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Antioxidant Activity and Phenolic Content of Soybean Seeds Extracts

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Abstract

Plants are a good source of natural antioxidants and could provide protection against harmful free radicals. Phenolic compounds were found to be an important part of human diet and are considered as active principles in many medicinal and agricultural plants. Detailed information about health-promoting components of different soybean cultivars could lead to a better understanding and an increased consumption of this crop, including its use in functional foods. The objective of this study was to determine total phenolics, total tannins, total flavonoids and antioxidant capacity with different assays of five Serbian soybean cultivars (Merkur, Sava, Valjevka, Venera and Victoria) extracted with three different solvents (70% acetone, 70% ethanol and 70% methanol). Total phenolics varied among cultivars and among applied solvents. Antioxidant properties highly depended on a solvent used for extraction. Such results highlight an existing variability in soybean seeds and emphasise the need to evaluate diversity and to support conventional breeding programs to improve soybean nutritional value.

Key words: antioxidant capacity, extraction solvents, flavonoids, *Glycine max* (L.) Merr., tannins

Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the major crops in the world and soybean seeds are a great source of oil and proteins for human and animal feed (Yin et al., 2011). Soybean seeds, besides oil and proteins, contain sugars and phenolics as well. Primary metabolites are essential for growth, development and reproduction, while secondary metabolites such as phenolics are associated with plant defence against pests and survival mechanisms under abiotic and biotic stress (Bellaloui, 2012). Phenolics are secondary plant metabolites characterized by having at least one aromatic ring with one or more hydroxyl groups attached. The distribution of phenolics differs by plant species and tissue, with many phenolics synthesized from carbohydrates via shikimate and phenyl propanoid pathways (Lattanzio et al., 2006). In plants these molecules are generally involved in fighting against aggression by pathogens, insects, herbivores, or ultraviolet radiation (Grassmann et al., 2002; Lattanzio et al., 2006; Báidez et al., 2006; Barbehenn & Constabel, 2011). Plant genetics and cultivar, growing conditions, maturity stage and post-harvest conditions are effective on quantity and quality of phenolic compounds present in plant foods. Phenolic compounds have antioxidant properties and can protect against degenerative diseases in which ROS-reactive oxygen species (superoxide anion, hydroxyl radicals, peroxyl radicals etc.) are involved (Ozcan et al., 2014).

The overall goal of this study was to investigate the antioxidant activities of soybean affected by different cultivars and extraction methods, and to determine the relation between antioxidant capacity of extracts and different phenolic groups in soybean seeds.

Material and Methods

The analysis was performed on 5 soybean cultivars: Merkur, Sava, Valjevka, Venera and Victoria supplied by the Institute of Field and Vegetable Crops at Rimski Šančevi, near Novi Sad, Serbia. Seeds for the *in vitro* experiments were collected at the full-maturity stage. The soybean seeds were hand-selected to eliminate those that were damaged or cracked. Whole seed material (1 g) was grounded to a fine powder in a mill and extracted overnight with 50 mL of 70% acetone, 70% ethanol or 70% methanol. The extracts were vacuum-filtered through sintered glass funnel and kept refrigerated until assayed.

The total phenolic content (TP) was determined using a Folin-Ciocalteu colorimetric method (Nagavani & Raghava Rao, 2010) and the results were expressed in milligrams of quercetin equivalents per 1 g of soybean seeds weight (mg QE/g). Data are reported as means for at least three replications. Total tannins (TT) content was determined by the Folin-Ciocalteu procedure, after removal of tannins by their adsorption on insoluble matrix PVPP (polyvinylpolypyrrolidone). Calculated values were subtracted from total phenolics content, and total tannin contents were expressed in milligrams of quercetin equivalents (QE) pep 1 g of dry seed weight (Nagavani & Raghava Rao, 2010). The total flavonoid (TF) content was determined spectrophotometrically (Saha et al., 2013). The amount of flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions (5-500 μg/mL).

DPPH radical scavenging activity. Scavenging of free radicals was tested in a DPPH (2,2-diphenyl-1-picrylhydrazyl) acetone solution (Lai & Lim, 2011). The degree of decoloration of solution indicates the scavenging efficiency of the substance added. Ferric-reducing antioxidant power (FRAP) assay was carried out according to the procedure described in the literature (Valentão et al., 2002). The standard curve was constructed using different concentrations of trolox, and the results were expressed as mg trolox equivalents per gram of seeds (mg TE/g). The ABTS assay was based on a method developed by Miller et al. (1993). Methanolic solution of known trolox concentrations were used for calibration and the results were expressed as mg trolox equivalents per g of seeds (mg TE/g).

The total antioxidant activity of plant extracts were evaluated by phosphomolybdenum method as reported by Kalaskar & Surana (2014). The standard curve for total antioxidant activity was plotted using trolox solution. A reducing power assay (total reduction capacity) was performed by method of Saha et al. (2013). Trolox was used as a standard. Inhibition of NO radical was performed by the method as described in Orčić et al. (2011). The radical inhibition capacity was expressed as % of inhibition compared with the blank control. The superoxide free radical scavenging activity was carried out by NBT (nitroblue tetrazolium) test (Kalaskar & Surana, 2014). The percent inhibition of superoxide anion generated was calculated using the following formula: scavenging activity (%) = (1 - absorbance of sample/absorbance of control) x 100.

Statistical analysis. Results were expressed as a mean value of determinations of 3 independent samples made in triplicates. Statistical significance was tested by analysis of variance followed by comparison of means by Duncan's multiple range test ($P < 0.05$) calculated using STATISTICA for Windows version 12.0 (StatSoft, Tulsa, OK, USA). Stepwise multiple regression analyses were used to determine correlation among variables.

Results and Discussion

The yield of phenolics extracted differs on the type of solvent, which exhibit varying polarities. Ethanol and methanol extracts of soybean seeds had lower TP content compared to acetone extracts. Natural phenolic compounds have been receiving increased attention due to their significant antioxidant activities. The unique structures make the phenolics inherently excellent hydrogen or electron donors, which enable them to readily stabilize or neutralize some harmful reactive oxygen species. Total phenolic content of the extracts from selected soybean cultivars are presented in table 1. The tested soybean seeds had a TP range of 2.228-5.251 mg QE/g. The TP content values were significantly different among different soybean genotypes and solvent used for extraction. In particular, the Venera cultivar had the highest TP content, while the Merkur had the lowest. These results are in accordance with the findings of some other authors concerning TP contents in soybean seeds (Malenčić et al., 2007, 2012; Bellaloui, 2012; Dajanta et al., 2013; Popović et al., 2013). However, some authors detected much higher (Taie et al., 2008) or lower (Mujić et al., 2011) content of TP in tested soybean seeds.

Tannins are common phenolic compounds in plants and are known for their ability to bind protein which reduces nitrogen availability in the diet (Zungu & Downs, 2015). Substantial accumulations of tannins may occur in almost any part of a plant: seeds, root, leaves and bark (Haslam, 2007). Tannins are also strong natural antioxidants (Figueroa-Espinoza et al., 2015). The total tannins content ranged from 0.685 mg QE/g (cv. Merkur, ethanol extract) to 2.256 mg QE/g (cv. Valjevka, acetone extract). These results are in agreement with the range found in literature (Popović et al., 2013) and within the range previously found for other yellow pigmented soybean seeds (Malenčić et al., 2007, 2012).

Flavonoids are a wide group of plant secondary metabolites, occurring in all parts of the plants. They have a variety of functions in plant biochemistry and physiology, acting as antimicrobials, antioxidants, UV protectors, photoreceptors, and also play an important role in nitrogen fixation. Flavonoids have been described as health-promoting agents as well (Karabin et al., 2015). The range of total flavonoids in all tested soybean seeds varied between 0.237 and 1.475 mg QE/g (Table 1).

Among different solvent extracts tested in this study, 70% acetone extract of seeds showed the highest TP, TT and TF contents, followed by 70% methanol extracts. The antioxidant activity of plant extracts may vary with assay performed. Therefore, a single assay could be inadequate (Yen et al., 2005).

Tab. 1. Content of total phenolics, tannins, flavonoids and proanthocyanidins in extracts of soybean seeds
Садржај укупних фенола, танина, флавоноида и проантоцијана у екстрактима сјемена соје

Cultivar *Сорта*	Solvent *Растварач*	Total phenolics[1] *Укупни феноли*	Total tannins[1] *Укупни танини*	Total flavonoids[1] *Укупни флавоноиди*
Merkur	70% methanol	$2.228^{a} \pm 0.052$	$0.925^{b} \pm 0.054$	$1.475^{a} \pm 0.087$
	70% ethanol	$2.255^{a} \pm 0.106$	$0.685^{a} \pm 0.071$	$0.237^{b} \pm 0.010$
	70% acetone	$3.278^{b} \pm 0.289$	$0.806^{a,b,c} \pm 0.233$	$0.755^{c,d} \pm 0.062$
Sava	70% methanol	$2.782^{c,d,e} \pm 0.133$	$1.146^{d} \pm 0.109$	$0.684^{c} \pm 0.033$
	70% ethanol	$3.042^{b,c,e} \pm 0.320$	$0.982^{a,b,d} \pm 0.298$	$0.416^{e} \pm 0.014$
	70% acetone	$3.967^{f} \pm 0.517$	$1.025^{b,d} \pm 0.238$	$0.567^{f} \pm 0.055$
Valjevka	70% methanol	$2.679^{d} \pm 0.083$	$1.082^{c,d} \pm 0.054$	$0.764^{c,g} \pm 0.069$
	70% ethanol	$2.695^{d} \pm 0.091$	$1.010^{b,d} \pm 0.183$	$0.636^{c,f,h} \pm 0.145$
	70% acetone	$5.251^{g} \pm 0.845$	$2.256^{e} \pm 0.214$	$0.747^{d,g,h,i} \pm 0.011$
Venera	70% methanol	$3.255^{b} \pm 0.199$	$1.529^{f} \pm 0.175$	$0.601^{f,j} \pm 0.039$
	70% ethanol	$3.113^{b} \pm 0.096$	$1.104^{c,d} \pm 0.108$	$0.340^{k} \pm 0.022$
	70% acetone	$4.862^{g} \pm 0.218$	$2.060^{e} \pm 0.069$	$0.678^{c.i,j} \pm 0.080$
Victoria	70% methanol	$2.896^{e} \pm 0.225$	$1.193^{d} \pm 0.040$	$0.670^{c} \pm 0.030$
	70% ethanol	$2.680^{d} \pm 0.517$	$0.822^{a,b} \pm 0.254$	$0.332^{b,e,k} \pm 0.119$
	70% acetone	$3.803^{f} \pm 0.310$	$0.882^{b} \pm 0.086$	$1.343^{l} \pm 0.070$

[1]mg quercetin equivalents (QE)/g
[a-l] values without the same superscript within each row differ significantly ($P < 0.05$)

For this reason, we checked antioxidant activities of different extracts of soybean seeds with seven different assays. Antioxidant activities measured in three different extracts obtained using DPPH, ABTS and FRAP assays are presented in Table 2.

Tab. 2. Antioxidant activity (DPPH, ABTS and FRAP assays) in extracts of soybean seeds
Антиоксидативно дејство (DPPH, ABTS і FRAP огледи) у екстрактима сјемена соје

Cultivar *Сорта*	Solvent *Растварач*	DPPH[1]	ABTS[1]	FRAP[1]
Merkur	70% methanol	$7.50^{a,c} \pm 0.18$	$14.70^{a} \pm 0.89$	$11.71^{a} \pm 0.10$
	70% ethanol	$10.98^{b} \pm 0.04$	$18.93^{b} \pm 2.17$	$12.64^{b} \pm 0.43$
	70% acetone	$6.37^{c} \pm 0.03$	$20.73^{b,c} \pm 2.25$	$12.85^{b} \pm 0.65$
Sava	70% methanol	$8.45^{a,f} \pm 0.04$	$23.45^{b,d,e} \pm 3.73$	$10.72^{a,c} \pm 0.62$
	70% ethanol	$9.73^{d} \pm 0.07$	$25.91^{e,i} \pm 2.14$	$11.10^{c,d} \pm 0.25$
	70% acetone	$5.10^{e} \pm 0.05$	$27.09^{e.f,h.i} \pm 2.89$	$10.79^{c,d} \pm 0.36$
Valjevka	70% methanol	$6.51^{c} \pm 0.05$	$22.37^{b,g} \pm 1.89$	$10.89^{a,d.e} \pm 0.72$
	70% ethanol	$7.12^{c,f} \pm 0.16$	$24.54^{d,g,h} \pm 1.99$	$12.57^{b} \pm 0.42$
	70% acetone	$4.68^{e} \pm 0.05$	$29.69^{i} \pm 2.48$	$11.41^{a,d} \pm 0.47$
Venera	70% methanol	$2.13^{g} \pm 0.10$	$26.49^{d,e,i} \pm 1.71$	$10.75^{a,d} \pm 0.61$
	70% ethanol	$2.35^{g,h} \pm 0.11$	$23.32^{c,d,e,g} \pm 1.60$	$11.48^{a,d} \pm 0.36$
	70% acetone	$3.76^{h} \pm 0.03$	$31.17^{f,i} \pm 2.18$	$11.40^{a,b,d} \pm 0.82$
Victoria	70% methanol	$8.39^{a,f} \pm 0.05$	$24.45^{c,d,e,g} \pm 2.12$	$10.53^{c,e} \pm 0.32$
	70% ethanol	$9.57^{d} \pm 0.06$	$23.17^{c,d,e,g} \pm 2.26$	$11.41^{a,d} \pm 0.47$
	70% acetone	$5.42^{e} \pm 0.07$	$26.54^{d,e,i} \pm 2.03$	$11.63^{a} \pm 0.12$

[1] mg trolox equivalents/g
[a-i] values without the same superscript within each row differ significantly ($P < 0.05$)

The antioxidant activity of extracts from soybean seeds as measured by DPPH assay ranged from 2.12 mg TE/g (cv. Venera, methanol extract) to 10.98 mg TE/g (cv. Merkur, ethanol extract). The best antioxidant activity measured with DPPH assay is obtained with 70% ethanol followed by 70% methanol. Differences for the ABTS radical cation scavenging capacities of each sample was recorded in this study. Among various samples acetone extract of cv. Venera possessed the highest ABTS radical scavenging activity (31.17 mgTE/g).

Tab. 3. Total antioxidant activity, total reduction capacity and scavenger activity of NO and O_2 radicals of soybean seeds extracts

Укупно антиоксидативно дејство, укупан капацитет редукције и јонизујућа активност NO и O_2 радикала из екстраката сјемена соје

Cultivar *Сорта*	Solvent *Растварач*	Total antioxidant activity[1] *Укупно антиоксидативно дејство*	Total reduction capacity[1] *Укупан капацитет редуковања*	NO radical inhibition *Без инхибиције слободних радикала*	Scavenging of O_2 radicals *Јонизација радикала O_2*
Merkur	70% methanol	$45.89^{a,e} \pm 2.17$	$15.59^{a,d,e} \pm 1.15$	$64.37^{a} \pm 3.33$	$48.16^{a} \pm 2.98$
	70% ethanol	$46.74^{a,b,e} \pm 2.40$	$16.12^{a,b,d,e} \pm 1.20$	$40.94^{b} \pm 4.71$	$13.57^{b,c} \pm 3.43$
	70% acetone	$52.39^{c,d,e} \pm 1.96$	$22.63^{c} \pm 0.13$	$19.59^{c} \pm 4.38$	$9.49^{c} \pm 0.68$
Sava	70% methanol	$44.21^{a} \pm 1410.53$	$13.63^{d,e} \pm 1.17$	$74.16^{d} \pm 4.11$	$36.86^{d} \pm 3.43$
	70% ethanol	$47.15^{a,d,e} \pm 0.53$	$14.91^{d,e,g,j} \pm 0.95$	$33.33^{b,e,f,g} \pm 6.47$	$16.70^{b,e} \pm 3.30$
	70% acetone	$52.17^{c,d,e} \pm 1.57$	$19.78^{f} \pm 0.19$	$17.75^{c,e} \pm 7.51$	$18.20^{b} \pm 4.45$
Valjevka	70% methanol	$45.53^{a,e} \pm 2.17$	$16.32^{a,g} \pm 0.15$	$72.85^{a,d} \pm 5.14$	$46.51^{a,d,f} \pm 5.03$
	70% ethanol	$46.99^{a,e} \pm 2.68$	$17.81^{a,f,h} \pm 1.33$	$47.07^{b,h} \pm 7.61$	$27.22^{d,e,g} \pm 6.99$
	70% acetone	$54.30^{c} \pm 0.67$	$21.20^{i} \pm 0.86$	$31.53^{f,i} \pm 1.58$	$5.73^{h} \pm 0.76$
Venera	70% methanol	$42.52^{a} \pm 3.07$	$16.14^{a,j} \pm 0.69$	$71.92^{d} \pm 2.60$	$42.82^{f} \pm 0.62$
	70% ethanol	$49.38^{e} \pm 1.20$	$18.41^{h} \pm 0.20$	$26.88^{b,c,i} \pm 4.56$	$14.27^{b} \pm 1.79$
	70% acetone	$50.72^{b,c,d,e} \pm 1.35$	$23.01^{k} \pm 0.10$	$42.15^{g,h} \pm 5.28$	$19.69^{b,g} \pm 3.03$
Victoria	70% methanol	$41.74^{a} \pm 2.10$	$15.59^{a,d,e} \pm 1.07$	$68.03^{a,d} \pm 4.62$	$50.51^{a} \pm 1.83$
	70% ethanol	$45.07^{a} \pm 1.26$	$17.63^{b,h} \pm 0.46$	$38.26^{b} \pm 3.36$	$11.84^{b,c,h} \pm 3.75$
	70% acetone	$52.52^{c,e} \pm 1.57$	$23.99^{l} \pm 0.44$	$20.31^{c} \pm 2.59$	$9.49^{b,c,h} \pm 2.23$

[a-l] values without the same superscript within each row differ significantly ($P < 0.05$)

Methanol extract of cv. Merkur showed the lowest ABTS radical scavenging activity (14.70 mgTE/g). Acetone extracts demonstrated higher scavenging activities then other extracts. FRAP test showed that soybean seeds have a significant reduction potential. However, all samples expressed similar activity. Our research shows that the FRAP method is independent of the solvent extraction polarity.

Results of total antioxidant activity, total reduction capacity, inhibition of NO radical and superoxide anion (O_2^-) radical scavenging activity are shown in Table 3. The phosphomolybdenum assay is a quantitative method to evaluate fat and water soluble antioxidant activity (total antioxidant activity), in which by transforming Mo(VI) into more stable Mo(V) non-reactive products occur (Kalaskar & Surana, 2014). Transformation of Fe^{3+} to Fe^{2+} in the presence of soybean seeds extracts was performed to measure the total reductive capability.

The total antioxidant activity and total reduction capacity in all tested genotypes was similar. The lowest bioactivity was measured in methanol extracts, while the total antioxidant activity of acetone extracts found to be the highest. On the contrary, the methanol extracts of all soybean cultivars expressed the highest scavenging activity for nitric oxide and superoxide radicals, while acetone extracts possessed the lowest scavenging activity for both radicals. There was a statistically significant correlation between TP content and TT content (r = 0.806), as well as, between TP content and antioxidant capacity measured with some, but not all, assays (DPPH: r = 0.578; ABTS: r = 0.828; total antioxidant activity: r = 0.702; total reduction capacity: r = 0.713). In this study, no statistically significant correlation was observed between antioxidant activity and TF content in soybean seeds. Positive correlation between amount of phenolic compounds in samples of different plant origin and antioxidant capacity is supported by work of other researchers (Usenik et al., 2008; Malenčić et al., 2010; Prvulović et al., 2012).

Conclusion

The results of the present investigation revealed that phenolic compound contents and antioxidant capacity of extracts of soybean seeds are significantly affected by the solvent system used for the extraction process. Out of the three solvent mixtures evaluated in the current study for the extraction of phenolic compounds, the use of 70% acetone yielded to the highest total contents of phenolics and exhibited the highest antioxidant activity in few assays (DPPH, total antioxidant activity and total reduction capacity.

The use of 70% ethanol solvent resulted in higher antioxidant activity measured by ABTS assay, while extracts in 70% methanol had higher scavenging activity of NO radicals. Data on phenolic compounds investigated in this study, as well as the antioxidant capacity of extracts of soybean seeds cultivars could be valuable to the food and pharmaceutical industries for the selection of varieties rich in nutriceuticals and could be also valuable for soybean producers in order to increase the biological value of commercial products.

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Андиоксидативно дејство и садржај фенола екстраката сјемена соје

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Сажетак

Биљке су добар извор природних антиоксиданаса, и могу имати заштитно својство против штетних слободних радикала. Фенолна једињења представљају важан дио људске исхране а налазе се у активном облику у многим биљкама које се користе у пољопривредној производњи и медицини. Детаљније информације о једињењима корисним у исхрани, изолованим из различитих сорти соје, омогућују разумијевање ове проблематике, као и повећано гајење овог усјева, укључујући коришћење усјева за обезбјеђење додатака исхрани. Циљ овог рада представља одређивање садржаја укупних фенола, укупних танина, укупних флавоноида и антиоксидативног дејства различитим методама, за пет сорти соје поријеклом из Србије (Меркур, Сава, Ваљевка, Венера и Викторија) и коришћењем три различита растварача (70% ацетон, 70% етанол и 70% метанол). Садржај укупних фенола је варирао за различите сорте и растваваче. Антиоксидативно дејство је значајно зависило од растварача кориштеног за екстракцију. Ови резултати истичу постојећи варијабилитет у сјемену соје и наглашавају потребу вриједновања ових различитости, као и подршке традиционалним програмима оплемењивања у циљу побољшања нутритивне вриједности соје.

Кључне ријечи: антиоксидативно дејство, растварачи за екстракцију, флавоноиди, *Glycine max* (L.) Merr., танини

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