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TOTAL PHENOLIC AND FLAVONOID CONTENTS AND BIOLOGICAL ACTIVITIES OF *CACHRYS CRISTATA* DC. EXTRACTS

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Abstract - The total phenolic/flavonoid contents and antioxidant potential of the methanol, ethyl-acetate, acetone and water extracts obtained from the aerial parts and fruits of *Cachrys cristata* DC. (Apiaceae) were compared. The total phenolic contents of the tested extracts were determined using Folin-Ciocalteu's reagent. The amounts per g of dry plant extract of gallic acid (GA) and quercetin (Qu) ranged between 22.60-166.97 mg, and 8.91-46.02 mg, respectively. The antioxidant activity, expressed as IC_{50}, ranged from 1.784-17.621 mg/mL and from 1.01-3.42 mg L(+)-ascorbic acid (Vitamin C)/g when tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS, respectively. The antimicrobial activity of the extracts was investigated by the microwell dilution assay, for the most common human gastrointestinal pathogenic bacterial strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC 25923 and yeast *Candida albicans* ATCC 10231. The results indicate that *C. cristata* can be regarded as a potential source of antioxidant and antimicrobial agents.

Key words: *Cachrys cristata;* Apiaceae; extracts; antioxidant activity; antimicrobial activity

INTRODUCTION

Many studies have been examined the chemical composition and biological activities of various extracts of plants from the Apiaceae family (Wang et al., 2012). Species from this family are characterized by the presence of furanocoumarins, flavones and flavonols. The most common flavonols are quercetin and kaempferol (Miean and Mohamed, 2001).

In various extracts of the Apiaceae species, coumarins are often detected as the most abundant group of secondary metabolites. Also known as benzopyrones, they represent a large group of phenolic compounds occurring in green plants, as well as in fungi and bacteria (Murray et al., 1982). They have antimicrobial, antitumoral and anti-aggregate activities and have been reported to be potent antioxidants with anti-inflammatory properties (Hoult et al., 1994, Paya et al., 1994, Ng et al., 1996, Hoult and Paya, 1996, Cai et al., 1997, Su et al., 1998, Kang et al., 1999). Furanocoumarins, alternatively known as furocoumarins, are a category of coumarins that occur as plant constituents predominantly in two families, the Apiaceae and the Rutaceae (Fujioka et al., 1999).

Cachrys cristata DC. (Syn. *Hippomarathrum cristatum* (DC.) Boiss.), belonging to the family Apiaceae, is a rare and critically endangered species in

the flora of Serbia (Nikolić, 1973; Ranđelović, 1999). The species *C. cristata* is a perennial plant, 30-80 cm high, with glabrous stems and opposite or whorled branches. Leaves are 2- to 4-pinnate, ovate in outline. Flowers are yellow, grouped in complex shields. Fruits are winged with 7-10 mm ridges (Tutin, 1968).

Essential oil isolated by hydrodistillation from the aerial parts and fruits of this species was analyzed using combined GC and GC/MS (Matejić et al., 2012). The aerial parts of *C. cristata* were rich in sesquiterpenes (45.7%) and oxygenated sesquiterpenes (32.9%), while the fruit oil consisted of a higher percentage of sesquiterpenes (48.3%) and oxygenated sesquiterpenes (36.7%). In the oil from aerial parts, dominant constituents were phytol (13.1%) and germacrene D (12.9%), while in the fruit oil of *C. cristata* most abundant were suberosin (19.7%) and germacrene D (12.3%) (Matejić et al., 2012).

In this work, we present a first report on the antioxidant and antimicrobial activities of *C. cristata* methanol, ethyl-acetate, acetone and water extracts.

MATERIALS AND METHODS

Chemicals

Organic solvents were purchased from "Zorka pharma" Šabac, Serbia; GA, 3-tert-butyl-4-hydroxyanisole (BHA) and DPPH were obtained from Sigma Chemicals Co., St Louis, MO, USA; Folin-Ciocalteu phenol reagent was purchased from Merck, Darmstadl, Germany; Sodium carbonate anhydrous (Na_2CO_3, $C_2H_3KO_2$, $K_2O_8S_2$); L(+)-ascorbic acid were purchased from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven Belgium; aluminum nitrate nonahydrate ($Al(NO_3)_3x9H_2O$) was purchased from Fluka Chemie AG, Buchs, Switzerland; ABTS and quercetin hydrate (Qu) were obtained from TCI Europe NV, Boerenveldsweg, Belgium. All other solvents and chemicals were of analytical grade.

Plant material

Aerial plant parts and fruits of *Cachrys cristata* were collected in July 2010 from Rujan Mountain, southeast Serbia. A voucher specimens for *C. cristata* (BEOU 16434), has been deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

Preparation of plant extract

Plant material was air dried in the dark and ground to a powder. The aerial plant parts and fruits (10 g) were powdered and extracted with 100 ml water, methanol, acetone and ethyl acetate. The mixture was exposed to ultrasound for 30 min and after 24 h standing in the dark was filtered. The methanol, ethyl-acetate and acetone solvents were removed by evaporation under reduced pressure, at maximum temperature of 40°C. Water extract was frozen and later dried by freeze-drying. After evaporation of the solvent, the crude extract was subjected to subsequent analysis.

Determination of total phenolic content

The total phenolic content of extracts was determined spectrophotometrically by the Folin-Ciocalteu method as described by Singleton et al. (1999), with some modifications. Briefly, 300 μl of different extract solutions and 1 500 μl of 1:10 Folin-Ciocalteu reagent were mixed. After 6 min in the dark, 1 200 μl of 7.5% Na_2CO_3 were added. After incubation for 2 h in the dark at room temperature, the absorbance at 740 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). The total phenolic concentration was calculated from the GA calibration curve (10-100 mg/l). Data were expressed as GA equivalents/g extract averaged from three measurements.

Determination of flavonoid content

The total flavonoid content was evaluated using aluminum nitrate nonahydrate according to the procedure reported by Woisky and Salatino (1998) with some modifications. The sample for determination was prepared by mixing 600 μl of extract solution and 2 580 μl of a mixture containing 80% C_2H_5OH,

Table 1. Total phenolic/flavonoid contents and antioxidant capacities by ABTS assay of aerial plant parts and fruits *C. cristata* extracts.

Cachrys cristata		Extract concentration (mg/ml)	Total phenolic content (mg GA/g)	Total flavonoid content (mg Qu/g)	ABTS assay (mg VitC/g)
Methanol extract	aerial parts	3	99.56±0.001	17.75±0.001	2.75±0.016
	fruits	2	89.87±0.005	9.30±0.001	2.23±0.023
Ethyl acetate extract	aerial parts	5	39.78±0.005	46.02±0.004	1.91±0.004
	fruits	5	22.60±0.003	10.02±0.001	1.01±0.017
Acetone extract	aerial parts	3	166.97±0.006	33.22±0.016	3.42±0.005
	fruits	2	129.22±0.015	8.91±0.003	2.30±0.003
Water extract	fruits	2	79.35±0.002	10.41±0.002	2.75±0.011
BHA		0.1	63.31±0.001	/	2.66±0.005
Vitamin C		0.1	40.91±0.002	/	/

Each value in the table was obtained by calculating the average of three analyses ± standard deviation.

Table 2. Antioxidant activities of *C. cristata* extracts using DPPH scavenging method.

Cachrys cristata		IC_{50} (mg/ml)
Methanol extract	aerial parts	4.058
	fruits	3.347
Ethyl acetate extract	aerial parts	9.743
	fruits	17.621
Acetone extract	aerial parts	9.605
	fruits	15.043
Water extract	fruits	1.784
BHA		0.1
Vitamin C		0.1

10% $Al(NO_3)_3x9H_2O$ and 1M $C_2H_3KO_2$. After 40 min of incubation at room temperature, the absorbance at 415 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). The total flavonoid concentration in the extract was calculated from the quercetin hydrate (Qu) calibration curve (10-100 mg/l) and expressed as Qu equivalents/g dry extract. Measurements were performed in triplicates.

Evaluation of DPPH-scavenging activity

The antioxidant activity of extract was evaluated by the radical scavenging method, using the stable radical DPPH as reagent (Blois, 1958). The effect of antioxidant compounds on DPPH radical scavenging is due to their hydrogen-donating ability or radical-scavenging activity. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, this gives rise to the reduced form diphenylpicrylhydrazine with the loss of its violet color (Molyneux, 2004). The water, methanol, ethyl-acetate and acetone solution of the investigated extract (300 µl) (the extract concentrations between 1 000 and 7 500 µg/ml) was added to 2 700 µl methanolic solution of DPPH (concentration 0.04 mg/ml), and after shaking, the reaction mixture was left to react in the dark for 30 min at room temperature. Absorbance of the remaining DPPH radical was measured

Table 3. Antimicrobial activity of aerial parts and fruits *C. cristata* extracts against pathogenic microbial strains using Micro-well Dilution Assay.

Cachrys cristata	Extract (MIC/MBC(MFC) in mg/ml)						Referent antibiotics
	Methanol (aerial parts)	Ethyl-acetate (aerial parts)	Methanol (fruits)	Ethyl-acetate (fruits)	Acetone (fruits)	Water (fruits)	MIC/MBC (MFC) in µg/ml
Gram-negative bacteria							Streptomycin
E. coli ATCC 25922	0.78/>50.00	25.00/50.00	25.00/50.00	25.00/50.00	25.00/50.00	>50.00/>50.00	16.00/16.00
P. aeruginosa ATCC 9027	0.78/>50.00	6.25/>50.00	0.78/>50.00	3.13/>50.00	25.00/50.00	>50.00/>50.00	8.00/8.00
S. enteritidis ATCC 13076	0.78/>50.00	12.50/12.50	1.56/>50.00	12.50/12.50	25.00/>50.00	>50.00/>50.00	4.00/4.00
Gram-positive bacteria							Chloramphenicol
Bacillus cereus ATCC 10876	0.78/>50.00	0.78/1.56	1.56/25.00	0.78/1.56	1.56/3.13	>50.00/>50.00	4.00/16.00
L. monocytogenes ATCC15313	12.50/12.50	0.78/1.56	6.25/>50.00	0.78/1.56	12.50/12.50	>50.00/>50.00	8.00/16.00
S. aureus ATCC 25923	0.39/12.50	0.78/6.25	3.13/25.00	0.78/6.25	25.00/50.00	>50.00/>50.00	1.00/8.00
Fungal strain							Nystatin
C. albicans ATCC 10231	6.25/>50.00	0.78/50.00	12.50/>50.00	0.78/50.00	6.25/50.00	>50.00/>50.00	16.00/16.00

at 517 nm. Every concentration was performed in triplicate and the same was done with Vitamin C and BHA which served as antioxidants. Blank probes contained methanol (A0). The decrease of absorption of the DPPH solution was calculated using the equation:

Percentage of absorption decrease (on 517 nm) = (A0-A1)x100/A0

Concentrations that decrease absorption of DPPH solution by 50% (IC_{50}) were obtained from the absorption curve of DPPH solution at 517 nm from the concentrations for each compound and standard antioxidant. For calculation of these values, Origin 7.0 software was used. Tests were carried out in triplicate.

Evaluation of ABTS radical scavenging activity

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical-scavenging activity was determined by the method of Miller and Rice-Evans (1997) with some modifications. The $ABTS^+$ solution was prepared by mixing 19.2 mg of ABTS with 5 ml of potassium persulfate (2.46 mM). The solution was kept at room temperature in the dark for 12-16 h before use. The $ABTS^+$ solution (1 ml) was diluted with 100-110 ml water in order to obtain an absorbance 0.7±0.02 at 734 nm. Fresh $ABTS^+$ solution was prepared for each analysis. Seventy five µl of antioxidant or standard solution was mixed with 3 ml of diluted $ABTS^+$ solution and incubated at 30°C for 30 s. The absorbance at 734 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). Water was

used as a blank. ABTS radical-scavenging activity in the different extracts was calculated from a vitamin C calibration curve (0-2 mg/l) and expressed as vitamin C/g dry extract. All experimental measurements were carried out in triplicate and were expressed as the average of three analyses ± standard deviation.

Microbial cultures

The antimicrobial activity of all tested samples was evaluated using laboratory control strains obtained from the American Type Culture Collection: Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076; Gram-positive bacteria: *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC 25923 and yeast *Candida albicans* ATCC 10231.

Micro-well dilution assay

The inocula of the microbial strains were prepared from the overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7-10^8 CFU/ml, depending on the genera) (NCCLS 2003). Serial dilutions of the tested samples (methanol, ethyl acetate and acetone extracts of *C. cristata* (100 mg/ml in a 30% ethanol and water extract; 100 mg/ml in 5% DMSO) were prepared in a 96-well microtiter plate over the range of 50.0-0.1 mg/ml in inoculated Mueller-Hinton broth. The final volume was 100 µl and the final microbial concentration was 10^6 CFU/ml in each well. The plates were incubated for 24 h at 37°C. All experiments were performed in triplicate. Two controls were included: medium with 30% ethanol (negative control) and medium with streptomycin, chloramphenicol and nystatin (positive control). Microbial growth was determined by adding 20 µl of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution (Sartoratto et al., 2004). The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the samples inhibiting visible growth (red-colored pellet on the bottom of the wells after the addition of TTC). To determine minimal bactericidal/fungicidal concentrations (MBC/MFC), broth was taken from each well without visible growth and inoculated in Mueller-Hinton agar (MHA) for 24 h at 37°C. MBC/MFC were defined as the lowest sample concentration killing 99.9% of bacterial/fungal cells.

RESULTS AND DISCUSSION

Total phenolic content

The total phenolic contents of the examined plant extracts (Table 1), expressed as gallic acid equivalents (GA) per gram of dry extract, ranged between 22.60 and 166.97 mg GA/g. The highest phenolic content was found in the acetone extract of aerial plant parts (3 mg/ml extract).

Flavonoid content

A summary of the quantities of flavonoids detected in the tested extract is shown in Table 1. The concentrations of flavonoids in plant extracts ranged from 8.91 to 46.02 mg Qu/g. The highest flavonoid content was measured in the ethyl-acetate extract of aerial parts (with 5 mg/ml extract concentration). Because of their presence in plants, flavonoids are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Stobiecki and Kachlicki, 2006).

DPPH-scavenging activity

The free radical-scavenging capacities of the tested extract were measured by the DPPH assay and the results are shown in Table 2. A lower IC_{50} value indicates higher antioxidant activity. The water extract of *C. cristata* fruits possessed the strongest antioxidant activity compared to the other extracts.

ABTS-scavenging activity

The results from the ABTS assay are shown in Table 1. The amount ranged from 1.01 to 3.42 mg vitamin C/g of *C. cristata* species. The highest content was identified in the acetone extract of *C. cristata* aerial

parts (3 mg/ml extract) and the lowest in the ethyl-acetate fruits extract (5 mg/ml extract).

Various solvents were used to achieve the extraction of active substances with diversity in their polarity. The acetone extract of *C. cristata* aerial parts showed relatively high antioxidant activity that is in accordance with the high concentration of its total phenols and flavonoids. Based on the results of this study, the extract with the highest antioxidant activity had the highest concentration of phenols. Phenolic compounds are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolics in plants directly contribute to their antioxidant action.

Antimicrobial activity

All tested extracts of *C. cristata* (except the water extract) showed inhibitory antimicrobial activity against all tested strains at concentrations of 0.78-12.5 mg/ml, but most of them did not show bactericidal effect at concentrations of 50.0 mg/ml (Table 3). As reference antibiotics, streptomycin and chloramphenicol, and antimycotic nystatin were used.

The ethyl-acetate extract of aerial plant parts and fruits showed the best activity against Gram-positive bacteria with an inhibitory and bactericidal concentration range of 0.78-6.25 mg/ml. The same extract had inhibitory effect on the *P. aeruginosa* strain at concentrations of 3.125-6.250 mg/ml, but the highest concentration of this extract did not show bactericidal effect on this treated bacterial strain. Similar results were observed for the *C. albicans* strain. Methanol extracts of aerial parts and fruits showed similar inhibitory effects to the ethyl-acetate extract, but the acetone extract had lower effectiveness compared to the others (except on *Bacillus cereus*). *B. cereus* was the most sensitive strain to extracts of *Cachrys cristata*. This is very important because *C. cristata* is a spice plant while *Bacillus cereus* is a bacterium that often causes food spoilage.

CONCLUSIONS

Methanol, ethyl-acetate, acetone and water extracts from *Cachrys cristata* are potential protective agents against oxidative stress provoked by DPPH and hydroxyl radicals. They expressed good antioxidant activity in comparison with the synthetic antioxidants BHA and Vitamin C. Ethyl-acetate and methanol extracts showed an inhibitory effect at low concentrations against a selected group of microorganisms that cause food spoilage. Our results point to their potential use as natural preservatives and in the production of food that could prevent gastrointestinal disorders.

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