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ORIGINAL PAPER

Binary ethanol–water solvents affect phenolic profile and antioxidant capacity of flaxseed extracts

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Abstract Flaxseed extracts are in focus because of their potential application as a food ingredient. Ethanol–water mixtures are recommended for preparation of plant extracts due to their acceptability for human consumption. However, there is a lack of comprehensive studies concerning solvent effect on phenolic profile (phenolic composition and content) and its impact on flaxseed extract antioxidant capacity. This study investigated the effect of ethanol concentration in ethanol–water extraction solvent on the changes in phenolic profile (HPLC analysis after alkaline or alkaline-acid hydrolysis) and the antioxidant activity ($DPPH^{\cdot}$ and ORAC_FL assays) of flaxseed extracts; various ethanol concentrations were investigated to prepare a suitable extract that could be safely introduced into food. The results showed relationships between the tested factors, i.e. the ethanol concentration and the phenolic content or the antioxidant capacity of flaxseed extracts. The content of phenolic compounds in extracts, including secoisolariciresinol diglucoside, phenolic acids and their glucosides, decreased with increasing concentration of the ethanol from 60 to 90 % in extraction solvent (total phenolics after alkaline hydrolysis were 106.5 and 7.7 mg g^{-1}, respectively). On the contrary, the content of phenolic acid esters was the highest in the 90 % ethanol flaxseed extract. Changes in the phenolic profile with ethanol increase in extraction solvent impacted negatively the radical scavenging activity of the extracts. This study indicates that the mixture of ethanol and water in proportion of 60:40 (v/v) is the most suitable to obtain flaxseed extract with the high content of phenolic compounds and antioxidant activity.

Keywords Binary ethanol–water solvent extraction · Flaxseed meal · Phenolic composition · Antioxidant capacity · Phenolic profile–antioxidant activity relationship

Introduction

Flaxseed (*Linum usitatissimum* L.) is a valuable oilseed crop with demonstrated health benefits [1, 2]. Besides its oleochemical application, the flaxseed is a source of some bioactive compounds, such as dietary fibre, unique proteins and phenolic compounds [3]. Flax phenolics (e.g. lignans) are particularly in focus because of their antioxidant and oestrogenic activity [4, 5]. Currently, the effective isolation of flax phenolic compounds is desirable due to their potential application as a food ingredient [4]. A solvent extraction is the most common method of phenolic compound recovery from plant material. However, some factors including extraction solvent type and concentration, as well as other extraction conditions, can significantly influence the extraction efficiency [6].

A plant extract composition, and consequently its biological activity, is strongly affected by applied solvent. The results of the previous studies [7, 8] indicated that the total content of phenolics (estimated with Folin–Ciocalteu reagent) extracted from flaxseed was affected by a solvent polarity. It was shown that the less polar ethyl acetate was less efficient than the more polar 70 % methanol [8]. Kasote et al. [9] studied the hydrophobic fraction obtained with n-butanol–water mixture (1:1, v/v) from flaxseed

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meal, and they did not find specific maximum absorption range (UV scanning) for lignans, the typical flaxseed phenolics.

Various solvents were utilised for extraction of phenolic compounds (mainly lignan secoisolariciresinol diglucoside—SDG, but also phenolic acids and their derivatives) from defatted flaxseed meal, flaxseed cake or hulls. Alcohols, i.e. methanol, ethanol or their various aqueous solutions, were the most often used for the extraction [7, 8, 10–13]. It was found [7] that a binary solvent extraction (alcohol–water) was more efficient for extraction of flaxseed phenolics than a mono-solvent one (with pure alcohol). Extractions with acetone–water and ethanol–1,4 dioxane (1:1, v/v) mixtures [14], as well as a pressurised low polarity water extraction [15], were also applied to obtain rich-in-SDG extracts.

Ethanol–water mixtures seem to be the most suitable solvents for the extraction because of different polarity of both solvents, possibility of mixing them in any proportion and their acceptability for human consumption [12]. Such ethanol–water flaxseed extracts could be safely introduced into food products without the risk of unacceptable level of hazardous solvent residues. Our previous study showed [16] that the extract addition protected meat products from lipid oxidation and deterioration of their nutritional quality. Moreover, Pag et al. [13] reported that the ethanol–water flaxseed extract exhibited an antibacterial activity against some gram-positive and gram-negative bacterial strains. These preservation capacities of flaxseed extracts can be valuable for food production.

It was shown that alcohol concentration was a very important factor among those affecting extraction of flaxseed phenolics [11–13]. The ethanol concentration (affecting solvent polarity) was more crucial for extraction yield than extraction temperature [11, 12] and flaxseed meal pre-treatment [17]. Westcott and Muri [11] suggested that alcohol should be mixed with minimum 50 % of water for effective flaxseed lignan extraction. Zhang et al. [12] studied the effect of extraction conditions such as ethanol concentration (50–100 %), extraction temperature (20–60 °C) and time on extraction yield upon ethanol–water solvent extraction of flaxseed followed by basic hydrolysis; they found that the ethanol concentration in the range of 56–83 % was the most efficient. The antioxidant activity and composition of the extracts were not investigated in the study. Therefore, there is still the need to search for the best ethanol concentration to obtain valuable flaxseed extracts that could be applied as functional food ingredients; such extracts should be rich in beneficial flaxseed phenolic compounds and also exhibit a good antioxidant capacity.

For studying composition–antioxidant relationship in crude flaxseed extracts, total phenolic contents were usually determined by the spectrophotometric method with Folin–Ciocalteu reagent in the earlier studies [7, 8, 10, 13]. However, the method is not specific for phenolic compounds because it is based on the red-ox reaction [18] and the results can be affected by various interfering substances present in crude plant extracts (e.g. sugars, proteins and other non-phenolic compounds). The analysis of lignan content (SDG or its aglycone secoisolariciresinol—SECO) was included only in few studies [13, 15]. An antioxidant activity of plant extract depends on a profile of phenolic compounds, i.e. both their composition and content. There is a lack of comprehensive studies concerning the effect of ethanol concentration in extraction solvent on the phenolic profile of flaxseed extracts and the relationship between the profile and the flaxseed extract antioxidant capacity. Therefore, the analysis of flaxseed extract phenolic profiles is included in the present study to show the effects and to clarify the problem.

The aim of the present study was to evaluate the effect of various ethanol–water solvents (ranging from 60 to 90 % of ethanol) on the composition and content of phenolics in flaxseed extracts as well as on their antioxidant activity measured using DPPH$^{\bullet}$ radical scavenging and ORAC_FL assays. Moreover, the correlations between the composition of binary solvent and the content of phenolics in flaxseed extracts or the extract antioxidant activity were established. We looked for the best ethanol concentration in extraction solvent to prepare valuable flaxseed extracts that could be safely introduced into food products.

Materials and methods

Materials

The seeds of Polish high-α-linoleate Oliwin flax variety (IHAR, Poland) were selected as a research material. Defatted flaxseed meal was prepared by cold extraction with hexane according to Waszkowiak and Rudzińska [19] and utilised for binary solvent extraction.

All solvents and reagents were of analytical (ACS) or HPLC grade. DPPH (2,2-diphenyl-1-picryl-hydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) were purchased from Sigma-Aldrich (Germany). Fluorescein sodium salt was purchased from Fluka (USA). Standards of lignans and phenolic acids (caffeic, *p*-coumaric and ferulic) were purchased from PhytoLab (Germany) and Sigma-Aldrich, respectively.

Binary solvent extraction

Binary solvent extraction was carried out according to Waszkowiak et al. [16]. Briefly, defatted flax meal was extracted with ethanol–water solvent (meal to extraction solvent of

1:7.5) under constant vigorous shaking (labscale orbital shaker GFL 3005, Germany) at ambient temperature (23 °C) for 1 h. Based on the previous results reported by Zhang et al. [12], five extraction solvents of various ethanol-to-water ratio were applied (60:40, 65:35, 70:30, 80:20 90:10; v/v).

The extract solution and meal residue were separated by filtration (Munktell & Filtrak filter paper no 1289, retention rate 8–12 μm, Germany). The residue was re-extracted and centrifuged (20 min, 1500×*g*; Centrifuge 5702 R, Eppendorf, Germany). The supernatants from both extractions were combined and evaporated applying rotary vacuum evaporator (Buchi, Switzerland) to remove ethanol, and then freeze-dried (Alpha 1-4 LSC Freeze dryer; Christ, Germany). The freeze-dried extracts were stored at 4 °C in dark for further use.

The extraction procedure was based on the previously reported results concerning the optimal conditions of ethanolic extraction from flaxseed [11–13].

Phenolic profile analysis

Analysis of phenolic composition of the ethanolic extracts was performed according to the Waszkowiak et al. [20] protocol. The protocol included alkaline and acid hydrolyses, followed by the high-performance liquid chromatography of the hydrolysed extracts.

Alkaline hydrolysis

An equal volume of 2 mol L^{-1} sodium hydroxide was added to a flaxseed extract solution (0.200 g mL^{-1} of appropriate binary solvent). Hydrolysis was carried out for 2 h at room temperature. The reaction was stopped by addition of 36 % hydrochloric acid (up to 1.2 mol L^{-1} final concentration in the sample). The sample was centrifuged (8000*g*, 5 min), and supernatant was submitted to HPLC analysis.

Acid hydrolysis

After alkaline hydrolysis, 1 mL of solution was taken and an equal volume of 4 mol L^{-1} hydrochloric acid was added. The sample was hydrolysed for 1 h at 85 °C, cooled and centrifuged (8000*g*, 5 min). The supernatant was submitted to HPLC analysis.

Qualitative and quantitative analysis of phenolic compounds by HPLC

After alkaline and alkaline-acid hydrolyses, the main flax phenolic compounds, i.e. lignans (SDG and SECO), phenolic acids and their derivatives, were identified and quantified by the HPLC method [20] using a Waters 600 high-performance liquid chromatograph (Waters, Millford, MA, USA) equipped with a Cadenza 5CD-C18 column (4.6 × 75 mm, 5 μm; Imtakt, Japan). A mobile phase gradient with acetonitrile (solvent A) and 0.1 % trifluoroacetic acid (solvent B) was developed: linear increment from 12 to 80 % of solvent A in 14 min at a flow rate of 0.7 mL min^{-1}, followed by a decrease in acetonitrile concentration to 12 % in 1 min at a flow rate of 1.0 mL min^{-1} which was kept for an additional 10 min to re-equilibrate the column; the next minute, the flow rate was decreased to 0.7 mL min^{-1}. The eluate was monitored using a photodiode-array 996 detector set at the wavelength characteristic for the tested compounds. The identification of phenolic compounds in the extract was done after alkaline and alkaline-acid hydrolyses by comparing their retention times with those of corresponding standards; additionally, a Waters 996 photodiode-array detector was applied to identify the compounds on the basis of their absorption spectra. The identification of compounds which standards are unavailable was done based on the elution order of flax phenolics and the formation or the decline under acid or alkaline hydrolysis (for details see [21]). Quantification of phenolics was done using the external standard method. Four independent samples were prepared for each extract. Each sample was injected two times. The results were expressed in mg per gram of extract.

Antioxidant activity analysis

DPPH assay

In the study, $DPPH^{\cdot}$ assay was performed according to the Sharma and Bhat method [22] based on the scavenging of $DPPH^{\cdot}$ free radical at the concentration of 50 μmol L^{-1} in buffered methanol (mixture of methanol and 100 mM acetic buffer, pH 5.57, in proportion of 3:2). Stock solutions of the extracts were prepared daily in buffered methanol, and then diluted to obtain various solutions at the concentrations within the assay activity range (final concentrations ranged from 0.03 to 0.12 g L^{-1}). Four independent experiments were performed for each extract.

The results were expressed as percentage of radical scavenging activity in comparison with the control without extract, and the log (concentration)–response curves were plotted. For comparison purposes and the correlation analyses, the EC50 value (i.e. the effective extract concentration needed to reduce $DPPH^{\cdot}$ radicals by half and expressed in g L^{-1}) was calculated based on the linear regression equation.

ORAC_FL assay

ORAC_FL assay (oxygen radical absorbance capacity assay with fluorescein as a fluorescent probe), which

measures antioxidant scavenging activity against peroxyl radical induced by AAPH, was carried out applying the method of Ou et al. [23] with a minor modification [24]. The assay was conducted at 37 °C in 75 mM phosphate buffer pH 7.4 with a blank sample in parallel. Briefly, 2.25 mL of fluorescein (48 nmol L^{-1} in phosphate buffer) was placed in a spectrophotometer polystyrene cuvette; then, 0.375 mL of sample and 0.375 mL of AAPH (153 mmol L^{-1} in phosphate buffer) were added. The fluorescence of fluorescein was recorded before and every 5 min after AAPH addition for 40 min (florescence spectrophotometer Hitachi F-2700, USA; 493-nm excitation and 515-nm emission wavelength).

Trolox solutions (0–100 μmol L^{-1}) were used as standards. Stock solutions of the extracts were prepared daily and then diluted to obtain appropriate concentrations within the assay activity range (final concentration: 0.03 g L^{-1}). At least four independent experiments were performed for each extract. The results were calculated applying the difference of areas under the fluorescein decay curves between the control without extract and the sample (net area) and were expressed as mmol of Trolox equivalents (TE) per gram of extract.

Statistical analysis

Statistical analyses were conducted using STATISTICA (version 9.0, StatSoft). Analysis of variance (ANOVA) for a CRD (completely randomised design) experiment was carried out, and then, Tukey's multiple range test at a significance level of $p \leq 0.05$ was applied to compare the means. Moreover, relationships between variables, i.e. between the extraction method (ethanol concentration in the extraction solvent) and phenolic compound contents or antioxidant activities of flaxseed extracts ($N = 4$ or 5 for ethanol concentration ranging from 60 to 80 % and from 60 to 90 %, respectively), were examined by linear regression analyses, and Pearson's correlation coefficients (r) were calculated.

Results and discussion

In the present study, ethanol–water solvents were applied to prepare extracts that could be safely introduced into food products. Zhang et al. [12] reported that the highest extraction yield was achieved with ethanol concentration in the range of 56–83 %, but the extraction yield decreased with lower ethanol content in extraction solvent. However, the activity and composition of extracts were not investigated. Our preliminary study concerning the effect of ethanol concentration in extraction solvent on flaxseed extract antioxidant activity showed that the activity significantly decreased when ethanol concentration in extraction solvent was lower than 60 %; the results of ORAC_FL assay amounted to 0.36 ± 0.01, 0.67 ± 0.04 and 1.07 ± 0.07 mmol TE g^{-1}, and DPPH assay results (expressed as half-maximal effective concentration—EC50) were 0.125 ± 0.007, 0.092 ± 0.001 and 0.081 ± 0.002 g L^{-1} for 30, 50 and 60 % ethanol extract, respectively. Based on these results, binary solvents with ethanol concentration from 60 to 90 % were selected to prepare flaxseed extracts and study the correlation between the ethanol–water solvent and the profile of extracted phenolics, and the antioxidant activity of flaxseed extracts.

Effect of ethanol concentration in extraction solvent on phenolic profile of flaxseed extracts

Typical HPLC chromatogram of phenolic compounds determined in flaxseed extracts is shown in Fig. 1. In Table 1, the phenolic contents of the flaxseed extracts are presented. The main phenolic compound of the 60–80 % ethanol extracts was flaxseed lignan SDG after alkaline hydrolysis (and its aglycone SECO after subsequent acid hydrolysis). The SDG concentration in the extract was found to be strongly negatively correlated with the ethanol concentration in extraction solvent ($r = -0.987$; Table 2); its content was the highest in 60 % ethanol extract and decreased with increasing ethanol concentration in the extraction solvent (Table 1). The SDG concentration was lower by about 11, 16, 44 and 94 % for the 65, 70, 80 and 90 % ethanol extract, respectively, as compared to the 60 % ethanol extract.

Phenolic acids, i.e. *p*-coumaric, caffeic, ferulic and hydroxybenzoic acids, as well as their derivatives (glucosides and esters), were also found in all extracts (Table 1; Fig. 1). Presence of these compounds in flaxseed was also reported in the earlier studies [25, 26]. Among non-glycosylated phenolic acids, only *p*-coumaric and ferulic acids were detected after alkaline hydrolysis of the extracts (the hydrolysis degraded the ester linkages but not glucosidic bounds); after subsequent acid hydrolysis, caffeic and *p*-hydroxybenzoic acids were also detected (Table 1).

The relationships between the extraction solvent and the content of phenolic acids or their derivatives in the flaxseed extracts were also observed. The content of phenolic acids and phenolic acid glucosides did not change significantly or slightly decreased with increasing ethanol concentration in the extraction solvent from 60 to 70 % (Table 1). Application of 80 % ethanol resulted in a considerable decrease in the content of these compounds. The increase in ethanol concentration up to 90 % in the solvent caused a further considerable drop of phenolic acid contents as well as their glucoside derivatives. In the 90 % ethanol extract, *p*-coumaric and ferulic acid contents after alkaline hydrolysis were about 16 % and 10 % of their amount in 60 % ethanol extract; in the

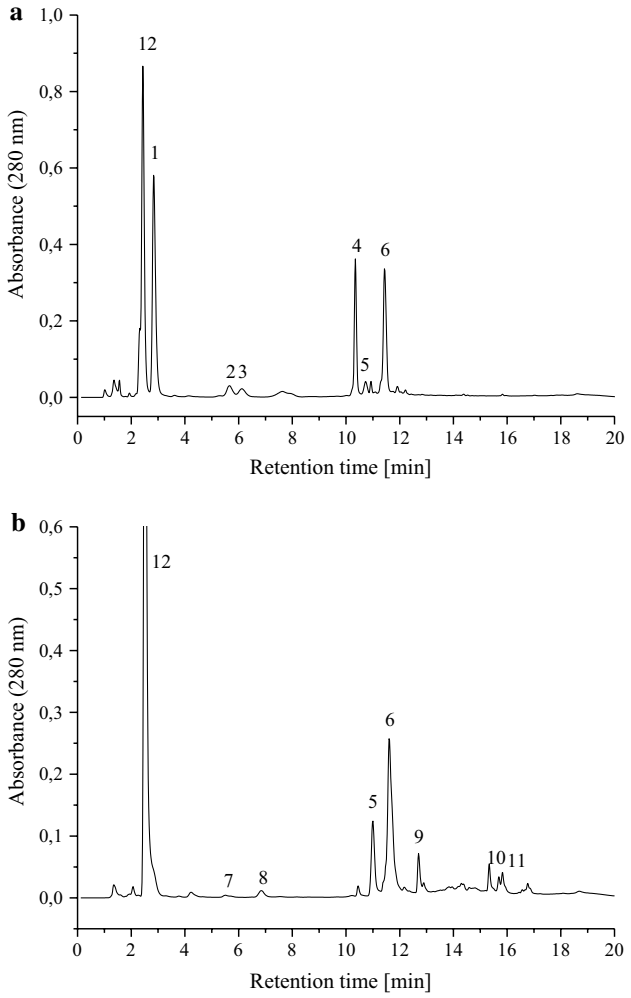

Fig. 1 HPLC chromatograms of **a** the alkaline and **b** alkaline-acid hydrolysates of 60 % ethanol flaxseed extracts: (*1*) *p*-coumaric acid glucoside, (*2*) caffeic acid glucoside, (*3*) ferulic acid glucoside, (*4*) SDG, (*5*) *p*-coumaric acid, (*6*) ferulic acid, (*7*) *p*-hydroxybenzoic acid, (*8*) caffeic acid, (*9*) SECO, (*10*) *p*-coumaric acid ester, (*11*) ferulic acid ester, (*12*) 5-hydroxymethylfurfural

case of *p*-coumaric, caffeic and ferulic acid glucosides, the amount dropped to 8, 10 and 11 %, respectively. The statistically significant negative linear correlations ($p < 0.05$) were found between the content of all phenolic acid glucoside derivatives and ethanol concentration in the applied extraction solvents, as well as between *p*-coumaric or *p*-hydroxybenzoic acid content and the ethanol concentration (particularly when it ranged from 60 to 90 %) (Table 2).

On the contrary, the extraction with 90 % ethanol increased significantly the concentration of phenolic acid esters in the extract (both *p*-coumaric and ferulic acid derivatives); their amounts were about 5 times higher than in the 60 % ethanol extract (Table 1).

The increase in ethanol concentration (from 60 to 90 %) in binary extraction solvents also changed the contribution of particular phenolics to total phenolics in the tested extracts. Having calculated the contribution of main phenolic compound groups to the total phenolics (after alkaline hydrolysis), it was found that the percentage of SDG decreased from 73 to 65 % when 60 and 90 % ethanol extracts were compared. The total phenolic acids and phenolic acid glucosides increased from 14 to 19 % and from 13 to 15 % of the total phenolics, respectively. It was also observed that the ratio of *p*-coumaric acid to ferulic acid (amount after alkaline hydrolysis) in 90 % ethanol extract (1:6) was half of this ratio in the other extracts (1:11–12); however, the ratios of *p*-coumaric acid, glucoside:caffeic acid, glucoside:ferulic acid glucoside were similar in all tested extracts.

The results of the study demonstrated that the phenolic profile of flaxseed extracts (i.e. the composition and content) significantly varied depending on the applied solvent. Our study showed that ethanol-to-water ratio of extraction solvent is an important factor affecting efficiency of phenolic compound extraction; an increase in ethanol volume in the tested solvents impacted negatively extraction of most flaxseed phenolic compounds. The 60 % ethanol extract was found to have a significantly higher content of SDG and total phenolics (after alkaline hydrolysis) than the other tested extracts. This is similar to the results of the previous studies concerning the effect of extraction conditions on phenolic content in extracts of other oilseeds [27, 28]. Taha et al. [27] studied the effect of various ethanol concentrations (ranged from 50 to 80 %) on the total content of phenolic compounds in sunflower meal extracts, and the results indicated that 60 % ethanol was the most efficient. Wettasinghe and Shahidi [28] showed that alcohol concentrations between 50 and 60 % were the best to prepare extracts from defatted borage meal.

It should be noticed that besides the differences in phenolic profiles of the tested flaxseed extracts, the differences in sensory quality were also observed. The 60 % ethanol extract was found to have a good texture quality (fine homogenous powder after freeze-drying with no changes in the quality after long-term storage in closed jar) when compared to 80 and 90 % ethanol extracts (they were difficult to dry and highly hygroscopic during storage; data not shown). The stability of extract sensory quality is important for its future application.

Effect of ethanol concentration in extraction solvent on antioxidant activity of flaxseed extracts

Two assays were selected to evaluate the changes in antioxidant activity of flaxseed extracts upon ethanol–water solvent extraction: ORAC_FL assay based on hydrogen atom transfer (HAT) reaction, i.e. radical quenching via H atom transfer and DPPH$^{\bullet}$ scavenging activity assay, classified as

Table 1 Effect of extraction method (ethanol–water solvent) on composition of phenolic compounds (mg g^{-1}) in flaxseed extracts

Phenolic compounds	Extraction solvent (% of ethanol)				
	60 %	65 %	70 %	80 %	90 %
After alkaline hydrolysis					
SDG	77.98 ± 1.04a	69.12 ± 0.58b	65.33 ± 0.68c	43.40 ± 0.46d	5.12 ± 0.24e
p-Coumaric acid glucoside[A, C]	9.77 ± 0.10c	10.58 ± 0.05a	10.06 ± 0.09b	6.77 ± 0.15d	0.78 ± 0.02e
Caffeic acid glucoside[A, D]	2.01 ± 0.01a	1.83 ± 0.04b	1.78 ± 0.02b	1.36 ± 0.03c	0.17 ± 0.01d
Ferulic acid glucoside[A, E]	1.82 ± 0.02a	1.73 ± 0.03b	1.64 ± 0.03c	1.28 ± 0.01d	0.17 ± 0.01e
p-Coumaric acid	1.26 ± 0.02a	1.16 ± 0.01b	1.13 ± 0.02b	0.96 ± 0.04c	0.21 ± 0.04d
Ferulic acid	13.70 ± 0.13a	14.04 ± 0.17a	13.67 ± 0.24a	11.96 ± 0.25b	1.24 ± 0.05c
Total after alkaline hydrolysis	106.53 ± 1.16a	98.45 ± 0.77b	93.62 ± 0.66c	65.72 ± 0.57d	7.70 ± 0.26e
After alkaline-acid hydrolysis					
SECO	15.84 ± 0.21a	14.07 ± 0.12b	13.30 ± 0.14c	8.90 ± 0.09d	6.43 ± 0.95e
p-Coumaric acid	7.80 ± 0.25a	7.52 ± 0.26a	7.47 ± 0.14a	4.83 ± 0.17b	3.21 ± 0.32c
Caffeic acid	1.28 ± 0.03b	1.61 ± 0.04a	1.66 ± 0.04a	0.98 ± 0.06c	0.45 ± 0.06d
Ferulic acid	14.46 ± 0.15b	17.14 ± 0.63a	16.64 ± 0.42a	12.28 ± 0.35c	8.09 ± 0.68d
p-Hydroxybenzoic acid	0.77 ± 0.03a	0.67 ± 0.03b	0.63 ± 0.01b	0.35 ± 0.01d	0.41 ± 0.02c
p-Coumaric acid ester[B, C]	0.71 ± 0.15bc	0.89 ± 0.02b	0.63 ± 0.15c	0.75 ± 0.14bc	3.53 ± 0.20a
Ferulic acid ester[B, E]	1.83 ± 0.39bc	2.14 ± 0.04b	1.45 ± 0.35c	1.77 ± 0.19bc	8.01 ± 0.68a
Total after alkaline-acid hydrolysis	42.67 ± 0.75ab	44.04 ± 0.99a	41.78 ± 1.14b	29.85 ± 0.72c	30.11 ± 1.12c

Mean ($n = 4$) ± SD; a, b, c, d—means with different letter in each row are significantly different at $\alpha = 0.05$ (one-way ANOVA and Tukey's test, a—the highest content)

SDG secoisolariciresinol diglucoside, *SECO* secoisolariciresinol

[A] Identified based on elution order of flax phenolics [21] and its decline after acid hydrolysis

[B] Identified based on elution order of flax phenolics and formation conditions [21]

[C] Quantified as *p*-coumaric acid

[D] Quantified as caffeic acid

[E] Quantified as ferulic acid

electron transfer (ET) reaction (i.e. direct radical reduction via ET) [18, 23].

The statistical analysis showed a significant effect ($p < 0.05$) of extraction solvent on the extract antioxidant activity (Fig. 2). It was found that the $DPPH^{\cdot}$ radical scavenging activity, expressed as the EC50 value, decreased with increasing ethanol concentration in the extraction solvent. EC50 values were 0.081, 0.103 and 0.134 g L^{-1} for 60, 70 and 80 % ethanol extract, respectively. The high positive correlation ($r = 0.978$; Table 2) was found between the concentration (60–80 % of ethanol) and the $DPPH^{\cdot}$ radical scavenging activity of the flaxseed extracts. Further increase in ethanol concentration up to 90 % drastically lowered $DPPH^{\bullet}$ radical scavenging activity of the extract (Fig. 2).

In the case of ORAC_FL assay results (showing antioxidant activity of the extracts against peroxyl radical), the differences among the activity of 60, 65 and 70 % ethanol extracts (Fig. 2) were not significant. The increase in ethanol concentration in extraction solvent to 80 % and then 90 % significantly lowered the antioxidant activity of the extracts. The statistical analyses confirmed our findings (Table 2); the correlation between the ethanol concentration and the antioxidant activity of flaxseed extracts was not statistically significant when the solvent effect was analysed for the range of 60–80 % of ethanol ($p \geq 0.05$); however, the significant correlation was established ($p < 0.01$) for the range of 60–90 % of ethanol.

Correlation between phenolic content and antioxidant activity of flaxseed extracts

The relationship between the changes in the content of phenolic compounds and the antioxidant activity of flaxseed extracts obtained with the various ethanol–water solvents was also evaluated (Table 3). High correlation coefficients were found between the $DPPH^{\cdot}$ radical scavenging activity and the SDG content. Moreover, the high negative linear correlation was established between the antiradical activity and the amount of phenolic acids and their glucoside derivatives determined after alkaline hydrolysis of the extracts. The significant correlations were established when the effect of binary solvent was studied both for the range of 60–80 % (with exception of *p*-coumaric acid glucosides) and 60–90 % of ethanol.

Table 2 Correlation between the extraction method (ethanol–water solvent) and the phenolic compound content or the antioxidant activity of flaxseed extracts

Factors	Correlation coeff. *r*	
	ET-OH 60–80 %	ET-OH 60–90 %
	$N = 4$	$N = 5$
Phenolic compound (mg g^{-1})		
SDG[a]	−0.987*	−0.974**
p-Coumaric acid glucoside[a]	-0.828^{NS}	−0.920*
Caffeic acid glucoside[a]	−0.983*	−0.941*
Ferulic acid glucoside[a]	−0.983*	−0.935*
Total phenolic acid glucosides[a]	-0.881^{NS}	−0.927*
p-Coumaric acid[a]	−0.990*	−0.917*
p-Coumaric acid[b]	-0.919^{NS}	−0.970**
Ferulic acid[a]	-0.870^{NS}	-0.860^{NS}
Ferulic acid[b]	-0.558^{NS}	-0.852^{NS}
Caffeic acid[b]	-0.510^{NS}	-0.831^{NS}
p-Hydroxybenzoic acid[b]	−0.982*	−0.912*
Total phenolic acids[a]	-0.897^{NS}	-0.865^{NS}
Total phenolic acids[b]	-0.735^{NS}	−0.908*
p-Coumaric acid ester[b]	-0.117^{NS}	0.781^{NS}
Ferulic acid ester[b]	-0.319^{NS}	0.769^{NS}
Total phenolic acid esters[b]	-0.265^{NS}	0.773^{NS}
Total phenolics[a]	−0.980*	−0.959*
Antioxidant activity		
$DPPH^{\cdot}$ assay—EC50[c] (g L^{-1})	0.978*	0.789^{NS}
ORAC_FL assay[d] (mmol TE g^{-1})	-0.897^{NS}	−0.965**

Linear regression: $y = ax + b$, ** $p < 0.01$, * $p < 0.05$, *NS* the finding is not statistically significant ($p \geq 0.05$); ET-OH 60–80 % and ET-OH 60–90 %—the analyses were performed for the ethanol concentration in extraction solvent ranging from 60 to 80 % and from 60 to 90 %, respectively

[a] After alkaline hydrolysis of extract

[b] After alkaline-acid hydrolysis of extract

[c] *EC50* The effective extract concentration needed to reduce the initial $DPPH^{\cdot}$ amount by half

[d] *TE* Trolox equivalent

The analysis of relationship between the changes in the content of main phenolics and the antioxidant activity of flaxseed extracts measured by ORAC_FL assay (Table 3) showed the linear correlation between the extract antioxidant activity and SDG as well as the total amount of phenolics; however, it was statistically significant only when the effect of binary solvent was studied for the range of 60–90 % of ethanol ($p < 0.01$). Similarly to $DPPH^{\cdot}$ scavenging activity, the correlations were also established in the case of total phenolic acid glucosides and total phenolic acid contents determined after alkaline hydrolysis of the extracts both for the range of

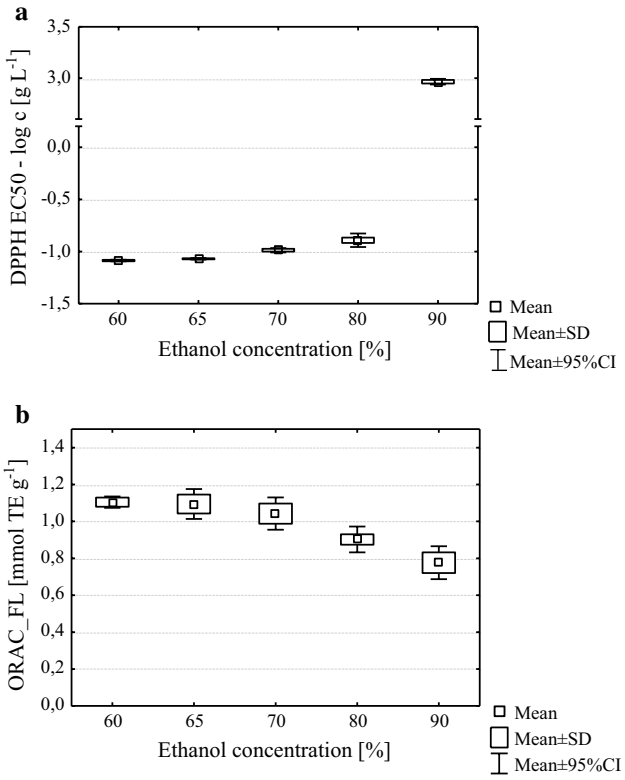

Fig. 2 Effect of extraction method (ethanol–water solvent) on the antioxidant activity of flaxseed extracts. **a** $DPPH^{\cdot}$ and **b** ORAC_FL assays. *EC50* and *TE* as in Table 2. Mean ($n = 4$) ± SD (as *square*); *error bars* represented the 95 % confidence intervals (CI) for mean (one-way ANOVA)

60–80 and 60–90 % of ethanol. This may suggest that the antioxidant activity measured by ORAC_FL assay is much more dependent on the content of the other phenolic compounds in the flaxseed extracts than SDG content. The ferulic acid presence should be highlighted since the relationship was found between the changes in ferulic acid content (after alkaline hydrolysis) and the antioxidant activity of flaxseed extract (Table 3). It was observed that the content of ferulic acid in the flaxseed extracts did not change when ethanol concentration in the extraction solvent increased from 60 to 70 % (Table 1); this corresponded with similar activity of the 60–70 % ethanol extracts against peroxyl radicals (ORAC_FL assay; Fig. 2) despite the significant decrease in SDG contents (Table 1). From the data, it can be stated that ferulic acid is an important element of the flaxseed extract antioxidant system.

The results of the study showed the relationships between the content of particular phenolic compounds and the antioxidant capacity of flaxseed extracts. Our results demonstrated the high correlation between the extract antiradical activity and the content of lignan SDG, as well as some phenolic acids and their derivatives. SDG molecule, as well as its aglycone SECO, has been previously reported

Table 3 Correlation between the phenolic compound content and the antioxidant activity of flaxseed extracts obtained with various ethanol–water solvents

Factors	Correlation coeff. *r*			
	DPPH· assay—EC50[c] (g L^{-1})		ORAC_FL assay[d] (mmol TE g^{-1})	
	ET-OH 60–80 %	ET-OH 60–90 %	ET-OH 60–80 %	ET-OH 60–90 %
	$N = 4$	$N = 5$	$N = 4$	$N = 5$
Total phenolics[a] (mg g^{-1})	−0.979*	−0.925*	0.948^{NS}	0.970**
Phenolic compound (mg g^{-1})				
SDG[a]	−0.973*	−0.903*	0.923^{NS}	0.973**
Phenolic acids and their derivatives after alkaline hydrolysis				
p-Coumaric acid glucoside[a]	−0.913^{NS}	−0.932*	0.982*	0.974**
Caffeic acid glucoside[a]	−0.965*	−0.947*	0.918^{NS}	0.952*
Ferulic acid glucoside[a]	−0.987*	−0.953*	0.956*	0.954*
Total phenolic acid glucosides[a]	−0.955*	−0.939*	0.996**	0.971**
p-Coumaric acid[a]	−0.956*	−0.967**	0.887^{NS}	0.931*
Ferulic acid[a]	−0.952*	−0.989**	0.994**	*
Total phenolic acids[a]	−0.958*	−0.988*	0.995**	0.909*
Phenolic acids and their derivatives after alkaline-acid hydrolysis				
p-Coumaric acid[b]	−0.948^{NS}	−0.808^{NS}	0.966*	0.988**
Ferulic acid[b]	−0.704^{NS}	−0.852^{NS}	0.854^{NS}	0.957**
Caffeic acid[b]	−0.648^{NS}	−0.835^{NS}	0.810^{NS}	0.942*
p-Hydroxybenzoic acid[b]	−0.972*	−0.488^{NS}	0.934^{NS}	0.857^{NS}
Total phenolic acids[b]	−0.840^{NS}	−0.844^{NS}	0.944^{NS}	0.983**
p-Coumaric acid ester[b]	−0.221^{NS}	0.997***	0.215^{NS}	−0.823^{NS}
Ferulic acid ester[b]	−0.377^{NS}	0.996***	0.310^{NS}	−0.816^{NS}
Total phenolic acid esters[b]	−0.336^{NS}	0.996***	0.286^{NS}	−0.818^{NS}

Linear regression: $y = ax + b$, *** $p < 0.001$, ** $p < 0.01$; * $p < 0.05$, *NS* the finding is not statistically significant ($p \geq 0.05$); ET-OH 60–80 % and ET-OH 60–90 %—the analyses were performed for the ethanol concentration in extraction solvent ranging from 60 to 80 % and from 60 to 90 %, respectively

[a] After alkaline hydrolysis of extract

[b] After alkaline-acid hydrolysis of extract

[c] *EC50* The effective extract concentration needed to reduce the initial DPPH· amount by half

[d] *TE* Trolox equivalent

to act as radical scavengers [29–31] and lipid peroxidation inhibitors [32, 33]. The 4-hydroxy-3-methoxy phenyl moiety of the lignan was suggested to be responsible for its antiradical activity [30, 34]. It was also proved that the presence of butadienol structure (oxygen-free benzylic position) enhances the radical scavenging activity of SECO in comparison with lignans with oxidised benzylic position (e.g. tetrahydrofuran-type lariciresinol [35] or butyrolactone-type matairesinol [34]); the importance of the structure for phenoxyl radical stabilisation was suggested [35].

Phenolic acids found in the flaxseed extracts, mostly classified as hydroxycinnamic acid derivatives, are also efficient antioxidants. Hydroxycinnamic acid has a simple phenylpropanoid (C_6–C_3) structure. Presence of a 2-propanoic acid side chain (–CH=CH-COOH) linked to the phenyl ring has been proposed to be a key for explanation of the higher antioxidant capacity of hydroxycinnamic acid derivatives than hydroxybenzoic acid derivatives with carboxyl group [36]; it was assumed that the side chain could play an important role in stabilisation of phenoxyl radical. Moreover, the red-ox potential and radical scavenging activity of hydroxycinnamic acid derivatives were related to the number and location of hydroxyl groups in the molecules [37]. Caffeic acid (3,4-dihydroxycinnamic acid) was found to be more active against DPPH· and peroxyl radicals than *p*-coumaric acid (4-hydroxycinnamic acid) [38, 39]. The methoxylation of 3-hydroxyl group in ferulic acid leaded to the increase in red-ox potential and the decrease in its antiradical activity when it was compared to caffeic acid activity [37].

The glucosylation effect on antioxidant activity of flaxseed phenolics, e.g. SDG versus SECO, was also studied,

but the results varied depending on the applied model system [30, 33]. The variability in the results was attributed to solubility and stability of the molecules which were dependent in part on polarity and pH of the environment of the model system under study. More polar SDG showed the higher antioxidant activity than SECO in PBS buffer environment of AAPH-derived peroxyl radical models [30] and the lower activity in lipid or liposomal systems [33]. In our research, difference in the changes of DPPH$^{\bullet}$ and ORAC_FL assay results could be also partially associated with differences in the assay conditions. However, the problem seems to be much more complex in the crude flaxseed extracts which are mixtures of compounds and further research should be performed to elucidate this finding.

The present results also showed that the increase in total concentration of phenolic acid esters in the flaxseed extract after extraction with 90 % ethanol correlated with a significant drop of the extract antioxidant capacity. High correlation coefficients (0.996–0.997) were established between the DPPH$^{\cdot}$ radical scavenging activity and the content of esterified phenolic acids in the 60–90 % ethanol extracts (Table 3). The linear correlation was also found in the case of ORAC_FL results (but it was not statistically significant). However, the results should be interpreted cautiously because simultaneous decrease in the content of other flaxseed phenolic compounds was observed with increasing ethanol content in extraction solvent. The study of Szwajger et al. [40] showed that phenolic acid esters had some antioxidant activity, although they were usually weaker radical scavengers and reducing agents in comparison with free forms. The exception was ferulic acid methyl ester which possessed the higher DPPH$^{\cdot}$ radical scavenging activity than ferulic acid [40]. It is worth to notice that esterified ferulic acid was found in a high amount in the 90 % ethanol extract. Therefore, the decrease in extract antioxidant activity seems to result rather from the drop of SDG and phenolic acid contents than from the increase in the content of ester derivatives.

In the present study, the phenolic profiles of flaxseed extracts were analysed both after alkaline hydrolysis and then subsequent acid hydrolysis. The higher correlation coefficients were observed between the antioxidant activity of flaxseed extracts and the content of phenolics detected after alkaline hydrolysis than those after alkaline-acid one (Table 3). In flaxseed, phenolic compounds are mostly present as large complexes (oligomers) which are composed of SDG molecules ester-linked with 3-hydroxy-3-methylglutaryl (HMG) residues and other phenolic compounds [41]. In our study, the alkaline hydrolysis of the tested extracts degraded the ester linkages but did not degrade glucosidic bounds (these were degraded after acid hydrolysis; Fig. 1). Therefore, the phenolics after alkaline hydrolysis were probably more closely related to the antioxidant activity of compounds in crude flaxseed extracts than those after alkaline-acid hydrolysis. Further research should be performed to elucidate this finding. An introduction of phenolic profile analysis after alkaline hydrolysis of flaxseed extract into future studies may help to explain a complex problem of composition–activity relationships.

Conclusions

The results of the study indicated relationships between the ethanol concentration in binary extraction solvent and the profile of extracted phenolic compounds, as well as the antioxidant capacity of flaxseed extracts. A proportion of ethanol to water in solvent mixture was crucial for the efficient extraction of phenolic compounds from flaxseed meal. The increase in ethanol concentration in binary extraction solvent from 60 to 90 % decreased the content of phenolic compounds in the extracts as well as changed the contribution of particular phenolic compounds to the total phenolics. Moreover, it was found that 60 and 65 % ethanol extracts showed the highest antioxidant activity (when both DPPH$^{\bullet}$ and ORAC_FL assays were applied); however, 60 % ethanol extract had significantly higher phenolic content (SDG and total phenolics after alkaline hydrolysis) than the other extracts. Therefore, the extraction method with 60 % ethanol can be an efficient and simple method to produce flaxseed extract that can be utilised as a valuable food ingredient.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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