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Evaluation of antibacterial effect of *Cyperus* species on typical food-borne pathogens

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ABSTRACT

Objective: To evaluate the antibacterial effect of *Cyperus* species as an important medicine plant in the study area.

Methods: The agar disk diffusion method was used to study the antibacterial activity of *Cyperus* extracts against 2 Gram-positive and 4 Gram-negative bacteria at concentration 400 and 600 mg/mL of methanol and the aqueous extract. Then minimum inhibitory concentrations and minimum bactericidal concentrations were determined by micro-dilution method.

Results: The results showed that the methanol extract of longus species presented the highest zone of inhibition against tested pathogens (15 mm inhibition zone). Other plants did not show significant inhibition zone. The methanol extracts of the plant against *Pseudomonas aeruginosa* (PTTC 1707) strains showed the best activities, with the lowest minimal inhibitory concentration of 3.125 mg/mL and minimum bactericidal concentration was 166.50 and 83.25 mg/mL, respectively for *Staphylococcus aureus* (PTTC 1431) and *Escherichia coli* (PTTC 1399).

Conclusions: The results showed that the methanol extract of the herb has antibacterial activity and therefore it could be used as a natural preservative ingredient in food and/or pharmaceutical industries.

1. Introduction

Since ancient times, all the aromatic and medicinal plants available worldwide have been used for their preservation and medicinal values, as well as to impart flavor to food and medicine formulations. Recently, there has been growing considerable interest in crude extracts and essential oils of medicinal and edible plants, herbs, vegetables and spices for the development of alternative food additives, in order to prevent the growth of food-borne pathogens or to delay the onset of food spoilage. The antimicrobial effects of aromatic oils extracted from a large number of plants have been evaluated and reviewed by many researcher.

Cyperus is a large genus of about 700 species of sedges, distributed throughout all continents in both tropical and temperate regions. Several *Cyperus* species are available worldwide and it is also available in some provinces of Iran like North Khorasan. Longus, difformis and rotundus are three important species of

Cyperus plant that belong to the family Cyperaceae and commonly known as weeds especially in rice fields and among them *Cyperus longus* (*C. longus*) and *Cyperus difformis* (*C. difformis*) are more common as herbal medicine between Iranian people. It is a multipurpose plant, widely used in traditional medicine around the world to treat stomach ailments, wounds, boils and blisters[1,2]. A number of pharmacological and biological activities including anti-candida, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, antioxidant, cytotoxic and apoptotic, anti-pyretic and analgesic activities have been reported for this plant[3-5]. Previous phytochemical studies on *Cyperus rotundus* (*C. rotundus*) revealed the presence of alkaloids, flavonoids, tannins, starch, glycosides and furochromones, and many novel sesquiterpenoids. In Asian countries, the rhizomes of *Cyperus* species are used as traditional folk medicines for the treatment of stomach and bowel disorders, and inflammatory diseases. *Cyperus* species (longus and difformis) are traditional herbal medicine used widely as analgesic, sedative, antispasmodic, antimalarial, stomach disorders and to relieve diarrhoea. They are used in the treatment of nausea and vomiting, dyspepsia, colic, flatulence, diarrhoea, dysentery, intestinal parasites, fever, malaria, cough, bronchitis, renal and vesical calculi, urinary tenesmus, skin diseases, wounds, amenorrhoea, dysmenorrhoea, deficient lactation, loss of memory, insect bites, food poisoning, indigestion, nausea,

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dysuria, bronchitis, infertility, cervical cancer and menstrual disorders, and the aromatic oils are made of perfumes and splash. Antibacterial activity of the extract of *C. rotundus* showed a remarkable activity against Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis*.

Another study stated that a marked inhibitory effect of *C. rotundus* was observed against *Salmonella enteritidis*, *S. aureus* and *Enterococcus faecalis* with total oligomers flavonoids and ethyl acetate extracts.

The most literature available reported the identification, characterization and determination of chemical compositions and antimicrobial properties of the essential oil or extracts of *C. rotundus* that is native to India. However, according to our knowledge, the fungi, yeasts, as well as pathogenic and saprophytic bacteria of the extracts obtained from *C. longus* and *C. difformis* have never been studied before by the scientist or researcher. It is also known that the antimicrobial effects of essential oils and the extracts of *Cyperus* herb and other medicinal plants may be subjected to change based on the variations in the chemical composition of an essential oil or extract which may be observed due to the geographical distribution, origin, the locality, the climate conditions, process, and the harvest time of the collected plant material. Thus the aim of this article was to study the antibacterial effect of *C. longus* and *C. difformis* that are native to Iran.

2. Materials and methods

2.1. Chemicals and plant materials

Gentamicin (Sinadaroo, Iran), methanol, ethanol and dimethyl sulfoxide (DMSO), Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) (Merck, Germany) were prepared. The aerial part (leaves) of *C. longus* and *C. difformis* species was collected in May 2016 from the mountains of North Khorasan Province in Iran. The plants were identified by the Research Center of Natural Products Health, North Khorasan University of Medical Sciences (Iran).

2.2. Preparation of extract

The plants leaves were dried at room temperature under shade[6], finely ground with a hammer mill, and the powdered sample from each plant was extracted with methanol (Merck, Germany) and distilled water (1.5 L) for 48 h at room temperature[7]. The extracts were filtered through filter paper, afterwards were dried in vacuum at 40 °C[8] and kept at 4 °C until further uses[9].

2.3. Organisms and inoculation conditions

Pure cultures of bacteria were obtained from the Persian Type Culture Collection (PTCC). They included Gram-positive bacteria: *Listeria monocytogenes* (*L. monocytogenes*) (PTCC 1304), *S. aureus* (PTCC 1431) and Gram-negative bacteria: *Salmonella enterica* (*S. enterica*) (PTCC 1709), *Escherichia coli* (*E. coli*) (PTCC 1399), *Pseudomonas aeruginosa* (*P. aeruginosa*) (PTCC 1707), *Campylobacter jejuni* (*C. jejuni*) (PTCC 1015). They were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar plate for 24 h prior to any antimicrobial test. MHA was used for the activation of bacteria and the MHB was used for

the minimum inhibitory concentration (MIC) determinations[10]. Finally, suspensions were adjusted to 0.5 McFarland standard turbidity. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU/mL (Library of Congress Cataloging-in-Publication Data, 2005).

2.4. Antimicrobial assay

The disk diffusion and micro-dilution methods were used for determining antibacterial activity of the extracts and fractions. All tests were performed in three replicate.

2.5. Disk diffusion method

The agar diffusion assay was performed according to the modified Kirby-Bauer disc diffusion method [11]. The extracts (methanolic and aqueous of two species) were dissolved in DMSO to a final concentration of 400 and 600 mg/mL as stock solution and sterilized by filtration through 0.45 µm Millipore filters. The discs (6 mm in diameter) were immediately placed on the surface plates (Petri dishes, 80 mm diameter) containing a suitable medium (MHA) seeded with the test organisms (1.5×10^8 CFU/mL)[6,12,13]. The amount of 15 µL of the extracts (methanolic and aqueous of two species) was poured onto discs. These plates were kept at low temperature (4 °C) for 15 min to allow maximum diffusion. Negative controls were prepared using the same solvent employed to dissolve the extract (DMSO) (10 µL). Gentamycin was used as standard antibiotic (positive control) (10 µL)[14]. The test plates were incubated at 37 °C for 24 h[15,16]. The test materials having antibacterial activity inhibited microorganism growth, and a clear, distinct zone of inhibition surrounding the discs was visualized[17]. Antimicrobial activity was evaluated by measuring the zone of inhibition ruler to an accuracy of 0.5 mm against the test organisms[11,17,18].

2.6. MIC test

The antibacterial activity of extracts was tested using the micro-dilution antibacterial assay for the MIC values[19] and minimum bactericidal concentration (MBC)[20]. The studied microorganisms included strains of *C. jejuni* (PTCC 1015), *S. aureus* (PTCC 1431), *S. enterica* (PTCC 1705) and *E. coli* (PTCC 1399). MIC was determined by the broth microdilution method in a 96-well micro-plate[21,22]. All tests were performed in MHB[23]. The microorganism inoculum was standardized with appropriate culture medium (MHB) to a final concentration of 1.5×10^6 CFU/mL by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV-120-01 spectrophotometer[3,22]. Each extract was dissolved in DMSO and added to MHB[3]. The final concentration of DMSO was lower than 2.5% and did not affect the microbial growth[21]. The extracts were serially diluted to give a concentration of 666, 333, 166.5, 50, 83.25, 41.62, 20.81, 10.40 and 5.25 mg/mL[24]. Then, 100 µL of each concentration was added in a well (96-well microplate) containing 95 µL of MHB and 5 µL of inoculum (1.5×10^6 CFU/mL)[25]. The microplate was incubated at (37 ± 1) °C for 24 h. Dilution of the extract corresponding to respective test organism showing no visible growth was considered as MIC[4] to determine MBC. 10 µL broth was taken from each well and inoculated in MHB for 24 h at 30 or 37 °C for bacteria. The MBC is defined as the lowest concentration of the methanol extracts at

which inoculated microorganism was completely killed (99.99%) [23].

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 11.5. Data were analyzed by ANOVA and reported as mean \pm SD.

3. Results

3.1. Results of disc diffusion test

The antimicrobial effect of extracts of *Cyperus* species against food-borne pathogens is shown in Tables 1 and 2. The diameters of inhibition zones varied from 0–15, 0–10 and 19–29 mm for methanolic extract of *C. longus*, *C. difformis* and gentamicin respectively. Among the six bacteria, *S. aureus* was the most sensitive (the diameter of inhibition zone was 5–15 mm) against both methanolic extract in all concentration. *E. coli* and *L. monocytogenes*, *P. aeruginosa* and *C. jejuni* were resistant to the *C. longus* and *C. difformis* extracts at all concentrations respectively. Also, the results indicated that *P. aeruginosa* was resistant to the *C. difformis* extract.

Table 1

Results of disc diffusion test and inhibition zones (mm) for methanolic extract of *Cyperus* species against Gram positive bacteria.

Concentration (mg/mL)	<i>L. monocytogenes</i>		<i>S. aureus</i>	
	<i>C. longus</i> extract	<i>C. difformis</i> extract	<i>C. longus</i> extract	<i>C. difformis</i> extract
400	0	0	10.00 \pm 2.83	5.00 \pm 1.35
600	0	0	15.00 \pm 3.25	8.00 \pm 0.33
Positive control (gentamicin)	28.88 \pm 2.35	27.99 \pm 2.05	26.10 \pm 2.50	28.10 \pm 1.87
Negative control (DMSO)	6.00 \pm 0.78	6.00 \pm 0.45	6.00 \pm 0.75	6.00 \pm 0.86

It should be noted that aqueous extract of longus and difformis did not have a significant effect against mentioned bacteria.

Table 2

Results of disc diffusion test and inhibition zones (mm) for methanolic extract of *Cyperus* species against Gram negative bacteria.

Concentration (mg/mL)	<i>E. coli</i>		<i>S. enterica</i>		<i>P. aeruginosa</i>		<i>C. jejuni</i>	
	<i>C. longus</i> extract	<i>C. difformis</i> extract	<i>C. longus</i> extract	<i>C. difformis</i> extract	<i>C. longus</i> extract	<i>C. difformis</i> extract	<i>C. longus</i> extract	<i>C. difformis</i> extract
400	0	7.00 \pm 0.55	0	0	8.00 \pm 1.89	0	0	0
600	0	10.00 \pm 3.80	0	0	11.00 \pm 0.89	0	9.00 \pm 2.20	0
Positive control (gentamicin)	28.88 \pm 1.13	27.99 \pm 2.08	26.10 \pm 0.87	28.10 \pm 2.32	21.00 \pm 2.07	19.00 \pm 1.75	20.00 \pm 1.23	21.00 \pm 2.20
Negative control (DMSO)	6.00 \pm 0.75	6.00 \pm 0.43	6.00 \pm 0.23	6.00 \pm 0.32	6.00 \pm 0.26	6.00 \pm 0.87	6.00 \pm 0.56	6.00 \pm 0.65

Table 3

MIC of methanolic and aqueous extracts of *Cyperus* species (mg/mL).

Microorganism		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>P. aeruginosa</i>	<i>C. jejuni</i>
<i>C. longus</i>	Methanolic extract	10.40 \pm 2.50	20.81 \pm 2.89	41.00 \pm 1.38	20.81 \pm 0.89	2.60 \pm 1.82	2.60 \pm 0.52
	Aqueous extract	41.00 \pm 3.29	41.00 \pm 28.00	0.25 \pm 3.42	41.00 \pm 2.89	2.60 \pm 0.89	20.81 \pm 0.42
<i>C. difformis</i>	Methanolic extract	41.00 \pm 4.19	20.81 \pm 2.25	83.25 \pm 2.00	41.00 \pm 3.75	2.60 \pm 0.71	20.81 \pm 0.85
	Aqueous extract	20.81 \pm 0.89	10.40 \pm 0.89	41.00 \pm 0.89	20.81 \pm 0.89	2.60 \pm 0.89	20.81 \pm 0.89

Table 4

MBC of methanolic and aqueous extracts of *Cyperus* species (mg/mL).

Microorganism		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>P. aeruginosa</i>	<i>C. jejuni</i>
<i>C. longus</i>	Methanolic extract	83.25 \pm 0.75	83.25 \pm 3.50	166.50 \pm 5.00	83.25 \pm 2.15	83.25 \pm 2.39	83.25 \pm 0.78
	Aqueous extract	83.25 \pm 2.37	83.25 \pm 3.20	166.50 \pm 5.00	166.5 \pm 3.00	166.50 \pm 3.22	41.00 \pm 4.30
<i>C. difformis</i>	Methanolic extract	–	166.50 \pm 0.93	–	–	5.25 \pm 0.43	–
	Aqueous extract	166.50 \pm 2.55	166.50 \pm 0.75	83.25 \pm 4.00	83.25 \pm 0.37	83.25 \pm 0.50	83.25 \pm 0.95

–: Not reported.

3.2. Results of MIC and MBC

Results obtained from the microdilution method are shown in Table 3. The MIC and MBC concentrations, two parameters that respectively quantify the bacteriostatic and bactericidal potential of bioactive compounds, were determined using dry extracts and the serial dilution method for the extracts with substantial antibacterial activity against the *C. jejuni*, *L. monocytogenes*, *P. aeruginosa*, *E. coli*, *S. enterica*, *S. aureus*. As is shown in Table 3, the obtained values for MIC were in the ranges 2.60–41.00 mg/mL for methanolic extract of *C. longus* and 2.60–83.25 for three other extracts (methanolic and aqueous of *C. difformis* and aqueous extract of *C. longus*), which showed the methanolic extract of *C. longus* had the strongest bacteriostatic effects on the tested bacteria. Also Gram-negative bacteria were the most sensitive to all of extracts. The values of MBC are reported in Table 4. The results showed that the values of MBC were in the ranges 5.25–166.50 mg/mL. Based on the results, most bactericidal effect was related to methanol extract of *C. difformis* against *L. monocytogenes* (MBC: 5.25).

4. Discussion

In recent year, the demand for natural products is increasing[25]. The antimicrobial properties of several naturally occurring compounds have been known for decades. Recently, many plants have received attention as sources of antibiotics[5]. Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects[6] and give them a good potential for using as a natural preservative in food[15].

Some studies show that Gram-negative bacteria are more resistant to herb extracts, but others claim the same for Gram-positive bacteria. In our study, the antibiotic drug and tested extracts had higher inhibitory activities against Gram-positive bacteria in

comparison with Gram-negative bacteria. Lipopolysaccharide layer of Gram-negative bacteria in outer membrane has a high hydrophobicity which acts as a strong permeability barrier against hydrophobic molecules. Hydrophobic molecules can pass through the cell wall of Gram-positive bacteria easier than the Gram-negative bacteria because the cell wall of the Gram-positive bacteria contained only peptidoglycan[9]. Also some studies showed that amongst the Gram-positive and Gram-negative bacteria, Gram-positive bacteria *S. aureus* were inhibited by plant extract[2,7].

Based on our experimental results, the extracts obtained from *Cyperus* species (*C. longus* and *C. difformis*) showed considerable antibacterial properties against typical food-borne pathogens especially Gram-positive bacteria. It should be noted that the methanolic extract of tested herbs was more active against mentioned bacteria, which indicated the presence of active compounds. Therefore, they could be used as possible food antimicrobial preservative in the food industry, but the *in vivo* studies should be done to evaluate the probable adverse effect on food sensory properties.

Conflict of interest statement

We declare that we have no conflict of interest.

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