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# Enhancement of antioxidant activity and bioactive compound contents in yellow soybean by plant-extract-based products

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**Abstract:** Polyphenols present in different plant cell organelles increase the resistance of plants to various types of environmental stresses. We investigated the possibility of increasing the content of bioactive compounds in the seed of yellow soybean variety Laura. The soybean was treated during vegetation with five products based on plant extracts, on the assumption of enrichment of plants with various nutrients. Soybean flour extracts were screened spectrophotometrically for total phenolic content and antioxidant activity. The antioxidant activity was evaluated using three methods. The content of phenolic acids was determined by HPLC, and the raw protein content was estimated by the Kjeldahl method. Depending on the treatment, variations in the quantity of individual phenolic acids with up to 90% higher concentration as compared to the control were observed. Controlled usage of certain plant extracts can increase the concentration of the target group of bioactive compounds in the samples. The synergistic effect of proteins and phenolic compounds on the antioxidant activity of extracts was detected. The results of this study are not only important from the aspect of plant resistance to various types of stress, but also when considering soybean as a functional food.

**Keywords:** antioxidant activity; plant extracts; phenolic acids; soybean seeds

## INTRODUCTION

Many compounds of natural origin activate plant metabolism by intensifying processes such as photosynthesis, respiration, adsorption and transport of ions, and consequently plant growth, contributing to greater bioproduction and yields of wild and cultivated plants.

Soybean (*Glycine max (L) Merrill*), as one of the major legume plants and one of the five most widespread crops in the world, is a suitable object for exploring the impact of different natural compounds on plants. Soybean is a plant with an extremely favorable chemical composition of grains, containing 36-42% protein and 15-23% of oil, which makes it species very useful for human consumption as well as animal feed. Soybean proteins are rich in essential amino acids (histidine, isoleucine, leucine, phenylalanine, threonine, tryptophan, valine and in small amounts of methionine and lysine), which are not found in animal proteins [1]. Soybean also contains vitamins of the B complex, β-carotene, minerals (mostly Ca and Fe) and small amounts of vitamins C, D, E, and K. These natural antioxidants prevent the oxidation of LDL cholesterol. Soybean seeds contain high concentrations of phenolic compounds



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[2], phenolic acids and flavonoids, most of which are isoflavones. Phenolic compounds are one of the largest, most widespread and biologically (physiologically and medically) important groups of secondary plant metabolites. They represent an integral part of the cell wall structure, mainly in the form of polymeric materials (e.g. lignin), providing a mechanical support for plant cells and a barrier to microorganisms [3,4]. They also participate in various biochemical processes related to photosynthesis, protect plants from bacteria, fungi and viruses, as well as from mechanical damage. Phenolic compounds, together with α-tocopherol and β-carotene, belong to the group of nonenzymatic antioxidants, and have found application in food and pharmaceutical industries [5,6]. In order to improve the chemical composition of soybean seeds, many studies have examined the influence of different methods of seed processing (fermentation and thermal treatment) on the phenolic compound contents [7,8], but there are very few reports on the effect of treatment during the vegetation period, on changes in phenolic compounds and the protein content in soybean seed.

The aim of this paper was to examine the changes in the contents of proteins, phenolic acids and the total phenolic content, and antioxidant activity of soybean seed as a result of the treatment of soybean plants with different products based on plant extracts. The results point to the possibility of producing soybeans with different classes of bioactive compounds, controlled by the application of natural products during the vegetative stage. It can be assumed that soybean seed with an altered chemical composition could positively affect the germination and early stages of plant growth.

## MATERIALS AND METHODS

### Chemicals and reagents

Gallic acid (97.5%), *trans*-cinnamic acid (97%), *p*-coumaric acid (98%), caffeic acid (≥98%), ferulic acid (99%); 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX, 97%), analytical grade water, acetonitrile (≥99.9%), methanol (≥99.9%), were purchased from Sigma-Aldrich (St. Louis, USA); 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ, ≥99%), was purchased from Fluka, (Büchi, Switzerland); L(+)-ascorbic acid (99%), was purchased from Acros Organics, (New Jersey, USA); chlorogenic acid (99.5%) was purchased from Chromadex, (USA); Folin-Ciocalteu reagent was purchased from Reagecon, (Ireland). Kjeldahl catalyst tablets were purchased from Fisher (UK). All other solvents and chemicals were of p.a. grade or higher purity.

### Plant material and plant-extract-based products

Experiments were conducted in 2016 in two experimental fields of the Maize Research Institute "Zemun Polje", Belgrade, Serbia, on slightly calcareous chernozem, with 3.3% organic matter and pH 7.17. The initial experimental plot area was 25 $m^2$ (4×6.25 m) with a spacing of 50 cm between rows and 17.5 cm between plants in a row. The experiments on each experimental field were carried out in three replications; a randomized plot design was applied. The soybean variety Laura (yellow grain) was used. This variety was selected for several reasons; it is characterized by the lack of Kunitz trypsin inhibitor (an inhibitor with significant antinutritive effects), which makes it very useful; the results of our previous research on this variety [9,10] indicated that the treatment with plant-extract-based products positively affected the contents of microelements and β-carotene, which together with phenolic compounds belong to the group of nonenzymatic antioxidants. Plants were treated with five plant-extract-based products of natural origin (plant and seaweed extracts), which are commercially used in plant production. The products used were: (1) "Vegard" (VEG) – extract of Chinese medicinal plants (with 1-2% $C_{org}$, 2-5% $N_{org}$, 5% humic acids, 10% fulvic acid, 1% $K_2O$, 1-2% amino acids); (2) "Ekofus" (EF) – a fucus algae (*Fucus vesiculosus*) extract (with 1.8% $N_{tot}$, 1% $P_2O_5$ (water-soluble), 2% $K_2O$ (water-soluble), 0.5% MgO, 1.8% Fe); (3) "Calbit-C" (CAL) – extract of wood chips treated with $H_2SO_4$ and $CaCO_3$ (with 15% CaO (water-soluble Ca in the form of Ca-lignosulphonate) and 4.5% $C_{org}$); (4) "Cropmax" (CRM) – plant extract (sugar cane molasses with 1.7% $N_{tot}$, 2% total amino acids, 2% $C_{org}$); (5) "Zircon" (ZIR) – extract of the medicinal plant *Echinacea pallida* (with 0.1 g/L phenolic acids: caffeic, chlorogenic and cichoric acids). Treatments were applied twice during the season, at the beginning and during the flowering stage, in accordance with producer's recommended doses. After the harvest and yield assessments, soybean seeds (100 g per treatment) were collected and stored in the freezer (-18°C±0.5°C) until analysis.

**Pre-extraction preparation of samples**

All samples were ground in a laboratory mill (IKA, IP-21, USA) and sieved through a laboratory sieve of 500-μm diameter. Flour samples were defatted in petrol ether in a Soxhlet extractor for 4 h at a sample/solvent ratio of 1:10 g/mL before extraction. The moisture content was determined by gravimetry. Defatted soybean flour (5 g per sample) was dried in an oven at 105°C until a constant mass was attained. This data was used to calculate the amounts of analytes in seed dry mass.

**Extraction of free phenolic acids**

Defatted soybean flour (0.5 g per sample) was mixed with 10 mL of the extraction solvent (a mixture of methanol and 10% hydrochloric acid at a ratio of 85:15% (v/v)). Selection of this extraction mixture was adopted on the basis of our previous research [11]. Extraction was carried out in a shaker (VXR basic VIBRAX, IKA) overnight. Samples were centrifuged (15 min at 12000×*g* in a MiniSpin Eppendorf centrifuge), filtered through a 45-μm syringe filter and subjected to HPLC analysis. Compounds in the supernatant are present in a soluble form.

**Alkaline hydrolysis**

A precipitate from the previous step was subjected to alkaline hydrolysis (with 10 mL 4 M NaOH, for 4 h at room temperature), to release phenolic acids covalently bound to other molecules such as fatty acids (soluble esters). A fraction of esterified phenolic acids was separated with 2×15 mL of a diethyl ether (DE) ethyl acetate (EA) mixture (1:1). After centrifugation, the DE/EA layers with isolated phenolic acids were evaporated to dryness in a rotary evaporator at 40-45°C, then dissolved in 2 mL methanol, filtered through a 45-μm syringe filter and subjected to HPLC analysis.

**Acid hydrolysis**

The lower aqueous layer, remaining after alkaline hydrolysis, was subjected to acid hydrolysis (with 2.5 mL concentrated HCl, for 30 min at 80°C), in order to break chemical bonds and isolate phenolic acids bound to cell wall substances, including pectin, cellulose, hemicellulose, arabinoxylans and structural proteins. Separation of the released phenolic acids was carried out with 2×15 mL of DE/EA (1:1). After centrifugation, the DE/EA layers with isolated phenolic acids were evaporated to dryness in a rotary evaporator at 40-45°C, dissolved in 2 mL methanol, filtered through a 45-μm syringe filter and subjected to HPLC analysis.

**Quantification of individual phenolic acids**

Quantification of six monitored phenolic acids was carried out by liquid chromatography. The Shimadzu Nexera HPLC system was used, and separation of phenolic acids was performed on an Agilent Zorbax SB C18 column (250×4.6 mm, id 5 μm). The column was thermostatically controlled at 25°C and the flow rate was set to 1 mL/min. A two-component mobile phase was used, comprised of 0.1% formic acid (A) and methanol (B), and the gradient was adopted from Đurović et al. [11]. Identification and quantification of the phenolic acids were performed at two wavelengths, at 280 nm for gallic and *trans*-cinnamic acid, and at 325 nm for *p*-coumaric, ferulic, chlorogenic and caffeic acids. Identification of phenolic acids was accomplished by comparing the retention time of the peaks to those of standard compounds. Quantitative analysis of phenolic acids was based on calibration curves constructed for each compound identified in the samples. Calibration curves were performed according to Đurović et al. [11]. The results were expressed as μg/g of dry matter (d.m.). A HPLC-DAD chromatogram of a standard mixture of six phenolic acids (concentration of 50 μg/mL), and HPLC-DAD chromatograms of all three fractions of phenolic acids are presented in Supplementary Fig. S1.

**Total phenolic content**

The total phenolic content (TPC) in all three fractions (free, esterified and bound) was determined by a Folin-Ciocalteu assay using gallic acid (GA) as the standard as described [12]. The absorbance was measured at 765 nm using a Shimadzu UV-2100 spectrophotometer. The concentrations of total phenolic compounds were expressed as mg GAE/g d.m.

**Determination of antioxidant activity by the DPPH assay**

For the DPPH assay, the same extracts were used as well as for determination of the TPC. To determine

the antioxidant activity of soybean seeds, the method of Chen and Ho [13] was used, slightly modified according to Đurović et al. [11]. The DPPH radical scavenging activity was determined spectrophotometrically by measuring the absorbance at 517 nm against methanol and calculated from the standard curve of Trolox reagent. The results were expressed as micromoles of Trolox equivalents (TE) per g of dry matter ($\mu$mol TE/g d.m.).

**Determination of antioxidant activity by the Briggs-Rauscher reaction method**

The dynamics of Briggs-Rauscher (BR) reaction were followed potentiometrically [14]. A Pt electrode was used as a working electrode, while the double junction Ag/AgCl was used as the reference electrode. The initial concentrations of reactants for the BR reaction were $[CH_2(COOH)_2]_0$=0.0789 mol/L, $[MnSO_4]_0$=0.00752 mol/L, $[HClO_4]_0$=0.0300 mol/L, $[KIO_3]_0$=0.0752 mol/ L and $[H_2O_2]_0$=1.2690 mol/L. The reaction volume was 25 mL. An aliquot (100 µL) of the soybean extract was added 30 s after hydrogen peroxide addition in a BR oscillatory regime. It is important to note that the experimental setup was adjusted to a human organism, with the temperature maintained at T=37±0.1°C and the BR reaction solution pH of stomach pH<2.

**Determination of antioxidant activity by the FRAP assay**

The FRAP method (ferric ion reducing antioxidant power assay) is based on the reduction of the $Fe^{3+}$-tripyridyl-triazine complex ($Fe^{3+}$-TPTZ) to the $Fe^{2+}$-tripyridyl-triazine complex. The amount of reduced $Fe^{2+}$-TPTZ complex was calculated from the equation of regression law, which was constructed by measuring the absorption of standard aqueous solutions of $FeSO_4\times7H_2O$ at 593 nm. FRAP was performed by the Benzie and Strain method [15] for all three fractions of phenolic acids (free, esterified and bound). Ten-times diluted plant extracts or $FeSO_4\times7H_2O$ solutions (150 µL) were mixed with 2.5 mL of FRAP reagent and incubated at 37°C in a water bath for 30 min. A calibration curve of $FeSO_4\times7H_2O$ was prepared at concentrations ranging from 10-1000 µmol/L. The results were expressed as µmol $Fe^{2+}$ eq/g d.m.

**Determination of total nitrogen (Kjeldahl method) and the protein content**

The Kjeldahl method is almost universally applied for the determination of the nitrogen content in plant material [16]. Total nitrogen is then multiplied by a factor to calculate the protein content. A factor of 5.71 was used to calculate the protein content in soybean seeds, according to literature data [17,18]. Soybean flour (0.5 g per sample) was digested in a Kjeldahl digestion flask by boiling with 10 mL of concentrated sulfuric acid and a Kjeldahl catalyst tablet until the mixture was clear (about 2 h) The digest was cooled and 20 mL of distilled water were added and connected for distillation. Ammonia was steam-distilled from the digest to which 50 mL of 32% sodium hydroxide solution were added. The distillate was collected in a conical flask containing 50 mL 0.1 M $H_2SO_4$ and Tashiro indicator. The ammonia that distilled into the receiving conical flask reacted with the acid and the excess acid in the flask was estimated by back-titration against 0.2 M NaOH with a color change from purple to green (endpoint). Determinations were made on all reagents alone (blank determinations). Total nitrogen was multiplied by 5.71 to arrive at the protein content.

**Oil determination**

The oil content was determined by the Soxhlet extraction method, in accordance with international standard EN ISO 659:2009. Subtraction of the mass prior to and after extraction provides the oil content in soybean seed.

**Statistical analysis**

Extraction procedures using each treatment were carried out in duplicate, all measurements were carried out in triplicate, and the values are reported as the means with standard deviation (SD). Analysis of variance (ANOVA), followed by Tukey's test, were performed to compare the effects of different plant-extract-based products and to determine significant differences between groups of results under a significance level of $p<0.05$. Principal component analysis (PCA) was used for evaluation of the interdependence of the phenolic acid contents and different treatments. All statistical analyses were performed by SPSS 15.0 (IBM Corporation, Armonk, New York, USA) for Windows Evaluation version.

## RESULTS

### Quantification of free, esterified and bound phenolic acids

The identification of phenolic acids in soybean seed extracts was based on external standards of phenolic acids; the compounds corresponding to the peaks were identified after comparing their retention times and HPLC-DAD spectra. HPLC-DAD chromatograms of standards and methanolic soybean extracts in all three fractions are presented in Supplementary Fig. S1. The amounts of six phenolic acids in three different fractions in soybean flour extracts are presented in Table 1 (free fraction), Table 2 (esterified solubilized fraction), and Table 3 (bound fraction). The highest amount of phenolic acids in soybean seed was detected in the free soluble fraction (Table 1). The biggest change was observed in the amount of *p*-coumaric acid, whose concentration was increased by "Calbit-C", "Cropmax" and "Zircon" treatments to 39.98%, 42.31% and 40.91% (w/w), respectively. In this fraction, the smallest effect on the variation in the amount of monitored phenolic acids was observed with the treatment with "Ekofus".

**Table 1.** Content of six individual phenolic acids in the free soluble fraction (μg/g d.m.) in soybean seeds influenced by treatments with different plant-extract-based products.

| | "Control" | "Vegard" | "Ekofus" | "Calbit-C" | "Cropmax" | "Zircon" |
|---|---|---|---|---|---|---|
| GaA | 55.85±0.60$^{a}$ | 55.97±0.74$^{a}$ | 63.61±0.53$^{c}$ | 70.25±0.52$^{e}$ | 67.21±0.50$^{d}$ | 58.86±0.42$^{b}$ |
| ChA | 80.21±0.47$^{c}$ | 76.76±0.65$^{b}$ | 70.17±0.74$^{a}$ | 89.16±0.76$^{ef}$ | 88.06±0.49$^{e}$ | 84.77±0.53$^{d}$ |
| CaA | 36.66±0.38$^{c}$ | 28.71±0.43$^{a}$ | 28.12±0.58$^{a}$ | 39.22±0.45$^{d}$ | 40.05±0.59$^{d}$ | 34.72±0.43$^{b}$ |
| CoA | 215.57±0.84$^{a}$ | 281.3±1.39$^{c}$ | 261.65±1.47$^{b}$ | 301.76±1.48$^{d}$ | 306.77±1.07$^{f}$ | 303.77±1.20$^{e}$ |
| FeA | 156.00±0.56$^{a}$ | 179.05±1.30$^{b}$ | 157.11±1.10$^{a}$ | 181.63±1.23$^{c}$ | 187.18±1.27$^{e}$ | 184.47±1.14$^{d}$ |
| TCA | 206.33±0.86$^{b}$ | 226.00±1.0$^{c}$ | 187.6±1.25$^{a}$ | 233.96±1.50$^{d}$ | 238.34±1.19$^{e}$ | 264.09±1.40$^{f}$ |

For each treatment, determinations of the phenolic acid contents were conducted in triplicate. Values are expressed as the mean with standard deviation (SD). Means followed by same letters within the same row are not significantly different at the 0.05 level.
Abbreviations: GaA – gallic acid; ChA – chlorogenic acid; CaA – caffeic acid; CoA – *p*-coumaric acid; FeA – ferulic acid; TCA – *trans*-cinnamic acid.

**Table 2.** Content of six individual phenolic acids in the esterified soluble fraction (μg/g d.m.) in soybean seeds influenced by treatments with different plant-extract-based products.

| | Control | "Vegard" | "Ekofus" | "Calbit-C" | "Cropmax" | "Zircon" |
|---|---|---|---|---|---|---|
| GaA | 57.69±0.64$^{c}$ | 53.98±0.24$^{b}$ | 52.55±0.64$^{a}$ | 60.85±0.47$^{d}$ | 60.45±0.32$^{d}$ | 57.53±0.53$^{c}$ |
| ChA | 52.1±0.51$^{b}$ | 56.13±0.36$^{c}$ | 45.86±0.44$^{a}$ | 58.4±0.57$^{d}$ | 58.75±0.35$^{d}$ | 59.59±0.48$^{d}$ |
| CaA | 53.77±0.48$^{c}$ | 56.02±0.36$^{d}$ | 48.7±0.64$^{a}$ | 51.68±0.44$^{b}$ | 51.59±0.47$^{b}$ | 55.68±0.61$^{d}$ |
| CoA | 177.07±0.73$^{a}$ | 342.77±0.66$^{f}$ | 247.39±1.58$^{c}$ | 264.56±1.27$^{d}$ | 223±0.99$^{b}$ | 268.39±1.29$^{e}$ |
| FeA | 146.28±0.38$^{a}$ | 266.24±0.73$^{f}$ | 201.7±1.53$^{c}$ | 223.73±1.03$^{e}$ | 185.63±1.35$^{b}$ | 207.05±1.09$^{d}$ |
| TCA | 45.79±0.54$^{a}$ | 47.36±0.36$^{c}$ | 45.86±0.59$^{a}$ | 48.64±0.57$^{d}$ | 48.51±0.45$^{d}$ | 46.49±0.54$^{b}$ |

For each treatment, determinations of the phenolic acid contents were conducted in triplicate. Values are expressed as the mean with standard deviation (SD). Means followed by same letters within the same row are not significantly different at the 0.05 level.
Abbreviations: GaA – gallic acid; ChA – chlorogenic acid; CaA – caffeic acid; CoA – *p*-coumaric acid; FeA – ferulic acid; TCA – *trans*-cinnamic acid.

**Table 3.** Contents of six individual phenolic acids in the bound insoluble fraction (μg/g d.m.) in soybean seeds influenced by treatments with different plant-extract-based products.

| | "Control" | "Vegard" | "Ekofus" | "Calbit-C" | "Cropmax" | "Zircon" |
|---|---|---|---|---|---|---|
| GaA | 69.41±0.63$^{a}$ | 71.1±0.57$^{b}$ | 75.95±0.59$^{c}$ | 80.16±0.54$^{d}$ | 80.68±0.56$^{d}$ | 80.56±0.46$^{d}$ |
| ChA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| CaA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| CoA | 45.05±0.49$^{b}$ | 42.75±0.21$^{a}$ | 44.61±0.50$^{b}$ | 49.26±0.47$^{de}$ | 46.97±0.54$^{c}$ | 48.73±0.51$^{d}$ |
| FeA | 43.79±0.37$^{b}$ | 43.08±0.23$^{a}$ | 44.8±0.52$^{c}$ | 51.35±0.50$^{f}$ | 47.66±0.58$^{e}$ | 45.58±0.59$^{d}$ |
| TCA | 43.06±0.30$^{a}$ | 44.19±0.23$^{b}$ | 45.59±0.52$^{c}$ | 46.91±0.43$^{d}$ | 51.92±0.48$^{e}$ | 46.71±0.53$^{d}$ |

For each treatment, determinations of the phenolic acid contents were conducted in triplicate. Values are expressed as the mean with standard deviation (SD). Means followed by same letters within the same row are not significantly different at the 0.05 level.
Abbreviations: GaA – gallic acid; ChA – chlorogenic acid; CaA – caffeic acid; CoA – *p*-coumaric acid; FeA – ferulic acid; TCA – *trans*-cinnamic acid; n.d. – not detected.

Esterified soluble *p*-coumaric and ferulic acids showed the biggest changes depending on the type of the treatments (Table 2). The treatment with "Vegard" led to an increase in the content of *p*-coumaric and ferulic acids by 93.58% (w/w) and 82% (w/w), respectively. Although "Vegard" and "Ekofus" exerted the smallest effect on the variations of free phenolic acids (Table 1), their impact was great on the esterified fraction (Table 2).

The smallest amount of phenolic acids was released after acid hydrolysis (Table 3). In this fraction, chlorogenic and caffeic acids were not detected in any sample, and changes in the amounts of other phenolic acids varied significantly less when compared to the free and esterified fractions. Parallel with the free fraction (Table 1), treatments with "Calbit-C", "Cropmax" and "Zircon" increased the amount of phenolic acids in this fraction (Table 3).

The results of ANOVA indicate a statistically significant difference ($p<0.05$) in the content of individual phenolic acids depending on the treatment in each fraction.

## Total phenolic contents and antioxidant activities of the extracts

The antioxidant activity cannot be fully described using a single method because it is influenced by many factors such as post-harvest storage, processing conditions, environmental conditions, physiological factors in plants, etc. [19,20]. Consequently, it is appropriate to use several assays instead of a single one to evaluate and compare the antioxidant activities of different substances of plant origin. Furthermore, antioxidant activities are also difficult to interpret because of the different viewpoints on the molecular mechanisms of free-radical scavenging, and the structural dependency of antioxidant action and different reaction rate constants towards radicals or molecules used in different antioxidant assessments. Thus, the antioxidant activities of the investigated soybean extracts were evaluated using different methods: TPC (Fig. 1), free radical scavenging activity by DPPH, reducing power assays FRAP (Fig. 2), and the BR reaction method (Fig. 3).

Comparing the outcomes of TPC, DPPH and FRAP with the results obtained by the BR reaction method,

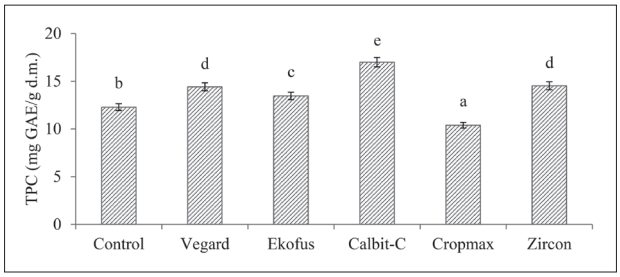


**Fig. 1.** Total phenolic content (TPC) in soybean seed influenced by treatments with different plant-extract-based products, determined as mg GAE/g dry matter (d.m.). * – Values followed by the same letters are not significantly different at the 0.05 level.

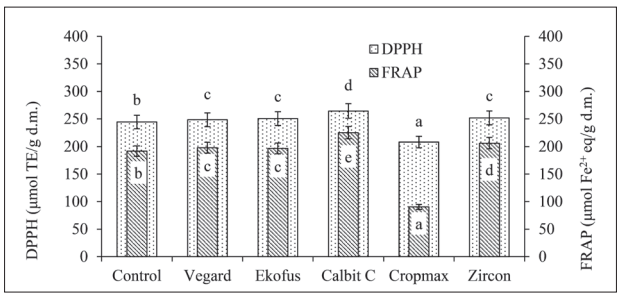


**Fig. 2.** Antioxidant activities determined by the DPPH and FRAP assays in soybean seed influenced by treatments with different plant-extract-based products. *– Values followed by the same letters are not significantly different at the 0.05 level.

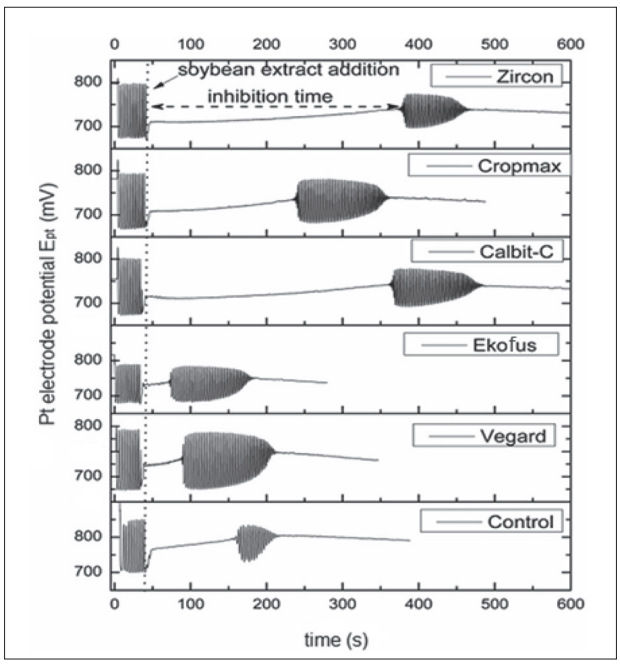


**Fig. 3.** The Briggs-Rauscher (BR) oscillograms obtained after the addition (100 μL) of a particular soybean extract, 30 s after beginning of the oscillatory reaction. The initial concentrations of reactants for BR reaction were as follows: $[CH_2(COOH)_2]_0$=0.0789 mol/L, $[MnSO_4]_0$=0.00752 mol/L, $[HClO_4]_0$=0.0300 mol/L, $[KIO_3]_0$=0.0752 mol/L and $[H_2O_2]_0$=1.2690 mol/L

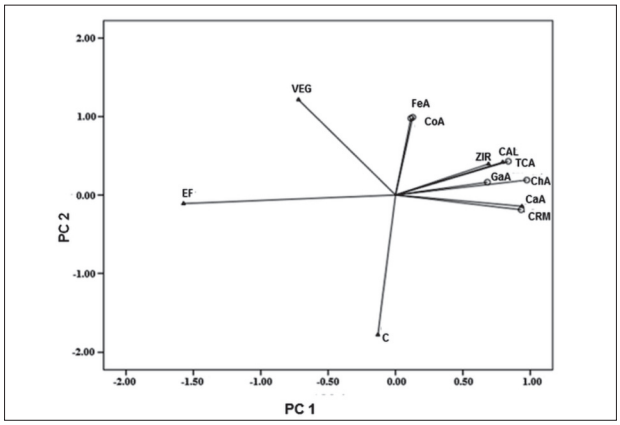


**Fig. 4.** PCA biplot of the phenolic acid profile in soybean seeds depending on the treatment with plant-extract-based products.

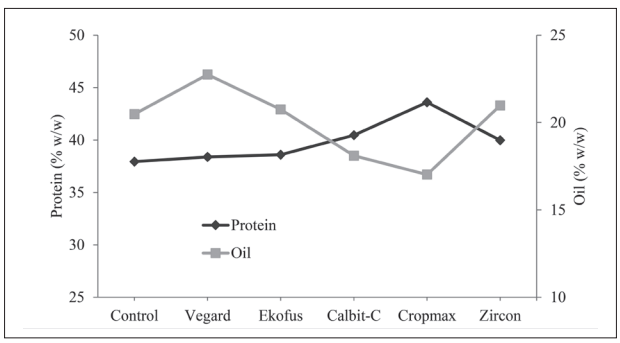


**Fig. 5.** Protein and oil contents in soybean seed influenced by treatments with different plant-extract-based products, expressed as % (w/w).

the best antioxidant activity (which means the highest inhibition time) was obtained for soybean treated with "Zircon" and "Calbit-C", while treatments with "Vegard" and "Ekofus" were significantly lacking (Fig. 3). Soybean treated with "Vegard" and "Ekofus" were very similar to each other and exhibited only slightly weaker antioxidant activities than the sample from control plants. According to the BR reaction method, the results obtained from soybean treated with "Cropmax" were between these two groups (Fig. 3).

According to the results of PCA (Fig. 4), PC 1 was explained by 57.31%, and PC 2 by 29.96% in total variability. PC 1 was explained by chlorogenic, caffeic and *trans*-cinnamic acids with 97.4%, 93.3%, and 83.7%, respectively, while PC 2 was explained by *p*-coumaric and ferulic acids with 99.1% and 97.8%, respectively.

## Protein and oil contents

Fig. 5 shows variations in the protein and oil contents (%, w/w) in soybean seeds from plants treated with five plant-extract-based products. Treatments with "Calbit-C" and "Cropmax" showed a negative influence on the oil content, but a positive influence on the raw protein content, especially "Cropmax", which increased the protein content for 5.66% (w/w) compared to the control sample.

## DISCUSSION

There are many studies on the phenolic compounds and their protective role in plants against a variety of stressors [21-23]. In the present paper, the antioxidant activity of soybean extracts was directly proportional to the total phenolic content. Also, treatment with different natural products (based mainly on plant and algae extracts) can contribute to the accumulation of certain groups of bioactive compounds and make the plant more resistant to the effects of external factors.

The content of all examined phenolic acids in seed samples varied in comparison to the control sample. Our results are in agreement with the Aludatt et al. [24], where the highest amount of phenolic acids in soybean seed was detected in the free soluble fraction. The authors also confirmed that the major predominant phenolic compounds in full-fat soybean and in defatted soybean meal were ferulic acid and *p*-coumaric acid, which is similar to the results of the present study. Kim et al. [25] confirmed that *p*-coumaric and ferulic acids were strongly bound in soybean with cell components, and released after alkaline hydrolysis. This result demonstrated that some phenolic acids were synthesized as macromolecular bound components (i.e. polyphenol, tannin, and lignin) to support structural cell wall development. Chlorogenic, caffeic and *p*-coumaric acids are intermediates during lignin biosynthesis, while ferulic acid is also probably linked to polysaccharides, lignin, and suberin [26,27]. The smallest amounts of phenolic acids were present in the bound fraction, which is also in accordance with Aludatt et al. [24].

HPLC-DAD chromatograms revealed differences in the phenolic compound profiles in three different fractions of methanolic soybean flour extracts. In ad-

dition to the examined phenolic acids, a large number of peaks that were not the subject of this study were observed. The esterified solubilized fraction is particularly rich in phenolic compounds, while in the bound fraction there is a compound that eluted in 11 min at a high content and which is not present in other forms. Results are in the accordance with the fact that phenolic compounds in soybean and related legumes were mainly presented in esterified-soluble and bound-insoluble forms [28,29]. We assumed that these peaks belong to other groups of phenolic compounds, especially to isoflavones. The results of many studies [30-33] confirm that isoflavones are the main class of phenolic compounds in soybean seed. This result could be a good basis for further investigation of the effect of plant-extract-based products on the contents of isoflavones and other groups of phenolic compounds in soybean.

According to the results of the *post-hoc* Tukey test, it is not possible to observe a regularity in the effect of individual treatments on the content of phenolic acids in soybean flour extracts. Their content randomly changed depending on the fraction in which they were found (free, esterified and bound).

Since antioxidants can act through different mechanisms, the antioxidant activities of soybean flour extracts were determined using different assays, DPPH, FRAP and BR. All examined plant-extract-based products expressed significant changes in the total phenolic contents and antioxidant activities of the soybean flour extracts. An exception was the treatment with "Cropmax", which only caused a decrease in TPC and antioxidant activities determined by the DPPH and FRAP methods. All other treatments showed a positive influence on the TPC, DPPH and FRAP methods, which is in accordance with several studies [34,35] that showed a positive influence of biofertilizers (based on plant extracts) on the yield, growth and antioxidant activities of different plant species. The results of the *post-hoc* Tukey test showed that all treatments significantly effected a change in TPC as compared to the control sample. A statistically significant difference in antioxidant activity was not found between the samples treated with "Ekofus" and "Vegard", while all treatments in relation to the control sample exhibited statistically significant differences in antioxidant activity as measured by both DPPH and the FRAP methods.

Inhibitory effects after the addition of aqueous extracts of soybean flour to an active BR mixture were reported [36]. However, to the best of our knowledge, this is the first time that soybean treated with products based on plant extracts during vegetation were analyzed in the oscillatory Briggs-Rauscher reaction. It is well-known that the BR reaction method provides a "larger antioxidant picture" and can also show a synergistic effect [37]. Therefore, the results obtained by the BR reaction method demonstrated the synergistic effect (of phenolic compounds and proteins) in soybean treated with "Cropmax", as well as a more favorable outcome of soybean treatment with "Calbit-C" and "Zircon". This indicated that not only phenolic compounds participated in the inhibition of the oscillatory regime, but also other molecular species (such as proteins and some ions), which influenced the BR reaction, possibly by building and/or stabilizing macromolecular structures in plant cells. In our study, the most positive influence on the TPC, DPPH, FRAP assays and BR reaction method was displayed by "Calbit-C", which contained water-soluble Ca-lignosulphonate. This is consistent with the fact that $Ca^{2+}$ in the form of functional Ca-pectate frames the cell walls (as well as many phenolic compounds), rendering them more resistant to different stressors. It also plays an important role in stabilizing the bond of phospholipids and cell membrane proteins, thus maintaining their functional stability [38,39].

PCA analysis is a very useful tool for reducing a large number of variables on a small number of composite variables (main components), explaining the variability of data in a most concise manner. In this way, it can reveal certain hidden connections and interdependence of data. The results of this study indicate that "Cropmax" induced the highest variability in caffeic acid concentration, and to a lesser degree in gallic and chlorogenic acid concentrations. "Calbit-C" and "Zircon" were responsible mainly for variations in the concentration of *trans*-cinnamic, gallic and chlorogenic acids. The other treatments, particularly "Ekofus" and the control, did not reflect variations in the investigated phenolic acids. This means that application of plant-extract-based products affected not only the yield but also the chemical composition of the grain, indicating that the phenolic composition and concentration can be altered in a certain way [40,41].

Another important group of biologically active compounds in soybean seed are proteins. In this study, we showed that using different types of natural products based on plant extracts can affect the protein content in soybean seed and that the best effect was achieved with "Cropmax". This was to be expected since "Cropmax" contains about 2% of amino acids, which were proven to have a positive effect on nitrogen metabolism and increased content of raw proteins in plant material [42,43]. The synergistic effect of proteins and phenolic compounds on the antioxidant activity of soybean seed was also observed using the Briggs-Rauscher method.

The use of products based on plant extracts during vegetative growth significantly influences the chemical composition of soybean seeds in terms of variability of the concentrations of phenolic acids in different cell components. A high variability in the protein content and antioxidant potential (determined by different methods) of the seed points to potentially large differences in the strengths of seedlings obtained from plants treated with these products.

## CONCLUSIONS

According to the results obtained by TPC, DPPH, FRAP and the BR reaction method, it can be concluded that different types of plant-extract-based products affect the amount of phenolic compounds and antioxidant activity of soybean seeds in different ways. Although there were variations in the quantity of individual phenolic acids in different fractions (free, esterified and bound), all applied products exhibited a positive effect on the increase in concentration of phenolic acids. This paper also shows that the high content of phenolic acids (primarily of *p*-coumaric and ferulic acids) in soybean seeds contributed to their high antioxidant activity. Controlled use of certain plant extracts can increase the concentration of different groups of bioactive compounds in the samples. Amino acid-based products (such as "Cropmax") increased the amount of raw protein in soybean seed. This is important from the aspect of plant resistance to various types of stress (environmental, mechanical, parasites, viruses), which is of great importance for the vigor of seeds and seedlings during germination and the early stages of vegetative growth, and also from the aspect of using soybean seeds as a potential source of antioxidants in functional foods and nutraceuticals. Although only one soybean variety (Laura) was tested in this study, the results are satisfactory, especially in light of the described synergistic effects of phenolic compounds and proteins on antioxidant activity. For this reason, they can serve as a good basis for extending research into the effects of plant-extract-based products on other groups of phenolic compounds, both in other soybean varieties and in related legumes.

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**Author contributions:** Sanja Đurović was the main contributor who performed the work and was responsible for conducting the experiments and for writing the manuscript. Bogdan Nikolić and Hadi Waisi performed the field treatments. Maja Pagnacco contributed to the Briggs-Rauscher antioxidant assay and data interpretation. Vesna Dragičević contributed to the PCA analysis and interpretation of the results. Nevena Luković helped in the protein analysis and interpretation of the results. Zorica Knežević-Jugović and Bogdan Nikolić supervised the data interpretation and manuscript writing.

**Conflict of interest disclosure:** The authors declare that they have no conflict of interest.

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## Supplementary Data

**Supplementary Fig. S1.**
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Djurovic%20et%20al_3913_Supplementary%20Fig.%20S1.pdf