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RESEARCH ARTICLE

Composition & biological activity of *Cyperus rotundus* L. tuber volatiles from Saudi Arabia

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Abstract

The present study was conducted to investigate the composition of the oil of *Cyperus rotundus* L. rhizomes collected in Saudi Arabia, and to evaluate *in vitro* cytotoxic, antimicrobial and mosquito mortality activities of both the oil and the alcoholic extract of the aforementioned plant. Nineteen compounds were identified by GC-FID and GC-MS, of which α-cyperone (21.1%) and 4-oxo-α-ylangene (12.8%) were the major components. The essential oil recovered with diethyl ether demonstrated potent cytotoxic activities against colon (HCT-116), hepatocellular (Hep G-2) and breast (MCF-7) human cancer cell lines with IC_{50}s of 1.06, 1.17 and 2.22 µg/mL, respectively. This oil also showed a comparable zone of inhibition against the gram positive bacteria *Staphylococcus epidermidis* and the gram negative bacteria, *Klebsiella pneumoniae*, *Shigella flexneri* and *Salmonella enteritidis* when compared to the standard drugs ampicillin and gentamycin. The ethanolic extract showed moderate activity against human cancer cells and pathogens. The essential oil was significantly more toxic to 1st instar *Aedes aegypti* larvae than the ethanol extract; however, the ethanol extract was more toxic to adult *Ae. aegypti* than the essential oil. Based on the results obtained, *C. rotundus* collected from Riyadh city could provide a potential and cheap source of biologically active compounds.

Keywords: *Cyperus rotundus*, Essential oil, GC-MS, Cytotoxicity, Antimicrobial, Larvicidal, Adulticidal, *Aedes aegypti*

Introduction

Cyperus rotundus L., (Cyperaceae), is a perennial glabrous herb commonly known as Nut Sedge, Purple Nut Sedge or as Nabtat Alsa'ad in Arabic. It is a highly invasive weed, widely distributed in tropical, subtropical, and temperate regions around the world (Parsons & Cuthbertson, 1992). The bulbous roots (tubers) store starch as a food reserve and give rise to new rhizomes. The tubers are around 1- 3.5 cm in length, reddish white inside and brownish black externally (Aghassi et al., 2013).The presence of *C. rotundus* in a field considerably decreases crop yield due to its strong competition for ground resources, and because the roots of *C. rotundus* release substances that are harmful to other plants ''allelopathic'' (El-Rokiek et al., 2010). Despite the allelopathic effects, the tubers have been used as an occasional nutrient source and have a long history in traditional medicine. Historically, Arabs used roasted *C. rotundus* tubers or hot ashes from burned tubers, to treat wounds, bruises, carbuncles, and other related complaints (Imam et al., 2014). Ayurvedic and Islamic herbal medicine practitioners have described *C. rotundus* tubers to treat uterine disorders, fever, delayed menstruation and dysmenorrhea, removal of obstructions and as stomachic and emollient plasters

(Mohsin et al., 1989; Pirzada et al., 2015). The tubers of *C. rotundus* are used in traditional Chinese medicine as an antidiarrheal, antidepressant, analgesic, antiinflammatory and antiemetic remedy for dysentery and women's diseases (Chen et al., 2014; Oh et al., 2015).

The results of a clinical study involved ninety one female volunteers suffering from androgenic hair and supported the traditional use of topical Egyptian *C. rotundus* essential oil for treatment of moderate degrees of hirsutism and axillary hair. The hair growth was effectively and safely decreased with no effect on serum testosterone (Mohammed, 2014). Furthermore, compounds isolated from *C. rotundus* tubers showed significant antidepressant (Zhou et al., 2016), anti-hepatitis B virus (Xu et al., 2015), antimalarial (Weenen et al., 1990), antidiarrheal (Uddin et al., 2006) and antioxidant activities (Yazdanparast & Ardestani, 2007).

Plant extracts as well as their essential oils have been the major source of natural products with potential biological importance to be used as alternative remedies for the treatment of infectious diseases (Hemaiswarya et al., 2008). Concerns about the toxicity of many synthetic insecticides and the development of resistance in insects are great impetus for the development of alternative insecticides and repellents from isolated natural products. Novel natural products can help manage disease vectors such as *Aedes aegypti* (L.), which transmits the pathogens of many diseases such as yellow fever, dengue, and zika virus (Campos, et al., 2015; Benelli & Mehlhorn, 2016). We conducted this study with the aim of finding naturally derived, safe, and easily obtainable substituents to be used medically for treating contemporary diseases. To the best of our knowledge, this is the first report evaluating the chemical composition and biological activities of the essential oil (EO) *C. rotundus* tubers growing in Saudi Arabia.

Materials and Methods

Plant Material

The fresh tubers of *Cyperus rotundus* Linn. were collected in March 2016 from a farm near Riyadh city. A voucher specimen (No. 19010) has been deposited in the Herbarium of the Department of Pharmacognosy, King Saud University.

Essential Oil Isolation and Extract Preparation

The fresh tubers (300 g) were crushed, directly immersed in water and hydrodistilled for 6-7 hrs using Clevenger apparatus. The oil was obtained by extraction with diethyl ether from the aqueous distillate, dried over anhydrous Na_2SO_4, and the ethereal layer was finally evaporated at room temperature. In parallel, the air dried tubers (100 g) were powdered and extracted with 85% ethanol by cold maceration until exhaustion. The ethanol extract was evaporated using a rotary evaporator to give a dark residue (2 g).

GC–MS Analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

GC Analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty & Stauffer, 1989; Koenig et al., 2004) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Joulain & Koenig, 1998; ESO 1999), was used for the identification.

Cytotoxic Activity

The mammalian cell lines HepG-2 (human liver cancer), HCT-116 (human colon cancer), and MCF-7 cells (human breast cancer) were obtained from the ATCC. The cells were propagated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St Louis, MO, USA), 1% L-glutamine, HEPES buffer, and 50 µg/mL gentamicin (Sigma Chemical Co., St Louis, MO, USA).

Cytotoxic activity was evaluated by the crystal violet staining (CVS) method in a three cell line-single concentration (50 mg/mL) anticancer assay by adapting the method described by Itagaki et al.-(Itagaki et al., 1991). Treated samples were compared with the negative cell control. All the experiments were carried out in triplicate. The standard antitumor drug used was doxorubicin.

Antimicrobial Activity

Antimicrobial tests were carried out by agar well diffusion according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria. Bacterial and fungal suspensions were prepared and cultivated on Mueller-Hinton agar and Sabouraud dextrose media punched with 6-mm diameter wells. Then, 100 µL of 10% of tested samples were added to the wells, with 10% DMSO used as the negative control. Amphotericin B, ampicillin and gentamicin (30 µg/mL) were used as standard agents against fungi, Gram-positive bacteria, and Gram-negative bacteria, respectively. After incubation of the plates at 37 °C for 18 to 24 h, the antimicrobial activity was evaluated by measuring the diameter of the inhibition zones. Each test was performed in triplicate and the average of the results calculated. The extraction solvents were used as negative controls (NCCLS, 2004).

Mosquito Activity

Mosquito colony: Aedes aegypti L. used for testing were pesticide susceptible and provided from the CMAVE insectary. The "Orlando" strain was collected near Orlando, Florida, USA in 1952 and has been in continuous laboratory colony for 64 years. Rearing procedures are standardized and have been described previously (Meepagala et al., 2015).

Larvicidal Activity Assay

Larvicidal activity testing was performed essentially as described previously, except with the noted exceptions (Meepagala et al., 2015). Embryonated eggs are hatched overnight from oviposition papers in

approximately 200 mL of deionized water with approximately 40 mg of a finely ground mixture (1:1) of alfalfa powder and pig chow. The next day, five first instar larva were gently aspirated by pipette in 180 µL of liquid and transferred to one well of a 96-well flat bottom tissue culture plate. The larva are provided with 10 µL of the supernatant from a 2% solution of 1:1 alfalfa powder: pig chow to stimulate natural feeding. Two microliters of a specific compound diluted to 100 µg/mL in ethanol or DMSO was added to the well and mixed gently with the larva. Further dilutions of each compound were made by addition of 1 µL, 0.5 µL, or 0.2 µL of stock into other wells. For each assay, a positive control of 41.36 ng/ml permethrin stock and a negative control of ethanol or DMSO was included. Assays were repeated at least three times on separate days using different hatches of eggs.

Adult Topical Activity

The toxicity of compounds was tested in assays against adult *Aedes aegypti* using cohorts of 3-6 day post-emergence females as described previously (Meepagala et al., 2015). Mosquitoes were cold anesthetized on ice and groups of 10 females sorted into individual plastic cups. Application of 0.5 µL of the appropriate dilution of the test chemical was made by repeater pipettor (Hamilton PB600) with a 25 µL blunt tip glass syringe (Hamilton 7100 series) to at least twenty females at each dose. Controls again included a solvent only negative and doses of 0.19 ng/organism and 0.86 ng/organism of permethrin as positive controls. After treatment, cohorts had access to a cotton ball saturated with 10% sucrose. Mortality was scored at 24 hours after application and a mosquito unable to right itself was scored as dead. The LD_{50} of the ORL strain was approximately 0.15 ng/organism, so a valid assay required complete mortality at 0.86 ng/org and approximately 50% mortality at 0.19 ng/organism in the Orlando strain positive controls and <10% mortality in the negative control. There was complete mortality in the higher permethrin dose and 60±10% mortality at the dose slightly above the LD_{50}. Assays were repeated at least three times on different days.

Results and Discussion

Oil Composition

Steam distillation of *C. rotundus* tubers yielded 0.2% of yellowish oil with a strong aromatic odor. The analysis of the essential oil by GC-MS allowed the identification of 17 compounds accounting for 55.0% of the total composition of oil. The oil composition is displayed in Table 1 and constituents are listed in order of their elution. Sesquiterpenes (41.2 %) and oxygenated monoterpenes (13.8 %) were the chief classes of identified compounds. The major compounds identified were the sesquiterpenes α-cyperone (21.1%), 4-oxo-α-ylangene (12.8%) and caryophyllene oxide (3.5%); as well as the monoterpene *trans*-pinocarveol (3.6%). The α-cyperone (11.0%) was also the major compound detected in the South African *C. rotundus* tubers essential oil (Lawal & Oyedeji, 2009). Cyperene and cyperotundone were the major compounds reported from *C. rotundus* tubers oil in the South of Tunisia as well as in Iran. In our current study, cyperene was found in minor amounts (0.3%), while cyperotundone was not detected (Lawal & Oyedeji, 2009; Aghassi et al., 2013). On the other hand, 4-oxo-α-ylangene was reported as one of the constituents in *C. rotundus* oil samples analyzed in Egypt and South Africa. However, in both studies, they were reported in smaller amounts (9.35 and 1.9 %) than those found in our study (12.8%) (El-Gohary, 2004; Lawal & Oyedeji, 2009).

Table 1. The composition of the essential oil of *Cyperus rotundus* L. rhizomes

RRI	Compound	%
1544	Cyperene	0.3
1586	Pinocarvone	0.2
1648	Myrtenal	1.7
1670	*trans*-Pinocarveol	3.6
1683	*trans*-Verbenol	1.5
1706	α-Terpineol	0.8
1725	Verbenone	1.7
1751	Carvone	0.1
1804	Myrtenol	2.8
1845	*trans*-Carveol	0.5
1864	*p*-Cymen-8-ol	0.9
1900	*epi*-Cubebol	0.4
1957	Cubebol	0.5
2008	Caryophyllene oxide	3.5
2071	Humulene epoxide-II	2.6
2289	4-oxo-α-Ylangene	12.8
2304	α-Cyperone	21.1
	Total	55.0

RRI: Relative retention indices calculated against n-alkanes, with % calculated from FID data

Cytotoxic Activity

Cytotoxic activity was determined for the total extract of *C. rotundus* tubers, as well as the essential oil against HepG-2, HCT-116 and MCF-7 carcinoma cell lines, using the CVS method employing doxorubicin as reference drug. The response parameter (IC_{50}) was calculated for each cell line. From the results shown (Table 2), both samples possessed a dose-dependent cytotoxic effect against the three cell lines. However, the volatile oil sample showed remarkable cytotoxic activity against all the cell lines, with IC_{50} values ranging from 1.06 to 2.22 µg/mL while the total alcoholic extract showed less selective cytotoxic activity (IC_{50} = 38.3–48.5 µg/mL) in comparison with doxorubicin (IC_{50} = 0.55–0.67 µg/mL). Several previous studies have reported equally remarkable cytotoxic and apoptotic activities for the oil of *C. rotundus* (Kilani et al., 2008a; Kilani et al., 2008b; Kumar & Khanum, 2013; Kilani et al., 2014).

Table 2. Cytotoxic activity of *C. rotundus* EO and total extract against three cancer cell lines

Sample concentration (µg/mL)		100	50	25	12.5	6.25	3.125	1.56	IC_{50}[2] µg/mL
Tumor cell line		% Inhibition[1]							
Hep G-2	EO	96.06±0.19	92.40±0.44	88.21±0.56	82.94±2.28	75.26±3.44	67.76±3.86	56.66±3.86	1.17
	Total extract	63.41±2.85	55.16±2.74	36.71±2.47	16.96±2.67	5.15±1.80	0.42±0.73	NT	43
	Doxorubicin	95.79±0.53	90.98±0.97	86.36±1.13	81.24±1.60	75.10±1.35	67.56±1.85	60.81±0.91	0.67
HCT-116	EO	95.96±0.46	92.72±0.90	90.06±1.60	85.14±3.57	80.00±1.60	75.16±1.30	61.01±1.95	1.06
	Total extract	68.09±2.85	57.17±2.65	41.90±3.45	28.94±3.10	16.34±3.10	7.21±1.47	NT	38.3
	Doxorubicin	96.21±0.28	93.67±0.44	89.38±1.29	82.64±0.77	76.89±1.27	71.19±1.81	65.13±1.75	0.55
MCF-7	EO	94.42±1.09	87.12±1.13	80.69±1.46	73.15±1.08	65.13±1.29	57.88±2.18	44.29±3.99	2.22
	Total extract	63.81±2.73	50.70±3.84	39.40±3.71	21.89±2.43	11.30±2.65	4.14±2.55	NT	48.5
	Doxorubicin	95.74±0.26	94.51±0.13	90.38±0.58	83.86±0.16	75.23±6.73	71.39±0.45	64.20±1.10	0.62

[1] The percent of cell survival inhibition at 50 µg/mL, compared to control; [2] IC_{50s} are expressed in (µg/mL); NT, not tested; $p < 0.01$, compared to reference drug.

Antimicrobial Activity

The antimicrobial activities of *C. rotundus* essential oil and the total ethanolic extract were evaluated by determining their zone of inhibition against four fungi, four Gram-positive bacteria and four Gram-negative bacteria (Table 3). The volatile oil exhibited noticeable *in vivo* antifungal efficacy against the pathogenic fungi *Absidia corymbifera*, *Geotricum candidum* and *Candida albicans* with 21.0, 24.0 and 23.0 mm zones of inhibition diameter, respectively. These results compared favorably to the standard drug amphotericin with 23, 27 and 26 mm zones of inhibition diameter, respectively. On the other hand, the maximum antimicrobial activity for the essential oil against the Gram positive bacteria *Staphylococcus epidermidis* (25±0.11 mm zone of inhibition diameter) and *Streptococcus pyogenes* (26.7±0.17 mm zone of inhibition diameter); and the Gram negative bacteria *Klebsiella pneumoniae* (26±0.33 mm zone of inhibition diameter) and *Salmonella enteritidis* (25.4±0.25 mm zone of inhibition diameter) showed results comparable to the standard drugs ampicillin and gentamycin.

These findings are in agreement with previous antimicrobial activity studies on volatile oil of *C. rotundus* tubers, which demonstrated inhibitory activity against a large number of microorganisms such as *S. aureus, S. pyogenes, E. coli, P. vulgaris, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Uddin et al., 2006; Pirzada et al., 2015). On the other hand, the total extract was moderately active against most of the tested organisms, but it was inactive against *Listeria innocua* and *Proteous vulgaris*.

Table 3. Antimicrobial activities (zone of Inhibition, mm) of *C. rotundus* EO and total extract against selected clinical pathogens

Tested microorganisms Fungi	**Test sample**	**Zone of Inhibition (mm) ±SD**	**Amphotericin B**
Absidia corymbifera (RCMB 02564)	EO	21±0.14	23±0.33
	Total extract	15±0.32	
Trichophyton mentagrophytes (RCMB 0925)	EO	18.67±0.11	24±0.24
	Total extract	17.34±0.12	
Geotricum candidum (RCMB 05097)	EO	24±0.34	27±0.22
	Total extract	21±0.14	
Candida albicans (RCMB 05036)	EO	23±0.27	26±0.18
	Total extract	20±0.23	
Gram Positive Bacteria			**Ampicillin**
Staphylococcus aureus (RCMB 010027)	EO	22.7±0.14	27.4±0.15
	Total extract	20.33±0.31	
Staphylococcus epidermidis (RCMB 010024)	EO	25±0.11	25±0.22
	Total extract	21.7±0.32	
Streptococcus pyogenes (RCMB 010015)	EO	26.7±0.17	26.4±0.25
	Total extract	20±0.10	
Listeria innocua (RCMB 010052)	EO	18±0.15	22.3±0.10
	Total extract	NA	
Gram Negative Bacteria			Gentamycin
Proteous vulgaris (RCMB 010085)	EO	16.4±0.18	23.3±0.26
	Total extract	NA	
Klebsiella pneumoniae (RCMB 0010093)	EO	26±0.33	26.67±0.31
	Total extract	16±0.16	
Shigella flexneri (RCMB 0100542)	EO	25±0.35	27±0.26
	Total extract	13.7± 0.19	
Salmonella enteritidis (RCMB 010084)	EO	25.4±0.25	25.3±0.17
	Total extract	20±0.31	

Data are expressed in the form of mean ± SD.

Larvicidal and Adulticidal Activity

Bioassay of the essential oil and total alcohol extract from *C. rotundus* was conducted on 1st instar permethrin-susceptible *Ae. aegypti* "Orlando" strain larvae and adult females (Table 4). In the larval assay, the essential oil produced 100% mortality at 1, 0.5, 0.25 and 0.1 μg/μL, whereas the ethanol extract was slightly less active. It produced 100% mortality at 1 and 0.5 μg/μL, 93% mortality at 0.25 μg/μL and significantly less mortality at 0.1 μg/μL. However, the ethanol extract had slightly higher toxicity than the essential oil in adult topical assays.

Table 4. Adulticidal and larvicidal activities of *C. rotundus* EO and total extract

samples	Adult	Larval			
		Dose (μg/μL)% mortality			
	5 μg/mosq.	1	0.5	0.25	0.1
EO	90 ± 10	100	100	100	100
Total extract	96.7 ± 5.8	100	100	93.3 ± 11.5	33.3 ± 23.1

Positive control permethrin at 41.36ppb resulted in 100% mortality and negative solvent control (DMSO) had 0% mortality for larval bioassays. In adult bioassays, permethrin at 0.86 ng/org resulted in 100% mortality. The acetone and untreated controls both had an average mortality of 6.7 ± 5.8 percent

Conclusion

The essential oil from *C. rotundus,* obtained from the Riyadh region, revealed some variations in the composition and percentage of compounds when compared with other *C. rotundus* essential oils from different areas around the world. This demonstrates chemical diversity within the *C. rotundus* species possibly due to chemotypicity, geographic location, harvesting period, and storage time. The essential oil of *C. rotundus* showed remarkable cytotoxic and strong antimicrobial activities against the tested cancer cell lines and microbial strains. This plant might provide a potentially available and cheap source of antibiotics and anticancer compounds and should be considered for further phytochemical investigation and pharmacological evaluation. The 1st instar *Ae. aegypti* larvae activity showed 100% mortality at the lowest dosage in initial screening assays. Further bioassay-guided studies are planned to follow up on compounds of significant interest. To the best of our knowledge, the current study highlights the chemical composition and biological activities of the essential oil and alcoholic extract of *C. rotundus* rhizomes growing in Saudi Arabia for the first time.

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