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Isolation and antioxidant activity of phenolic compounds of evening primrose (*Oenothera biennis* L.)

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SUMMARY. Ethanol-ethyl acetate extract of evening primrose (*Oenothera biennis* L.) meal was separated into six fractions using Sephadex LH-20 gel column chromatography. The content of total phenolics and antioxidant activity in sunflower oil in each isolated fraction was determined. A pronounced antioxidant effect was noted at the fractions with high content of total phenolics. Gallic acid, protocatechuic acid and their methyl and ethyl esters were identified in these fractions by gas chromatography and mass spectrometry.

KEYWORDS: natural antioxidants; evening primrose; phenolics; column chromatography; lipid oxidation

Development of rancidity in edible oils is a serious problem in the food industry. Oxidation of lipids not only produces rancid odour and flavour, but also can decrease the nutritional quality and safety of food products by the formation of secondary oxidation products in food after cooking and processing. Effective methods against lipid oxidation include processing by minimizing the loss of natural tocopherols, by eliminating metal contamination and by using antioxidants. In the last 15–20 years, a special attention has been given to the use of natural antioxidants because of the worldwide trend to avoid or minimize the use of synthetic food additives. The interest in natural antioxidants grows because they are presumed to be safe since they occur in foods and have been used for centuries [1]. Currently, there are many studies investigating alternative natural sources of antioxidants that could substitute synthetic compounds and play a role in preventing many diseases.

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Recently, evening primrose (*Oenothera biennis* L.) seeds and their extracts and fractions have been reported to be antioxidative in various systems [2-11]. We recently found out in our research that an ethanol-ethyl acetate extract of evening primrose meal possessed very pronounced antioxidant properties. Using this extract, longer oxidation stability of edible oils was observed than using ethanol, acetone or ethyl acetate extracts [10].

The objectives of the present study were to separate phenolic antioxidant components present in the ethanol-ethyl acetate extract using Sephadex LH-20 column chromatography and identify them as their trimethylsilyl derivatives using gas chromatography - mass spectrometry.

Materials and methods

Reagents and standards

Chemicals, (+)-catechin hydrate, quercetin dihydrate, *trans*-3,4-dihydroxycinnamic acid (caffeic acid), *trans*-phenylacrylic acid (cinnamic acid), *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) and 3,4,5-trihydroxybenzoic acid (gallic acid) were obtained from Fluka, Steinheim, Germany.

Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany, ascorbylpalmitate (AP) was obtained from Klaus Evers, Chemie-Handel GmbH, Hamburg, Germany, and Grindox-118 (10% ascorbylpalmitate, 20% propylgallate, and 70% monoglyceride ester of citric acid) was obtained from Danisco Ingredients, Brabrand, Denmark. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Sigma Chemical Co, St. Luis, Missouri, USA.

Preparation of ethanol-ethyl acetate extract

Evening primrose meal, after industrial extraction of oil by supercritical CO_2, was obtained from Flaveko, Pardubice, Czech Republic. Extraction of phenolic compounds was carried out under reflux conditions. Evening primrose meal (100 g) was mixed with a solvent (900 ml; 96-% (v/v) ethanol), refluxed for 30 min and left to cool down overnight at ambient temperature. The suspension was filtered through a filter paper (Filtrak No. 390, Spezialpapierfabrik, Niederschlag, Germany) in a Büchner funnel, the residue was washed twice with 100 ml of the same solvent. The filtrates were pooled and the solvent was removed under vacuum using a rotary evaporator (Model 350, Unipan, Warsaw, Poland) and water bath (Model 356 H, Unipan, Warsaw, Poland). Evening primrose ethanol extract (5 g from 6.84 g total)

was re-extracted with ethyl acetate (95 ml) at occasional shaking in a conical flask at ambient temperature for 4 hours. The mixture was filtered through a filter paper and the solvent was removed under vacuum using the rotary evaporator at 40 °C.

Column chromatography

The ethanol-ethyl acetate extract (500 mg from 645 mg total) was dissolved in 5 ml of 96-% (v/v) ethanol and applied to a chromatographic column (3.0 x 45 cm) packed with Sephadex LH-20 (Pharmacia, Uppsala, Sweden). The column material was suspended in 96-% ethanol, and it was allowed to swell for 24 h [12]. Elution was carried out with 96-% ethanol at a flow rate of 1 $ml.min^{-1}$. Sixty fractions, 10 ml each, were collected and their absorbance at 280 nm was measured using the spectrophotometer UV/VIS PU 8700 Philips (Pye Unicam, Cambridge, UK). Based on their absorbance data, eluates were then pooled into six major fractions and the solvent was evaporated under vacuum at 40 °C. The weight of each fraction was recorded and the content of phenolics determined.

Determination of total phenolics content

The total phenolics content in each fraction was estimated using Folin-Denis reagent [13]. The isolated fractions were as gallic acid equivalents, dissolved in ethanol to obtain a concentration of 0.5 $mg.ml^{-1}$. The Folin-Denis reagent (0.5 ml) was added to a centrifuge tube containing the fraction (0.5 ml). The contents were mixed and a saturated sodium carbonate solution (1 ml) was added into the tube. The volume was adjusted to 10 ml by adding 8 ml of distilled water and the contents were mixed vigorously. The tubes were allowed to stand at ambient temperature for 25 minutes and then centrifuged for 5 min at 4 000 *g*. Absorbance of the supernatants was measured at 725 nm. The total phenolics content in each isolated fraction was determined using a standard curve prepared for gallic acid [14]. Total phenolics were expressed as milligrams of gallic acid equivalents per gram of fraction. All measurements were replicated three times; mean values with ± standard deviations were reported for each case.

Evaluation of antioxidant activity

Sunflower oil, which was used as a substrate for an evaluation of antioxidant activity of the isolated fractions at a level of 0.02 % (wt-%), was obtained from the local fat factory Palma-Tumys, Bratislava, Slovak Republic. The oil was analysed by determining its iodine [15, Cd 1-25] and peroxide [15, Cd 8-53] values plus the fatty acid composition was determined. Fatty

acids methylesters (FAMEs) were prepared [16] and then analysed using a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame-ionization detector [10]. Sunflower oil used in this study had good initial qualities (Tab. 1).

TAB. 1. Parameters and fatty acid composition of the sunflower oil.
TAB. 1. Parametre a zloženie mastných kyselín slnečnicového oleja.

Parameter[1]				
Iodine value [g iodine per 100 g of oil][2]	126.8			
Peroxide value [mmol ½O_2 per 1 kg of oil][3]	0.429			
Oxidation stability at 110 °C - IP[4] [h]	3.65			
Fatty acid composition[5] [%]	C 14:0	0.08	C 18:2	57.38
	C 16:0	5.90	C 18:3	0.10
	C 16:1	0.10	C 20:0	0.31
	C 18:0	4.65	C 20:1	0.36
	C 18:1	30.37	C 22:0	0.75

1 - parameter, 2 - jódové číslo [g jódu na 100 g oleja], 3 - peroxidové číslo [mmol ½O_2 na 1 kg oleja], 4 - oxidačná stabilita pri 110 °C - IP, 5 - zloženie mastných kyselín.

An Oxidograph apparatus (Mikrolab, Aarhus, Denmark) was employed to monitor oxidation of the sunflower oil with an addition of isolated fractions at 110 °C. The Oxidograph can simultaneously analyse six samples, each reaction vial uses 5-g samples and 100 ml of oxygen. The oxygen consumption (drop of oxygen pressure) is monitored continuously and the analysis is finished within one working day. Oxygen pressure showed nearly no changes during the induction period (IP) and started to decrease rapidly after its end [10]. The IP was determined by the method of tangents to two parts of the oxidation curve. Measurements on Oxidograph apparatus were replicated three times and mean values with ± standard deviations were reported for each case. The effectiveness of antioxidants was expressed as a stabilisation factor F:

$$F = \frac{IP_A}{IP_0}$$

where IP_A is the induction period of oil in the presence of the evaluated compounds and IP_0 is the induction period of oil in the absence of additives.

The oil without any additive or with commercial antioxidants such as BHT (0.01 %), AP (0.02 %), Grindox-118 (0.02 %), catechin (0.02 %), caffeic acid (0.02 %) and gallic acid (0.02 %) was used for comparative studies.

Gas chromatography/Mass spectrometry

Standard phenolic compounds, ethanol-ethyl acetate extract of evening primrose and its fractions II–IV (2–5 mg) were silylated with 0.5 ml of bis(trimethylsilyl)trifluoroacetamide at 125 °C for 10 min in a sealed vial [17]. After cooling down, 1 μl of the reaction mixture was directly injected into a gas chromatograph. The gas chromatograph HP 5890II coupled to HP 5971A mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA) was employed. Samples were injected into a fused silica capillary column HP-1, 12 m × 0.20 mm × 0.33 μm (Hewlett-Packard) by split technique at 270 °C of injector temperature and carrier gas split ratio 1:30. Helium was used as a carrier gas. The column temperature was programmed from 100 °C to 270 °C with a gradient of 20 °C/min and held 12 min isothermically. The ionization energy (EI) was 70 eV.

Statistical analysis

Statistical analysis was carried out with Statgraphics Plus programme (version 1.4 for Windows, Manugistic Inc., Rockville, USA). The significance of differences among the mean values were determined at $p < 0.05$ using one-way analysis of variance and t-test. Linear regression analysis was carried out to evaluate the relationship between antioxidant activities and total phenolic contents.

Results and discussion

Column chromatography

Six fractions (I-VI) were separated from the ethanol-ethyl acetate extract of evening primrose meal using Sephadex LH-20 column chromatography according to their absorbance readings at 280 nm (Fig. 1). The chromatogram was characterized by one sharp intensive peak (II), two broad peaks (V and VI) and three medium-sized peaks (I, III and IV). The relative contents of fractions I and II were much higher (56.7 % and 29.9 %, respectively) than those of fractions III-VI (3.1-3.7 %) (Tab. 2).

Sephadex LH-20 is known to be an ideal medium for the fractionation of organic compounds on the basis of molecular size, but fractionation of aromatic compounds (especially phenolic compounds) might be also

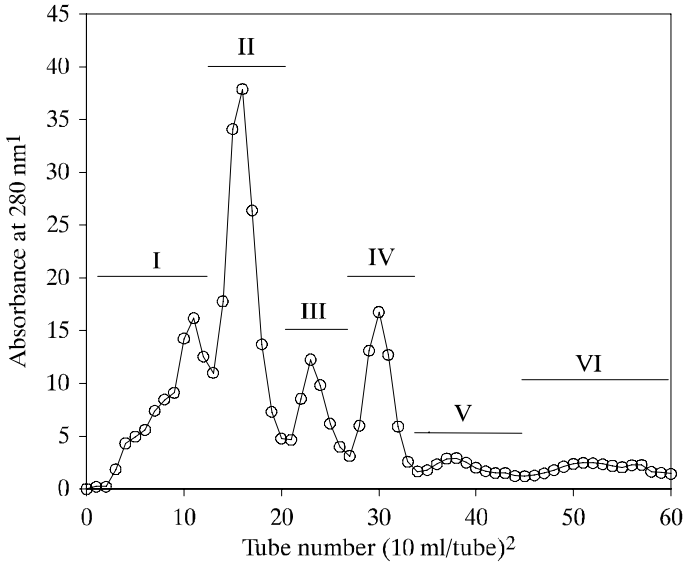

FIG. 1. Separation of evening primrose ethanol-ethyl acetate extract fractions by Sephadex LH-20 column chromatography with 96-% (v/v) ethanol as a solvent.

OBR. 1. Separácia etanol-etylacetátového extraktu pupalky dvojročnej kolónovou chromatografiou na Sephadexe LH-20 (96% obj. etanol ako rozpúšťadlo). 1 - absorbancia pri 280 nm, 2 - číslo skúmavky (10 ml/skúmavka).

TAB. 2. Distribution of separated phenolic fractions and their content of total phenolics as gallic acid equivalents.
TAB. 2. Relatívne zastúpenie a obsah celkových fenolických látok v chromatografických frakciách pupalkového extraktu (vyjadrené ako kyselina galová).

Fractions[1]	Relative content[2] [%]	Total phenolics[3]* [mg.g^{-1}]
I	56.7	8.1 ± 0.2
II	29.9	32.4 ± 0.4
III	3.3	284 ± 4
IV	3.3	290 ± 4
V	3.7	236 ± 1
VI	3.1	298 ± 3

* - each value is the mean of three determinations ± standard deviation.
* - každá hodnota je priemerom troch stanovení ± štandardná odchýlka. 1 - frakcia, 2 - relatívne zastúpenie, 3 - celkové fenolické látky.

associated with the free phenolic hydroxyl groups [12]. An ethanolic [4] and aqueous acetone [2, 5, 9] extracts of evening primrose have also been fractionated by Sephadex LH-20 column chromatography. Antioxidant activity of the so obtained fractions was dependent on the content of total phenolics in the individual fractions.

Total phenolics in the fractions

The total content of phenolics in the ethanol-ethyl acetate extract of evening primrose meal fractions as determined by the Folin-Denis reagent ranged from 8 to 298 $mg.g^{-1}$ (Tab. 2). Folin-Denis reagent is non-specific, so it detects all phenolics (phenolic acids, flavonoids and tannins) present in the extract [12]. In fractions III–VI the content of phenolics was significantly ($p < 0.05$) higher than in the fractions I and II.

The total phenolics content in the ethanol-ethyl acetate extract before the column chromatography was 168 ± 13 $mg.g^{-1}$, almost two times higher in comparison with the crude ethyl acetate extract, and 2.4 times lower in comparison with the crude ethanol extract.

Antioxidant activities of fractions

The effect of collected fractions (I–VI) on oxidation of sunflower oil (SO) was evaluated using Oxidograph apparatus at 110 °C. Results of their antioxidant properties are presented in Fig. 2. The antioxidant activity of the fractions determined by the Oxidograph method was expressed as the stabilization factor (F) used by Marinova and Yanishlieva [18]. The higher value of F indicated the stronger antioxidant activity, value of one indicated that the sample had no activity, a sample that showed oxidation effect produced a value below one. Fractions I and VI of the ethanol-ethyl acetate extract of evening primrose had oxidation effects, fractions III and IV had strong antioxidant activity and fractions II and V did not affect the oxidation of SO. Strong antioxidant activity of fractions III and IV compared to those of other examined fractions may be due to the diversity in structural characteristics of phenolic antioxidants present in the fractions. Although it is believed that higher amount of phenolic compounds offers better antioxidative properties, the relationship between phenolic contents and antioxidant activities for fractions was not significant ($R^2 = 0.488$, $\alpha = 0.05$). This indicates that factors other than total phenols play a major role in the antioxidant activity of plant materials. Particularly, the chemical structure of phenolic compounds is very important for their antioxidant activity [19-21]. The fraction III of the ethanol-ethyl acetate extract of evening primrose meal (0.02 %), containing 284 $mg.g^{-1}$ of phenolics, had

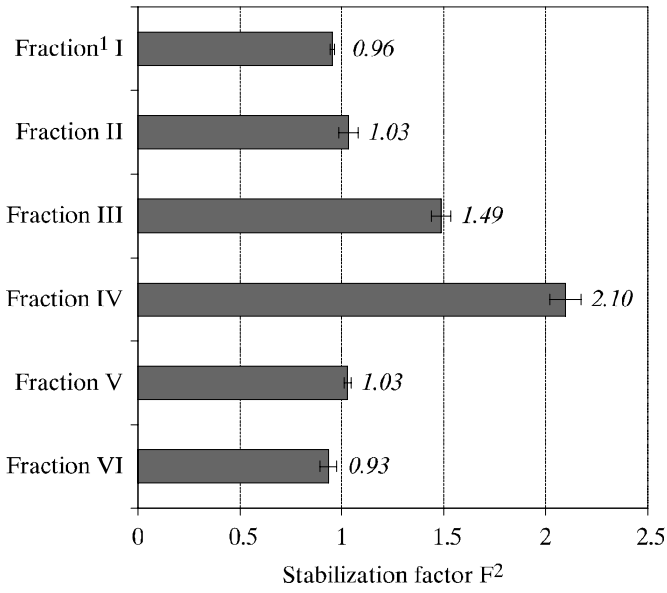

FIG. 2. Antioxidant activity of evening primrose ethanol-ethyl acetate extract fractions after column chromatography in sunflower oil as measured by oxygen consumption using Oxidograph apparatus at 110 °C (0.02% (wt-%) addition of each fraction).

OBR. 2. Antioxidačná aktivita frakcií z chromatografie etanol-etylacetátového extraktu pupalky dvojročnej v slnečnicovom oleji (0,02 % hm. prídavok frakcií, merané spotrebou kyslíka na prístroji Oxidograph pri 110 °C).
1 - frakcia, 2 - stabilizačný faktor F.

1.3–1.4 times higher activity than butylated hydroxytoluene (BHT, 0.01 %) or ascorbylpalmitate (AP, 0.02 %) and the fraction IV (0.02 %), containing 290 mg.g^{-1} of phenolics, had as much as 1.8–2 times higher activity than BHT (0.01 %) or AP (0.02 %).

Identification of antioxidant effective compounds

The total ion current chromatograms of trimethylsilyl derivatives of the crude ethanol-ethyl acetate extract of evening primrose meal, fraction III and IV, are shown in Fig. 3. Phenolic compounds were identified by comparison of their retention times with standards and by comparison of their mass spectra with those in the Wiley 138 and NIST/EPA/NIH mass spectra libraries. Methyl ester of protocatechuic acid, protocatechuic acid, methyl ester of gallic acid, ethyl ester of gallic acid, gallic acid and catechin were

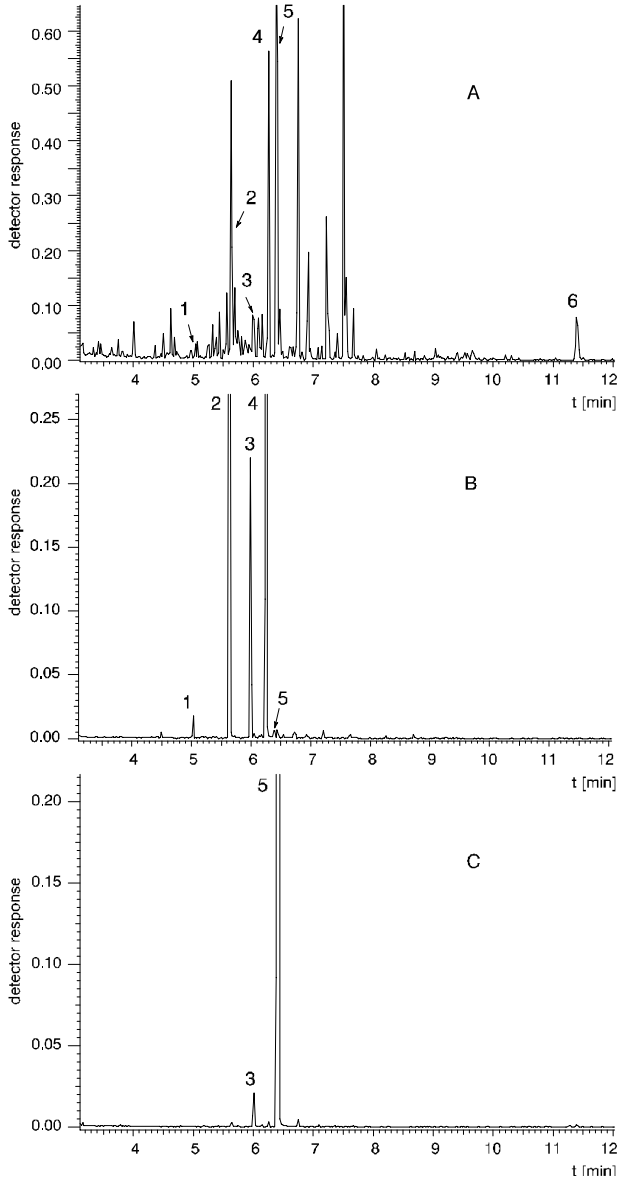

FIG. 3. Total ion current chromatograms of trimethylsilyl derivatives of ethanol-ethyl acetate crude extract constituents (A), fraction III (B) and fraction IV (C).
1 - methyl ester of protocatechuic acid, 2 - protocatechuic acid, 3 - methyl ester of gallic acid, 4 - ethyl ester of gallic acid, 5 - gallic acid, 6 - catechin.

OBR. 3. GC-MS chromatogramy trimetylsilyl derivátov zložiek etanol-etylacetátového surového extraktu (A), frakcie III (B), a frakcie IV (C).
1 - metylester kyseliny protokatechovej, 2 - kyselina protokatechová, 3 - metylester kyseliny galovej, 4 - etylester kyseliny galovej, 5 - kyselina galová, 6 - katechín.

identified in the crude extract. Methyl ester of protocatechuic acid, protocatechuic acid, methyl ester of gallic acid, ethyl ester of gallic acid and gallic acid were found in fraction III and methyl ester of gallic acid and gallic acid were found in fraction IV. Both gallic acid and protocatechuic acid may occur in the natural material as free acids, glycosides, esters (e. g. ethyl esters of protocatechuic and gallic acids were detected in wine recently [22], methyl gallate was found in chestnuts [23]), and insoluble bound phenolic acids [24]. However, it is unclear whether methyl and ethyl derivatives of gallic and protocatechuic acids were present in the raw material or produced by reactions during the process of isolation (extraction or column chromatography).

Fractional separation of evening primrose extracts indicates that these extracts consist of many phenolic substances other than phenolic acids such as flavonoids (catechin, epicatechin and procyanidin gallate) [2, 4] and

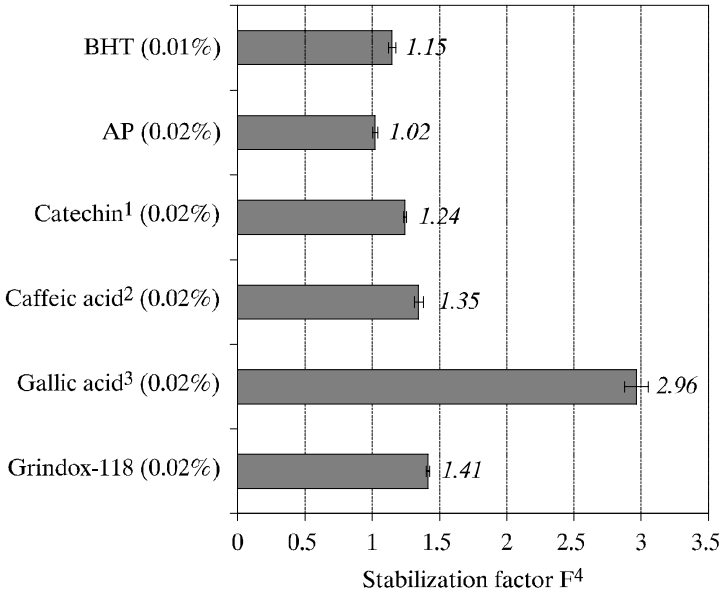

FIG. 4. Antioxidant activity of commercial antioxidants in sunflower oil as measured by oxygen consumption using Oxidograph apparatus at 110 °C. Addition of antioxidants in wt-%. BHT - butylated hydroxytoluene, AP - ascorbylpalmitate.

OBR. 4. Antioxidačná aktivita komerčných antioxidantov v slnečnicovom oleji meraná spotrebou kyslíka na prístroji Oxidograph pri 110 °C. Prídavok antioxidantov v % hm. BHT - butylhydroxytoluén, AP - askorbylpalmitát. 1 - katechín, 2 - kyselina kofeínová, 3 - kyselina galová, 4 - stabilizačný faktor F.

tannins [9]. These compounds also may have various antioxidant and synergistic effects depending on the used testing methods and systems, in which they are tested.

Results on antioxidant properties of commercial antioxidants are presented in Fig. 4. Commercial chemicals (+)-catechin hydrate (0.02 %) and caffeic acid (0.02 %) stabilized sunflower oil as well as commercial antioxidant Grindox-118. However, the oil with commercial gallic acid (0.02 %) had almost 3 times higher oxidation stability than the control oil and 2.1–2.9 higher stabilization factor than the other evaluated commercial antioxidants. These experimental results show that particularly gallic acid and its derivatives isolated from the extract of evening primrose could be the phenolic compounds suitable for stabilization of sunflower oil.

Based on presented results research continues with the aim to isolate and identify other co-occurring phenolic components present in evening primrose meal. The potential application of extracts and fractions of evening primrose in different systems is also being examined.

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Izolácia a antioxidačná aktivita fenolických látok z pupalky dvojročnej (*Oenothera biennis* L.)

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SÚHRN. Etanol-etylacetátový extrakt múky z pupalky dvojročnej (*Oenothera biennis* L.) sa rozdelil gélovou kolónovou chromatografiou na Sephadexe LH-20 na šesť frakcií. Frakcie sa podrobili testom na celkový obsah fenolických látok a na antioxidačnú aktivitu v slnečnicovom oleji. Výrazný antioxidačný efekt mali frakcie s vysokým celkovým obsahom fenolických látok. V týchto frakciách sa metódou GC-MS identifikovali: kyselina galová, kyselina protokatechuová a ich metyl- a etylestery.

KĽÚČOVÉ SLOVÁ: prírodné antioxidanty; pupalka dvojročná; fenolické zlúčeniny; kolónová chromatografia; oxidácia lipidov