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Impact of the duration of ultrasound-assisted extraction on total phenolics content and antioxidant activity of lupin seeds

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The influence of the duration of ultrasound-assisted extraction on antioxidant activity and total phenolics content of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') was investigated for the first time. Lupin seeds were with German origin but introduced in Bulgaria. They were extracted for 10, 20 and 30 min with absolute methanol and the obtained extracts were evaluated for total phenolic content and antioxidant activity. The polyphenol content of the extracts was found to be in the range of 1.65 – 2.03 mg gallic acid equivalents (GAE)/g dry weight sample, depending on the duration of the extraction process. The antioxidant activity was estimated by ABTS•+ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)) (2.28 – 2.89 mmol Trolox Equivalent (TE)/g dw), DPPH• (1,1-diphenyl-2-picrylhydrazyl radical) (2.01 – 2.71 mmol TE/g dw), FRAP (ferric reducing/antioxidant power) (3.76 – 4.36 mmol TE/g dw) and CUPRAC (cupric ion reducing antioxidant capacity) (3.07 – 4.69 mmol TE/g dw) methods. Generally, methanol extracts with 30 min of extraction displayed the highest total phenolic contents, while 10 min of extraction time was the least efficient ultrasound-assisted method. On the other hand, antioxidant activity of the extracts was highest in 20 min of extraction apart from CUPRAC method where 30 min of extraction depicted more antioxidant capacity of the methanol extract.

Keywords: Lupin seeds, antioxidant activity, total phenolic content, extraction duration.

INTRODUCTION

Phenolic compounds are found in all plant species and represent secondary metabolites which may possess resistance to oxidation processes [1, 2]. They have antioxidative, antiallergic, anti-inflammatory, and anticarcinogenic activities. Phenolic compounds can also protect against cell damage and prevent the risk of degenerative diseases [3-5].

There are several methods for extracting the phenolic compounds from different parts of the plants including hot continuous extraction method (Soxhlet), liquid-liquid extraction, solid-liquid extraction, supercritical fluid extraction, solid-phase micro extraction, microwave extraction, sonication, etc. [6]. Ultrasound-assisted extraction is often performed in order to enhance the molecular interaction and to reduce the extraction time of polyphenols. On the other hand, many authors report that the total polyphenol amounts from the same plant and its antioxidant activity may vary widely, depending on the applied extraction conditions – type of solvents and duration of extraction [1, 7].

Due to their biologically active molecules many leguminous plants (in particular lupin) are considered to have antioxidant activity. Lupin, which belong to family Fabaceae, is a good source of valuable nutrients such as proteins, lipids, dietary fibres, minerals and vitamins, as well as phytochemicals (polyphenols, mainly tannins and flavonoids) which possess antioxidant capacity [8-11]. Few researchers reported that lupin seeds are a source of polyphenols and exhibit antioxidant activity [11-13]. On the other hand, there is scarce information about the impact of the extraction time on antioxidant activity and total phenolic content of lupin seeds. For that reason, the aim of the present study is to examine the total phenolic content and antioxidant activity of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') using ultrasound-assisted extraction performed with absolute methanol for different time intervals (10, 20 and 30 min).

MATERIAL AND METHODS

Samples

Lupin (*Lupinus angustifolius* L. cultivar 'Boregine') is a German variety introduced in the southern part of Bulgaria. The plant was harvested in June 2018, the seeds were removed, air-dried and used for the subsequent analysis.

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Chemicals and reagents

Chromatographic grade methanol was used for the analyses (VWR, Austria). Ammonium acetate, copper(II) chloride, gallic acid, glacial acetic acid, sodium acetate trihydrate, ferric chloride hexahydrate, hydrochloric acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox,) and reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), Folin–Ciocalteu reagent, phosphate buffered saline, pH 7.4, (PBS), were purchased from Sigma-Aldrich.

Preparation of extracts

The milled plant material was weighed with 0.0001 g precision and 2 g were used for analysis. The initial milled material was extracted *via* ultrasonication for 10, 20 and 30 min with 40 mL of methanol at room temperature. All samples were filtered under vacuum. The extraction was repeated three times. The supernatants were combined and evaporated under vacuum at a temperature of the water bath 40°C. The volume of all samples was adjusted to 60 mL and passed through a membrane filter with pore size of 0.45 μm prior to analysis.

Total phenolic content (TPC)

TPC in the extracts was determined by a colorimetric method using Folin-Ciocalteau's reagent [14] with slight modifications. Calibration curve was obtained using as standard an ethanolic solution of gallic acid at concentrations between 25 and 1000 μg/mL. Briefly, 100 μL of extract or gallic acid standard was mixed with 2.4 mL of distilled water, 500 μL of 0.2 M Folin-Ciocalteu's reagent and 2 mL of 7.5 % sodium carbonate solution. The tested samples were incubated for 2 h in dark at room temperature. The absorbance of the samples was measured at 765 nm with a spectrophotometer Camspec M508, England, using a blank sample. The total phenolics content was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g dw) based on the calibration curve.

Trolox Equivalent Antioxidant Capacity (TEAC)

ABTS method. The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by the colorimetric method reported by Re *et al*. (1999) [15]. For this assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical (ABTS•+) solution was prepared by dissolving 7 mM of ABTS in 2.45 mM $K_2S_2O_8$. This mixture was shaken for 12 – 16 h at ambient temperature in the dark until obtaining a stable oxidative state. For the study of the extracts, the ABTS•+ stock solution was diluted with PBS until absorbance became 0.70±0.02 at 734 nm. Sample analysis was performed as follows: 2 mL of ABTS solution and 20 μL of sample or standard were mixed. Absorbance of sample was measured at 734 nm with a spectrophotometer Camspec M508, England after samples incubation at 25°C for 5 min. The calibration curve was plotted by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as a standard. The results were expressed as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Cupric ion reducing antioxidant capacity (CUPRAC) method

The lupin seeds extracts were investigated by cupric ion reducing antioxidant capacity (CUPRAC) method described by Apak *et al*. (2006) [16]. In test tubes were mixed 1 mL copper(II) chloride solution (10 mM), 1 mL neocuproine alcoholic solution (7.5 mM) and 1M ammonium acetate buffer solution (pH = 7), 0.2 mL tested extract or Trolox and 0.9 mL water (final volume, 4.1 mL). Absorbance against a blank sample was measured at 450 nm with a spectrophotometer Camspec M508, England after 30 min in dark at room temperature. Calibration curve was achieved using Trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Ferric reducing antioxidant power (FRAP) method

All samples were investigated by ferric reducing antioxidant power (FRAP) method described by Benzie and Strain (1996) [17]. The FRAP reagent was freshly prepared before analyzes by mixing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-*s*-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in dd H_2O in a ratio 10:1:1. In test tubes were mixed 0.15 mL tested extract or standard Trolox and 2.85 mL FRAP reagent. Absorbance against a blank sample was measured at 593 nm with a spectrophotometer Camspec M508, England after 15 min in dark at room temperature. Calibration curve was achieved using Trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

DPPH method

Antioxidant activity was measured according to Brand-Williams *et al*. (1995) [18] procedure. In the test tubes were mixed 150 μL of extract or Trolox and 2.85 mL of 0.12 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, which was prepared with 4.8 mg DPPH dissolved in 100 mL CH_3OH). The mixtures were shaken and then incubated for 30 min at room temperature. The absorbance was recorded at 517 nm with a spectrophotometer Camspec M508, England. To quantify the antioxidant activity a standard Trolox curve was used in the concentration range from 0.045 to 1.5 mmol Trolox. The results were expressed as mmol TE/g dw.

Statistical analysis

All measurements were performed in triplicate (n = 3) and the results were presented as mean value with the corresponding standard deviation (SD). Significant differences were determined by analysis of variance (Duncan test) with a significance level $p<0.05$ using IBM SPSS Statistics 19.

RESULTS AND DISCUSSION

Plant extracts were obtained by ultrasound-assisted solvent extraction with methanol for 10, 20 and 30 min and were subjected to estimation for their total phenolic content and antioxidant activity.

Total phenolic content (TPC) of the lupin seeds extracts is shown in Figure 1.

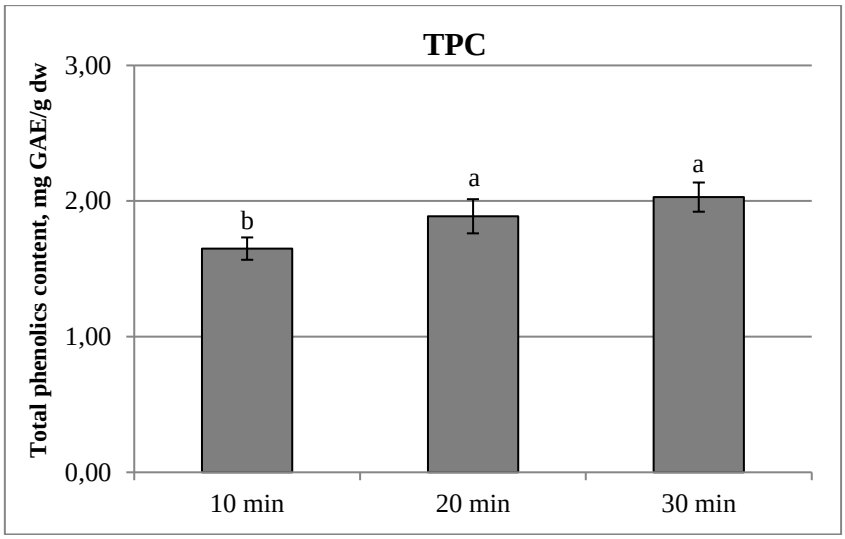

Fig. 1. Total phenolic contents (TPC) of extracts from lupin seeds. * Different letters indicate significant difference at $p < 0.05$ levels by Duncan's Multiple Range Test

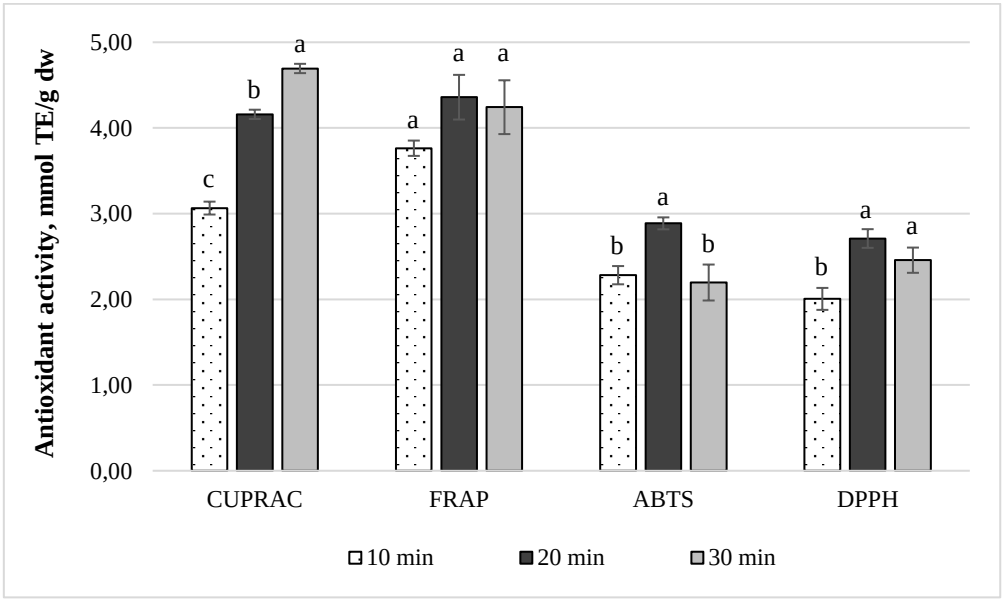

Fig. 2. Antioxidant activity of methanol extracts of lupin seeds using CUPRAC, FRAP, ABTS and DPPH methods. *Different letters for the same method indicate significant difference at $p < 0.05$ levels by Duncan's Multiple Range Test

TPC of the samples ranged from 1.65 to 2.03 mg GAE/g dw. It was observed that the content of the total phenolics increased with increasing the time of the extraction. The analysis of variance using Duncan test (with a significance level $p < 0.05$) revealed that there were significant differences in total phenolics in 10 min and the other extraction times. On the other hand, the differences between 20 min and 30 min of extraction were not significant ($p > 0.05$). Similar results were obtained by Martínez-Villaluenga *et al.* (2009) [8] and Fernandez-Orozco *et al.* (2006) [19] who reported the total phenolics content to range from 1.8 to 2.5 mg ferrulic acid equiv/g and from 1.43 to 3.55 mg (+) catechin/g, respectively. Karamać *et al*. (2018) [20] observed that total polyphenols in different wild and cultivated *Lupinus albus* L. seeds were slightly higher (from 4.36 to 7.24 mg GAE/g) than the reported in the present study. Significantly higher results for eight *L. angustifolius* genotypes grown in western Canada were reported by Oomah *et al*. (2006) [11] (from 12.75 to 14.65 mg (+) catechin/g).

Four different tests were used for the analysis of the antioxidant capacity of methanol extracts of the examined seeds. The scavenging activity of the extracts towards DPPH, ABTS, FRAP and CUPRAC was expressed in mmol TE/g dw.

The CUPRAC values for the lupin seeds ranged from 3.07 to 4.69 mmol TE/g dw, where the lowest value was for the shortest time of extraction and the highest was observed for 30 min of extraction. There were significant differences between the antioxidant activities of the extracts for all durations, which signified that the best extraction time for lupin seeds using CUPRAC method was 30 min.

The FRAP values ranged between 3.76 – 4.36 mmol TE/g dw, but the analysis of variance using Duncan test (with a significance level $p < 0.05$) revealed that there were not significant differences of the antioxidant activity of lupin seeds extracted for 10, 20 and even 30 min. These results were much higher than those reported for lupin seeds by Karamać *et al*. (2018) [20].

The antioxidant capacity by ABTS radical cation of the methanol extracts of lupin seeds ranged from 2.20 (30 min) to 2.89 mmol TE/g dw (20 min). No significant differences between the ABTS values for lupin seeds extracted for 10 and 30 min were noticed, but they were significantly different for 20 min of extraction where the highest value was observed. The results obtained were much higher than those reported by Martínez-Villaluenga *et al*. (2009) [8] who established that the antioxidant capacity of raw seeds of *L. angustifolius* cv. Troll, *L. angustifolius* cv. Emir and *L. albus* cv. Multolupa, measured by TEAC assay, were as follows: 47.9, 47.0 and 71.4 μmol Trolox/g d.m., respectively.

The DPPH radical scavenging activity is used for estimation of the antioxidant capacity of extracts against oxidation caused by free radicals [21] and the values for the lupin extracts ranged from 2.01 (10 min) to 2.71 mmol TE/g dw (20 min). It was observed that the antioxidant activity of lupin seeds using DPPH assay were not significantly different for 20 and 30 min of extraction, even though the value in 30 min was lower. The results from the present study were much higher than those reported by Martínez-Villaluenga *et al.* (2009) [8] who established that the antioxidant capacity of raw seeds of *L. angustifolius* cv. Troll, *L. angustifolius* cv. Emir and *L. albus* cv. Multolupa were as follows: 3.09, 3.06 and 2.83 μmol Trolox/g dw, respectively.

As it is observed, CUPRAC and FRAP antioxidant capacity assay of lupin seed extracts showed higher values than ABTS and DPPH methods.

It is well known that the antioxidant activity is also related to the content of polyphenols [22]. The results from the present study confirmed this statement – the highest total phenolic content was observed in the lupin seeds extracted for 20 and 30 min and the highest values for antioxidant capacity were noticed in the seeds for the same duration of extraction. The analysis of variance using Duncan test depicted that the duration of the ultrasound-assisted extraction did influence the total polyphenol content and the antioxidant activity of lupin seeds when they were extracted for 20 and 30 min in absolute methanol.

CONCLUSION

The impact of the duration of ultrasound-assisted extraction on antioxidant activity and total phenolic content of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') was examined for the first time. The phenolic contents of the seeds during 30 min of extraction did not differ significantly from those during 20 min of extraction. The same tendency was observed in the antioxidant activity of the extracts determined by FRAP and DPPH methods. The methanol extracts of the seeds exhibited higher antioxidant activity in 30 min using CUPRAC method which was significantly different from the extraction for 10 and 20 min. The extraction for 20 min showed higher antioxidant activity for ABTS method, while those for 10 and 30 min they were not significantly different.

Overall, the best methods for evaluating the antioxidant capacity of lupin seeds were CUPRAC and FRAP, and the most suitable time for extraction was 20 min.

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