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Full Length Research Paper

Phenolic compounds and biological activity of *Capsicum annuum* L.

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The objective of this study was to evaluate antifungal and antioxidant activities of vegetable extracts (*Capsicum annuum* L. cv. Dora, cv. Strizanka, cv. Morava), grown in Serbia. Different experimental models have included the determination content of total phenolics, total flavonoids, antioxidant capacity and minimum inhibitory concentration of the extract. The phenolic composition of different extracts was determined by the high-performance liquid chromatography (HPLC) method. The highest amounts of phenols and highest antioxidant capacity were found in the cultivar Dora extracts. The highest phenolic content has been shown in cultivar Strizanka ethanol extract. All of the extracts showed strong antimicrobial activity. On the basis of the results obtained, the extracts were found to serve as a potential source of natural antioxidants and antimicrobial due to their marked activity. The obtained results may be useful in the evaluation of new dietary and food products.

Key words: High-performance liquid chromatography (HPLC), phenolic components, antimicrobial activity, antioxidant activity.

INTRODUCTION

The medicinal properties of plants have been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities and low toxicity (Vaquero et al., 2010). Antimicrobial activity of herbs has been known and described for several centuries (Begamboula et al., 2003). Many naturally occurring compounds found in edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and could serve as a source of antimicrobial agents against bacteria and fungi (Deans and Ritchie, 1987; Janssen et al., 1985; Kim et al., 1995). Several studies have pointed out the possibility to use essential oils and/or their components in medical and plant pathology as well as in the food industry for the control of microorganisms pathogenic to consumers and/or responsible for food spoilage (Cantore et al., 2009). Human organism is exposed to a large number of foreign chemicals everyday (Santhakumari et al., 2003). The most of which are man-made and our inability to properly metabolize them negatively affects our health by the generation of free radicals.

Free radicals are also generated during normal metabolism of aerobic cells (Carmen and Florin, 2009; Ghaseme et al., 2009). The oxygen consumption inherent in cells growth leads to the generation of series of oxygen free radicals. Highly active free radicals and their uncontrolled production are responsible for numerous pathological processes such as cell tumor (prostate and colon cancers) and coronary heart diseases (Duh and Yet, 1997). Antioxidants can significantly delay or prevent the oxidation of easily oxidizable substances (Cao et al., 1997). Natural antioxidants are classified according to their mechanism of action as chain-breaking antioxidants which scavenge free radicals or inhibit the initiation step or interrupt the propagation step of oxidation of lipid and as preventive antioxidants which slow the rate of oxidation by several actions but do not convert free radicals (Prieto et al., 1999). However, there have been

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concerns about synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) because of their possible activity as promoters of carcinogenesis (Iqbal et al., 2005). There is growing interest toward natural antioxidants from herbal sources (Nobuyuki et al., 1985).

MATERIALS AND METHODS

Chemicals used

All standards for high-performance liquid chromatography (HPLC) analysis were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA) and Alfa Aesar (Karlsruhe, Germany). Acetonitrile and phosphoric acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade (Aldrich Chemical Co., Steinheim, Germany).

Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV-VIS spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).

Plant collection

Capsicum annuum L. was cultivated under open field conditions during the autumn growing cycle in 2010 on an experimental field at Trbušani, near Čačak, Serbia (Abbasi et al., 2010). In the experiment three varieties of peppers were presented: 1. Cultivar Dora, plant: medium early variety, very long and large elongated fruits; fruit: light yellow in technological and intensively red in botanical, fleshy, elongated,narrow and flattened, easy for roasting and peeling. 2. Cultivar Strizanka, plant: strong, wellbranched, medium high, with upright fruits; fruit: semi bell type, yellow in technological and intensively red in biological maturity, medium large. 3. Cultivar Morava, plant: strong, well branched, medium high, upright fruits; fruit: semi bell type, yellow-green in technological and red in biological maturity, medium large.

Preparation of the extracts

Plant sample (10.0 g) was extracted by concentration of 70%$_{vol}$ ethanol or ethanol (100.0 ml) as a solvent. The extraction process was carried out using ultrasonic bath (Brason and Smith-Kline Company, B-220, (Smith-KlineCompany, USA)) at the room temperature for 1 h. After filtration, 5 ml of liquid extract was used for extraction yield determination. Solvent was removed by rotary evaporator (Devarot, Elektromedicina, Ljubljana, and Slovenija) under vacuum, and was dried at 60°C to the constant mass. Dry extracts were stored in the glass bottles at 4°C to prevent oxidative damage until analysis.

Test microorganisms

The antimicrobial activity of the plant extract was tested *in vitro* against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi; *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The fungi were cultured on potato-glucose agar for seven days at room temperature of 20°C under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another seven days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined by micro-dilution method in 96 multi-well microtiter plates (Satyajit et al., 2007). All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µl stock solutions of oil (in methanol, 200 µl/ml) and cirsimarin (in 10% DMSO, 2 mg/ml) was pipetted into the first row of the plate. 50 µl of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of oil) was added to the other wells. A volume of 50 µl from the first test wells was pipetted into the second well of each microtiter line, and then 50 µl of scalar dilution was transferred from the second to the twelfth well. 10 µl of resazurin indicator solution (prepared by dissolution of a 270 mg tablet in 40 ml of sterile distilled water) and 30 µl of nutrient broth were added to each well. Finally, 10 µl of bacterial suspension (10^6 CFU/ml) and yeast spore suspension (3×10^4 CFU /ml) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas ketoconazole was used as control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 24 h for the bacteria and at 28°C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of three values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

Determination of total phenolic content

Total phenols were estimated according to the Folin-Ciocalteu method (Singleton et al., 1999). The extract was diluted to the concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7.5 %). After 15 min at 45°C, the absorbance was measured at 765 nm using a spectro-photometer against a blank sample. Total phenols were determined as gallic acid equivalents (mg GAE/g extract), and the values are presented as means of triplicate analyses.

Determination of flavonoid content

Total flavonoids were determined according to Brighente et al. (2007). A total of 0.5 ml of 2% aluminium chloride ($AlCl_3$) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 h of staying at room temperature, the absorbance was measured at 415 nm in a spectrophotometer against the blank sample. Total flavonoids were determined as rutin equivalents (mg RE/g dry extract), and the values are presented as means of triplicate analyses.

Table 1. Minimum inhibitory concentrations (MIC) of the ethanolic extracts of the different cultivars.

Extract/microbial strain	Strizanka (MIC µgml^{-1})	Dora (MIC µgml^{-1})	Morava (MIC µgml^{-1})	Amracin (MIC µgml^{-1})	Ketoconazole (MIC µgml^{-1})
Staphylococcus aureus ATCC *25923*	312.50	156.25	156.25	0.97	-
Klebsiella pneumonia ATCC 13883	156.25	78.12	78.12	0.49	-
Escherichia coli ATCC 25922	78.12	78.12	78.12	0.97	-
Proteus vulgaris ATCC 13315	78.12	156.25	78.12	0.49	-
Proteus mirabilis ATCC 14153	156.25	78.12	156.25	0.49	-
Bacillus subtilis ATCC 6633	78.12	39.10	39.10	0.24	-
Candida albicans ATCC 10231	39.10	78.12	78.12	-	1.95
Aspergillus niger ATCC 16404	39.10	19.53	39.10	-	0.97

MIC, Minimum inhibitory concentrations.

Determination of total antioxidant capacity

The total antioxidant activity of the vegetable extracts was evaluated by the phosphomolybdenum method (Satyajit et al., 2007). The assay is based on the reduction of Mo (VI) – Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A total of 0.3 ml of sample extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm using spectrophotometer against the blank after cooling to room temperature. Methanol (0.3 ml) in place of extract was used as the blank. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry extract.

HPLC analysis

Quantification of individual phenolic compounds was performed by reversed phase HPLC analysis, using a modified method of Mišan et al. (2011). HPLC analysis was performed by using a liquid chromatography (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies, USA), a binary pump, an online vacuum degasser, an autosampler and a thermostated column compartment, on an Agilent, Zorbax Eclipse Plus-C18, 1.8 µm, 600 bar, 2.1 × 50 mm column, at a fow-rate of 0.8 ml/min. Gradient elution was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) as follows: initial 0 to 2 min, 100% B; 2 to 4 min, 98 to 100% B; 4 to 6 min, 95 to 98% B; 6 to 7 min, 73 to 95% B; 7 to 10 min, 48 to 75% B; 10 to 12 min 48% B; 12 to 20 min, 40 to 48% B. The total running time and post-running time were 21 and 5 min, respectively. The column temperature was 30°C. The injected volume of samples and standards was 5 µl and it was done automatically using autosampler. The spectra were acquired in the range 210 to 400 nm and chromatograms plotted at 280, 330 and 350 nm with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm.

RESULTS AND DISCUSSION

Fruits and vegetables contain antioxidant compounds broadly called polyphenols that are known to reduce oxidative stress and prevent chronic diseases (Ames et al., 1993; Diaz et al., 1997; Giacosa et al., 1997; Gariballa and Sinclair, 1998; Miller et al., 1998; Muller et al., 1999). The antioxidant properties of these compounds are responsible for their anticancer, antiviral and antiinflammatory properties (Ames et al., 1995). They can also prevent capillary fragility and platelet aggregation (Benavente-Garcia et al., 1997; Aviram, 1999).

Antimicrobial activity

The results of antimicrobial activity obtained by the dilution method are given in Table 1; MICs were determined for eight selected indicator strains. The results presented in Table 1, reveal antimicrobial activity of the vegetables extracts (*C. annuum* L. cultivar Dora, *C. annuum* L. cultivar Strizanka, *C. annuum* L. cultivar Morava), within the concentration range from 19.53 to 312.5 µg/ml. Extract of *C. annuum* L. cultivar Dora, showed the highest antimicrobial susceptibility of fungi *A. niger* (MIC = 19.53 µg/ml). Intermediate antimicrobial susceptibility were shown: extract of *C. annuum* L. cultivar Strizanka, by yeast *C. albicans* and fungi *A. niger*, extract of *C. annuum* L. cultivar Dora, by *B. subtilis* and extract of *C. annuum* L. cultivar Morava, by *B. subtilis* and *A. niger* fungi (MIC = 39.1). The lowest antimicrobial susceptibility was shown by the extract of *C. annuum* L. cultivar Strizanka, by *S. aureus* (312.5).

Determination of antioxidant compounds

As a chemical structure of phenolic compounds is responsible for their antioxidant activity, so measurement of total phenolics content could be related to antioxidant properties of investigated material. Total phenolics content, total flavonoids content and total flavonoids/total phenolics ratio (TF/TP) are presentand in the Table 2. In all tested samples, content of total phenolics was higher than the content of total flavonoids. The highest content

Table 2. Total phenolics, flavonoids, total flavonoid and total phenolics content ratio (TF/TP) and total antioxidant capacity in the different cultivar extracts.

Type of extract	Total phenolics (mg GAE g^{-1} d. e.)	Flavonoids (mg RE g^{-1} of d. e.)	TF/TP × (100 %)	Total antioxidant capacity (µg AA g^{-1} d. e.)
Dora	50.41 ± 0.0265*	23.18 ± 0.5437	45.99	48.50 ± 1.1872
Morava	32.95 ± 0.6583	17.43 ± 0.3898	52.93	72.20 ± 0.7255
Strizanka	33.34 ± 0.3412	25.14 ± 0.3245	75.41	98.00 ± 0.7259

*Results are mean values ± SD from three experiments; d. e., different exdtracts.

Table 3. Quantitative and qualitative contents of phenolic components in Dora.

Sample	Component	Content (mg g^{-1})
Dora	Gallic acid	0.086
	Protocatehuic acid	0.343
	Caffeic acid	0.064
	Vanillic acid	0.146
	Chlorogenic acid	0.799
	Ferulic acid	0.072
	Rosmarinic acid	0.648
	Sinapic acid	0.250

Table 4. Quantitative and qualitative contents of phenolic components in Morava.

Sample	Component	Content (mg g^{-1})
Morava	Gallic acid	0.369
	Caffeic acid	0.545
	Rutin	0.089
	Rosmarinic acid	0.060
	Naringenin	0.044
	Siringic acid	0.045

Table 5. Quantitative and qualitative contents of phenolic components in Strizanka.

Sample	Component	Content (mg g^{-1})
Strizanka	Gallic acid	0.164
	Protocatehuic acid	0.519
	Rosmarinic acid	4.019
	Cinnamic acid	0.040
	Naringenin	0.022
	Quercetin	0.041
	Miricetin	3.315

of phenolic compounds was detected in *C. annuum* L. cultivar Dora extract, 50.42 mg GAE/g. Similar content of total phenoilc compounds was observed in cultivars Morava and Strizanka extracts, ~ 30 mg GAE/g of dry extract. Flavonoids are considered as phenol compounds with highest antioxidant activity due to their chemical structure (Kähköne et al., 1999). The lowest total flavonoid content was shown by cultivar Morava extracts, 17.45 mg RE/g of dry extracts. Higher and similar content of total flavonoid compounds was observed in cultivars Dora and Strizanka extracts (~ 23 mg RE/g) than cultivar Morava extracts. The ratio TF/TP cultivar Strizanka extract was much higher than in the other two extracts. This could contribute to its total antioxidant activity because flavonoids are considered as the most active antioxidant phenolic compounds.

Phenolics and flavonoids can play a double role in reducing the rate of oxidation, as they participate in iron chelation and trapping radicals (Carmen and Florin, 2009). Table 2 shows antioxidant capacity of the different extracts (lower values indicate more powerful antioxidant capacity). Effectiveness in reducing powers was in a descending order of cultivar Dora > cultivar Morava > cultivar Strizanka. A strong correlation between free radical scavenging and the phenolic contents has been reported for many plants (Singleton et al., 1999). Extract of cultivar Dora showed the best antioxidant capacity and the highest content of antioxidant compounds. Content of plant phenolics in vegetables extracts, expressed as mg/g of dry extracts are shown in Tables 3 to 5. In crude extracts, the following compounds were identified and quantified: gallic acid, protocatehuic acid, caffeic acid, vanillic acid, chlorogenic acid, rosmarinic acid, ferulic acid, sinapic acid, siringic acid, naringenin, miricetin, rutin and quercetin. The dominant components of cultivar Dora extracts were chlorogenic and rosmarinic acids. In the extract of cultivar Dora were less common acids: protocatehuic acid, sinapic acid and vanillic acid. Content of gallic, caffeic and ferulic acids were lower than 0.1 mg/g of dry extracts. In cultivar Morava extract, dominant components were gallic (0.369 mg/g) and caffeic acids (0.545 mg/g). In cultivar Strizanka extract, six phenolic compounds were detected: gallic acid, protocatechuic acid, rosmarinic acid, cinnamic acid, naringenin quercetin and miricetin. Rosmarinic acid (14.019 mg/g) was the richest.

Conclusion

Insensitive research for new, unexplored, natural

antioxidant and antimicrobial source is very significant and can bring new natural products in pharmaceutical and food industry for their every day battle with reactive oxygen species. Discovering a natural source of antioxidants could be significant and for artificial toxic antioxidants replacement in food industry. The results of this study clearly indicate that vegetable extracts can be use as antioxidant and antimicrobial products. Also, they all possess reductive capabilities. They are all adequate source of phenolic and flavoniods compounds, compounds well known as an antioxidants with high antioxidant activity. This study demonstrates good antioxidant and antimicrobial properties of all investigated vegetables extracts prepared from *C. annuum* L. cultivars Dora, Strizanka and Morava.

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