalents) in the following order: TOF > ethyl acetate > methanolic > aqueous

Table 4
Superoxide anion (O_2^-) radical-scavenging activity of extracts from arial parts of *C. rotundus*[a].

Extracts	Dose µg/assay	% inhibition	CI_{50} (µg/ml)
Ethyl acetate extract	1000	74.34±4.6	50
	300	66.7±3	
	100	56.7±2.8	
	30	47±0.3	
	10	0.7±1.05	
TOF extract	1000	72.1±2	60
	300	64.2±6	
	100	47±2.4	
	30	42.8±9	
	10	35.8±2.4	
Methanolic extract	1000	66.5±3.2	90
	300	50.8±2.2	
	100	50.52±2.1	
	30	38.5±1.5	
	10	12.1±7.5	
Aqueous extract	1000	53±7.1	370
	300	47±0.9	
	100	29.9±7.2	
	30	25.7±1.5	
	10	5±3	
Quercitin[b]	1000	64.96±2	360
	300	34.5±3	
	100	20.1±1.6	
	30	9.17±1.3	
	10	1.38±2.2	

[a] Values expressed as means±SD (n=3).
[b] Positive control.

extracts. The antioxidant order of the TOF and ethyl acetate tested extracts, follows almost their total polyphenol and total flavonoid content. The correlation observed between the activity of extracts and their concentrations, suggests the involvement of active constituents that can scavenge superoxide anions. In fact, polyphenols are considered to be the most active antioxidant derivatives in plants (Bors et al., 2001). However, it has been shown in other studies (Meda et al., 2005) that the phenolic content of some plant extracts does not necessarily correlate with their antioxidant activity. This latter is generally the result of the combined activity of a wide range of compounds, including phenolics, peptides, organic acids and other components (Gallardo et al., 2006).

Regarding to their protective effect against free radical damage, that may be the cause of many diseases including cancer, genotoxic and antigenotoxic activities of *C. rotundus* extracts were invested in the present study.

3.4. Genotoxicity assay

In a series of experiments preceding the antigenotoxic study, it was ascertained that the different amounts of extracts added to the indicator bacteria do not influence its viability. In fact, we tested the effects of different concentrations of extracts on the viability of the bacteria using the disk method. We observed no inhibiting effect of bacterial growth on Luria Broth medium around the disk soaked with the tested extract, up to 500 μg/assay.

The results of the genotoxicity test are shown in Table 5. Experiments realized with aqueous, ethyl acetate, methanolic and TOF extracts prepared from *C. rotundus* aerial parts, revealed no effect on the induction factor at the different tested concentrations. In fact, according to Kevekordes et al. (1998), compounds are classified as non-genotoxic if the induction factor remains <1.5, as not conclusive if the induction factor ranges between 1.5 and 2 and as genotoxic if the IF exceeds 2. Based on this, the four extracts were found to be non genotoxic as well as with or without metabolic activation system. The absence of genotoxicity of the different tested extracts indicates that DNA does not seem to be a relevant target for these extracts. In fact, extracts should not produce DNA lesions that block DNA synthesis, leading to the induction of SOS system. However, we believe that extracts exhibiting IF values around 0.5 and alkaline phosphatase activities in the same range of that obtained with the negative control may reflect a protective effect against spontaneous mutagenicity by stimulating SOS DNA repair. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. Whereas, extracts exhibiting low IF values and low alkaline phosphatase activities should be slightly toxic against E. Coli PQ37 at a given concentration (500 μg/assay with TOF extract) but not above, this could be indicative of the presence of hormesis. The absence of genotoxicity is not a characteristic of all natural products in use, since other medicinal plants assayed with the SOS chromotest in the presence or not of the S9 metabolisation system, have resulted positive for genotoxicity (De Carvalho et al., 2003).

3.5. Antigenotoxicity assay

Doses of 10 μg/assay of nifuroxazide (directly acting mutagen), and 10 μg/assay of AFB1 (indirectly acting mutagen) were chosen for the antigenotoxicity studies, since these doses were not toxic, and since they induced a significant SOS response.

The inhibitory effects of the tested extracts on the genotoxicity induced by AFB1 using the SOS chromotest are presented in Table 6. Increased concentrations of TOF and ethyl acetate extracts decreased AFB1-induced genotoxicity. Indeed, the highest inhibition percentages of genotoxicity obtained with the above-mentioned extracts were respectively 87.16% (at a concentration of 200 μg/assay) and 73.68% (at 500 μg/assay). In contrast, aqueous and methanolic extracts showed a relatively weak efficiency in reducing AFB1-induced genotoxicity, the IF of this mutagen was decreased respectively about 43.11% and 46.19% at the same concentration of 500 μg/assay. The four

Table 5
Genotoxicity induced by *C. rotundus* extracts in the absence and presence of the exogenous metabolic activation mixture (S9)[a].

Extract	Dose μg/assay	−S9			+S9		
		β gal (UE)	AP (UE)	IF	β gal (UE)	AP (UE)	IF
NC	0	5±0.02	22.03±0.01	1	9.54±0.002	12.58±0.007	1
Aqueous extract	50	3.91±0.002	23.8±0.0005	0.72	9.42±0.004	12.5±0.003	1
	200	4.2±0.007	22±0.002	0.86	9.85±0.007	14±0.02	0.93
	500	7.32±0.02	24.63±0.001	1.09	9.48±0.01	13.5±0.002	0.94
TOF enriched extract	50	4.12±0.002	22.07±0.017	0.72	5.3±0.02	8±0.01	0.72
	200	2.43±0.004	20.53±0.02	0.52	3.86±0.007	4.35±0.009	1.02
	500	2.25±0.01	19.07±0.02	0.48	2.31±0.001	5.25±0.02	0.5
Ethyl acetate extract	50	5.34±0.01	24.6±0.02	0.98	6.48±0.004	11.91±0.005	0.72
	200	6±0.015	30.26±0.01	0.9	6.85±0.009	14.91±0.003	0.61
	500	6.45±0.005	30.06±0.02	0.97	8.37±0.002	21.25±0.04	0.52
Methanolic extract	50	4.57±0	24.06±0.006	0.86	5.91±0.005	14.16±0.002	0.55
	200	4.62±0.01	25.73±0.02	0.81	6.57±0.01	13.66±0.003	0.64
	500	5.37±0.007	31.53±0.02	0.77	6±0.005	12.25±0.01	0.65

NC: negative control; UE: enzymatic unit; ß-gal: ß galactosidase; Ap: alkaline phosphatase; IF: Induction factor.
[a] Values expressed as means±SD (n=3).

Table 6
Inhibition of AFB1 (10 µg/assay)-induced mutagenicity in *E. Coli* PQ37 assay system, in the presence of metabolic activation (S9), using the SOS chromotest.

Extract	Dose µg/assay	ß gal (UE)	Ap (UE)	IF	% of inhibition
NC	0	5.8±0.02	7.62±0.006	1	–
Aflatoxin B1	10	47.88±0.03	5.43±0.01	11.6	–
Aqueous extract	50	41.38±0.01	3.72±0.01	14.63	−28.5
	200	32±0.01	4.65±0.01	9.05	24.05
	500	33.27±0.01	6.22±0.007	7.03	43.11
TOF enriched extract	10	48.5±0.05	6.06±0	10.53	10.09
	50	32.11±0.01	12.44±0.02	3.39	77.45
	200	7.05±0.006	3.93±0.02	2.36	87.16
Ethyl acetate extract	50	27.22±0.005	6.58±0.006	5.44	58
	200	23.91±0.01	6.84±0.01	4.59	66
	500	19.66±0.001	6.81±0.002	3.78	73.68
Methanolic extract	50	29.77±0.008	4.82±0.005	8.10	33.01
	200	25.11±0.004	4.63±0.005	7.11	42.35
	500	24.55±0.001	4.82±0.01	6.70	46.19

NC: negative control; UE: enzymatic unit; ß-gal: ß galactosidase; Ap: alkaline phosphatase; IF: Induction factor.

tested extracts induced a decrease of mutagen genotoxicity as a function of extract concentration. The weak antigenotoxic activity of aqueous and methanolic extracts when large excess of each one was added, to the assay system, could be explained by the inhibition of the penetration through the cell membrane at the high doses, of extracts or molecules which are implied in the mutagenic inhibitory effect towards AFB1 (Bouhlel et al., 2007).

Induction of SOS response with nifuroxazide, was also affected by the four tested extracts. As shown in Table 7, the IF of nifuroxazide decreased by 51.13%, 68.91%, 53.37% and 68.21% in the presence of 500 µg/assay of aqueous, ethyl acetate and methanolic extracts, and 200 µg/assay of TOF enriched extract, respectively. This study showed that, TOF enriched extract was the most effective against nifuroxazide induced genotoxicity, while aqueous extract was the weakest one. In fact, we can notice that all the tested *C. rotundus* extracts induced a decrease of nifuroxazide genotoxicity in a dose response manner.

Anti-genotoxic activity of the tested extracts may be ascribed to flavonoids (Calomme et al., 1996), coumarins (Choi et al., 1997) and tannins (Baratto et al., 2006; Lee et al., 2003) detected in aqueous, TOF, ethyl acetate and methanol extracts. Besides, all the tested extracts showed more or less important antigenotoxicity towards AFB1 (10 µg/assay) as well as nifuroxazide (10 µg/assay). This suggests that these extracts inhibit microsomal activation or that they directly protect DNA strands from the electrophilic metabolite of the mutagen. They may inhibit several metabolic intermediates and reactive oxygen species (ROS) formed during the process of microsomal enzyme activation which are capable of breaking DNA strands. The observed protective effect of aqueous, TOF, ethyl acetate and methanol extracts towards nifuroxazide and AFB1, may correspond to a synergic participation of several components in each extract. In comparison, sterols which are the main constituents of ethyl acetate extract, seem to be the most likely candidates for providing the antigenotoxic activity of this extract. However, we cannot exclude the possibility that other compounds, participate in the anti-genotoxic effect of *C. rotundus* extracts. On the other hand, TOF and ethyl acetate extracts from *C. rotundus* inhibited more strongly AFB1-induced genotoxicity than nifuroxazide, suggesting that these extracts did necessitate metabolic activation to give better antigenotoxicity effect. Besides, the highest antigenotoxic activities were observed with TOF and ethyl acetate extracts against nifuroxazide as well as AFB1. This result could be explained by the presence of active compounds diluted and/or masked by various other components in the whole crude aqueous and methanolic extracts and which are more accessible in the TOF and ethyl acetate extracts.

In conclusion, the present study has demonstrated that *C. rotundus* extracts possess potent antimicrobial, antioxidant and antigenotoxic activities, which could be derived from compounds such as flavonoids and phenols. The antigenotoxic activity could be ascribed, at least in part, to their antioxidant properties but we cannot exclude other additional mechanisms. Results we obtained in this work with SOS chromotest are in accordance with those revealed with some strains used in another DNA damage detecting assay, called the Ames assay, which we performed previously with the same plant extracts. Nevertheless, we believe that further studies will be required to fully investigate the *in vitro* and *in vivo* antigenotoxic activities of *C. rotundus* extracts in mammalian cells and animal experiments using different tests as: (i) micronuclei, (ii) chromosome aberrations and (iii) comet assay data…. Antimicrobial, antioxidant and antigenotoxic activities exhibited by the tested extracts should have contributed, at least partly, to the therapeutic benefits of certain traditional claims. Furthermore, *C. rotundus* extracts may give rise to anticarcinogenic agents and could be promising candidates for further studies designed to obtain more evidence on their potential chemopreventive activity.

Table 7
Inhibition of nifuroxazide (10 µg/assay)-induced mutagenicity in *E. Coli* PQ37 assay system, in the absence of metabolic activation (S9), using the SOS chromotest.

Extract	Dose µg/assay	ß gal (UE)	Ap (UE)	IF	Inhibition of percentage (%)
NC	0	3.89±0.001	21.53±0.007	1	–
Nifuroxazide	10	14.68±0.01	9.1±0.007	8.96	–
Aqueous extract	50	15.66±0.03	12.71±0.007	6.84	26.6
	200	15.83±0.05	13.71±0.01	6.41	32.03
	500	10.95±0.009	12.42±0.005	4.89	51.13
TOF enriched extract	10	10.70±0.001	10.21±0.001	5.82	39.41
	50	9.18±0.0005	9.96±0.005	5.11	48.36
	200	6.16±0.01	9.67±0.06	3.53	68.21
Ethyl acetate extract	50	6.42±0.01	8.46±0.08	3.99	53.06
	200	4.67±0.01	7.70±0.008	3.19	65.62
	500	6.29±0.01	11.1±0.01	2.98	68.91
Methanolic extract	50	13.46±0.02	12.26±0.01	5.77	25.11
	200	10.84±0.008	10.03±0.01	5.68	26.53
	500	9.04±0.06	11.96±0.01	3.87	53.37

NC: negative control; UE: enzymatic unit; ß-gal: ß galactosidase; Ap: alkaline phosphatase; IF: Induction factor.

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