POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF PHYTOESTROGEN CONTAINING FOOD AND DIETARY SUPPLEMENTS: EVALUATION OF DPPH FREE RADICAL SCAVENGING ACTIVITY BY HPLC

Ilija Klarić[1*], Daniela Amidžić Klarić[2], Ana Mornar[2], Biljana Nigović[2], Mario-Livio Jeličić[2], Edvin Brusač[2]

[1]*Public Health Brčko DC, Department of Health Ecology, R. Dž. Čauševića 1, 76000 Brčko DC, B&H*
[2]*University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića 1, 10000 Zagreb, Croatia*
[*]*klaric67@gmail.com*

INTRODUCTION

Soybeans, red clover, chastetree, hop and flax have all been found to contain a wide range of phytoestrogenic compounds, and a large number of dietary supplements contain their extracts as principal ingredients. Since polyphenolic compounds are responsible for the potential antioxidant activity and radical scavenging capacity of plant food, this study aimed to evaluate total polyphenol content (TPC) and antioxidant activity (AA) of phytoestrogen-containing food and dietary supplement (DS) formulated products prepared.

MATERIALS AND METHODS

Sample

Four commercially available food samples of soybeans and flax (F1-F4) were collected from a local health food store, while twenty phytoestrogen-containing dietary supplements (DS1-DS20) were obtained from a public pharmacy in Zagreb, Croatia. Briefly, fifteen dietary supplements were botanical monopreparation products containing following herbal extracts: soy (DS1-DS4), chasteberry (DS5-DS9), red clover (DS10 and DS11), hop (DS12-DS14), and flax (DS15). Five samples were classified as multi-botanical dietary supplement products containing several bioactive ingredients: soy and chasteberry (DS16 and DS17), soy and red clover (DS18), soy and flax (DS19), and red clover and hop (DS20). The dietary supplements analysed in this study were in multiple dosage forms, including liquid extracts (2 samples), tablets (5 samples), and capsules (13 samples). Liquid products contained only ethanolic herbal extract, while other products contained additional ingredients, such as vitamins and minerals. Two different batches of each dietary supplement product were analysed to investigate batch-to-batch variability.

The extraction procedures

A sample portion of 25 ± 2 mg was accurately weighed in a screw capped 15-mL centrifuge tube. After adding 10 mL of methanol:ultra-pure water (80:20, *v/v*), the samples were sonicated for 15 min at room temperature in Elmasonic X-TRAH ultrasonic bath (Elma, Singen, Germany). The clear solution was obtained by centrifugation (Hermle Z 306, Wehingen, Germany) at 3000 g for 10 min at 25 °C and supernatant was further filtered through a 0.45 µm Chromafil membrane filter (Macherey-Nagel, Düren, Germany) before UV-Vis and HPLC analysis.

Determination of the total polyphenol content

The determination of the TPC in phytoestrogen-containing samples was based on the procedure using the Folin–Ciocalteu reagent [Singleton et al., 1999].
Briefly, 0.20 mL of methanolic extract was pipetted into a 5-mL volumetric flask and diluted with ultra-pure water to 2.5 mL. Following this, 0.25 mL of Folin-Ciocalteau reagent was added in the volumetric flask, and after 3 min, 0.5 mL of 10% sodium carbonate solution was added. Then the mixture was well shaken using vortex mixer (ZX3, Velp scientifica, Usmate, MB, Italy) and made up to volume with ultra-pure water. The solution was allowed to stand in the dark at room temperature for 2 hours before reading at 740 nm using a UV–Visible spectrophotometer (model Lambda 25, Perkin–Elmer, Waltham, MA, USA). The blank was prepared as prescribed, the same volume of methanol (80:20, *v/v*) was pipetted instead of 0.20 mL of methanol extract. Gallic acid was used as a standard and the calibration curve of gallic acid was prepared in the range from 0.4 to 6.0 µg/mL at 5 concentration levels (r = 0.9999). The results were expressed as gallic acid equivalents (GAE) (mg of GAE per g of sample).

Determination of antioxidant activity

The antioxidative activity of the phytoestrogen-containing sample was determined using the HPLC-DPPH assay method. Briefly, 400 µL of DPPH solution was added to aliquot of 1 mL of sample extract solution. Following this, the mixture was shaken well on a vortex mixer in an Eppendorf tube and placed in the dark, at room temperature. The reaction time was 30 min. Afterward, the solution was filtered through Minisart RC4 0.20 µm injection filter (Sartorius, Göttingen, Germany) and placed in amber-glass HPLC vials. The chromatographic conditions were a modification of those by Chandrasekar et al. [2006] and used for monitoring of DPPH assay (Table 1). Sample preparation and analysis was performed in triplicate.
The ability of a sample to scavenge the 'stable' free DPPH radical was determined from the difference in the peak area of the initial solution of the radical itself and the solution of the radical after reaction with the sample (Figure 1). TROLOX was used as a standard antioxidant and the calibration interval was in the range from 0.05 to 0.30 mM at 6 concentration levels. The results were expressed as TROLOX equivalent antioxidant capacity (TEAC) determined from a standard calibration curve obtained by standard concentration versus difference in peak area.

Table 1. HPLC-DPPH method

Assay method	
Instrument:	Dionex chromatographic system: • P680 pumping system • ASI 100 automated sample injector • TCC-100 thermostatted column compartment • UVD170S detector • Chromeleon 6.8 software
Column:	XBridge C18 column (4.6 x 150 mm, particle size 3.5 µm)
Mobile phase:	methanol:ultrapure water (80:20, *v/v*)
Mode:	isocratic elution
Injection volume:	20 µL
Column temperature:	25 ± 0.1 °C
Flow:	1 mL/min
Detection λ:	517 nm
Retention time:	3.8 min
Total run time:	5.0 min

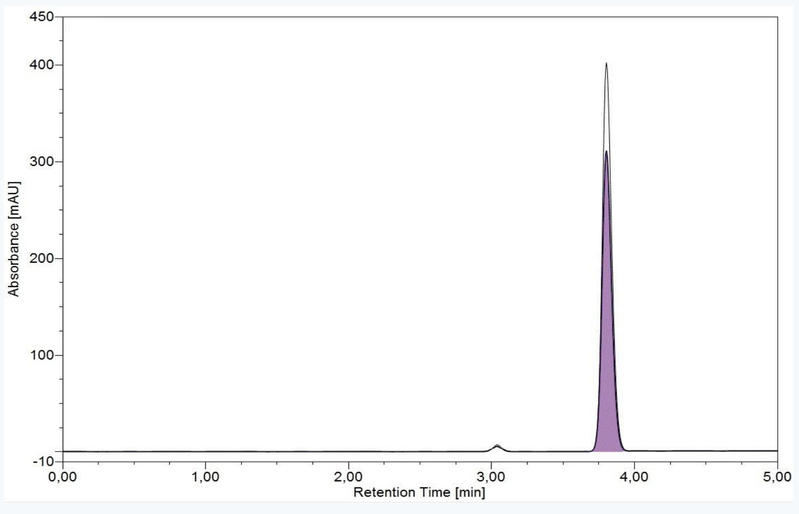

Figure 1. The chromatogram of the DPPH solution before (white) and after the sample (DS19) reaction (purple) recorded at 517 nm.

RESULTS AND DISCUSSION

Polyphenol content and antioxidant activity in formulated DS (TPC: 17.65±10.28 mg GAE/g; AA: 103.01±58.42 mM TEAC/g) was higher than in functional food samples (TPC: 6.18±1.37 mg GAE/g dry matter; AA: 40.01±15.80 mM TEAC/g dry matter). Also, the results indicate that multi-botanical products (TPC: 23.70±9.09 mg GAE/g; AA: 143.49±45.63 mM TEAC/g) have higher polyphenol content and antioxidant activity than monopreparation (TPC: 15.63±10.11 mg GAE/g; AA: 89.52±57.07 mM TEAC/g).
Considering the average weight of individual tablet or capsule, polyphenol administration following single solid dosage form is in the wide range from 1.87 mg (DS10) to 51.95 mg (DS9), as presented in Table 2. According to products' labels, there was a large diversity among the serving size of each product (up to 6 solid dosage forms or 30 drops for liquid extract). Hence, the daily administration of polyphenols that patients would ingest if they followed the daily serving recommendation by the manufacturer varied over the range 1.87 (DS10) – 103.9 (DS9) mg/day, for the adult population.
Based upon the results for analysed samples they can be divided into three categories according to their TPC in the recommended daily dose: (i) above 100 mg of polyphenols (1 sample), (ii) 10 - 100 mg polyphenols (10 samples) and (iii) 1 - 10 mg polyphenols (9 samples).

Table 2. Total polyphenol content and antioxidant activity of phytoestrogen containing samples.

Sample	Total phenolic content			DPPH	
	mg GAE/g sample	Total amount of phenolic content per unit of the dosage form*** [mg]	Total amount of phenolic content per recommended daily servings**** [mg]	mM TEAC / mL extract	mM TEAC /g sample
All samples					
Mean values	15.74			0.235	92.51
Median	13.43			0.185	72.23
Range of values	2.34 – 38.21			0.046 – 0.537	20.37 – 208.5
Interquartil	7.60 – 24.52			0.112 – 0.364	44.22 – 143.4
RSD [%]	0.07 – 2.72			0.91 – 6.97	
Food samples*					
Mean values	6.18		284.1	0.093	40.01
Median	6.24		278.2	0.092	40.34
Range of values	4.52 – 7.72		57.24 – 608.0	0.046 – 0.141	20.37 – 58.99
Interquartil	5.42 – 6.99		154.9 – 391.1	0.077 – 0.108	34.41 – 45.94
RSD [%]	0.15 – 0.94			1.78 – 4.12	
Dietary supplement samples					
Mean values	17.65	12.18	49.47	0.263	103.01
Median	15.93	9.26	37.96	0.216	84.09
Range of values	2.34 – 38.21	1.87 – 51.95	6.28 – 154.0	0.054 – 0.537	21.30 – 208.5
Interquartil	8.95 – 25.67	5.79 – 13.17	28.89 – 61.48	0.130 – 0.385	51.30 – 153.3
RSD [%]	0.07 – 2.72			0.91 – 6.97	
Batch-to-batch variability** RSD / [%]	0.11 – 57.93				

Legend:
*expressed in mg /g of dry matter; **batch-to-batch variability is given to dietary supplement products; ***unit of the dosage form – 1 tablet or capsule; 1 ml liquid extract; ****recommended daily servings – 1 to 6 tablets or capsules; 30 ml for liquid extract; 40-90 g for soya, 10-50 g for flaxseed

The statistical analysis of obtained results has demonstrated a significant correlation between the TPC and antioxidant activity estimated HPLC-DPPH method (Figure 2). Pearson correlation coefficient indicated a relationship for all investigated samples ($r=0.9837$; $p< 0.0001$).

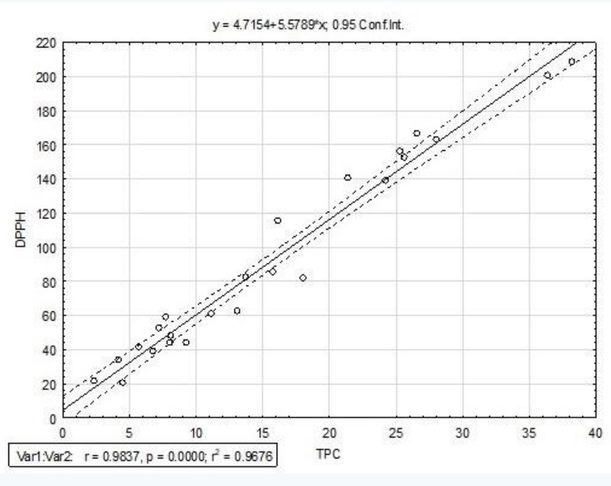

Figure 2. Correlation between total polyphenol content and antioxidant activity estimated by HPLC-DPPH method in phytoestrogen containing food and dietary supplements samples.

CONCLUSION

The results of this study contribute to a better understanding of the phenolic profile and antioxidant properties of phytoestrogen containing dietary supplements. Polyphenol content and antioxidant activity in formulated DS products was higher than in functional food samples. Also, the results indicate that multi-botanical preparations have higher polyphenol content and antioxidant activity than mono-botanical ones. The correlation between polyphenol content and the antioxidant effect is strongly statistically significant, so it can be concluded that antioxidant activity is proportional to the content of secondary metabolites. The most eye-catching batch-to-batch deviations were represented by one chasteberry and one red clover-based products.