In the recent years, there has been growing interest in the use of natural antioxidants rather than of synthetic ones. Many plant extracts have been studied for their potential antioxidant activity. Very important and inexpensive sources of natural antioxidants are industrial by-products, especially from agriculture and food producers (Schmidt & Pokorný 2005). They are found to be rich sources of bioactive compounds such as flavonoids, tannins, phenolic acids, terpenoids, and many others. Evening primrose meals are one of these studied by-products that are gaining attention (Hagerman et al. 1998; Moure et al. 2001; Schmidt et al. 2003a; Zahradníková et al. 2007).

Evening primrose (Oenothera biennis L.) or evening star, is a biennial flowering plant growing to the height of 1.2 m with easy germination. Its hermaphrodite monocious, pale yellow flowers bloom for most of the summer, from June to September. They open in the evening, hence the name “evening primrose”. Mature seeds contain approximately 7–14% γ-linolenic acid, a rare essential fatty acid (Hudson 1984). Therefore the seeds have been used for the production of edible oil with a high biological activity. Evening primrose seed oil is used to reduce the pains of premenstrual stress syndrome, it relieves obesity, diabetes mellitus, hypercholesterolemia, and others (Meyer et al. 1998). Attention is also given to polyphenols present in the plant seeds. Polyphenols of the plant seeds prevent oxidation of lipids by scavenging free radicals and the reactive forms

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Fractionation and Identification of Some Phenolics Extracted from Evening Primrose Seed Meal

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Abstract


Crude ethanol and ethyl acetate extracts of evening primrose (Oenothera biennis L.) seed meal were separated into fractions using Silicagel L 100/200 column chromatography and methanol as the mobile phase. Six fractions were obtained from the ethanol extract and five fractions from the ethyl acetate extract and their contents of total phenolics were determined. By means of HPLC, the active compounds were identified as catechin, gallic acid, caffeic acid, syringic acid, and ferulic acid.

Keywords: evening primrose; phenolic acids; HPLC; phenolic compounds

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of oxygen. The activated oxygen forms such as peroxide, hydrogenperoxide, hydroxyl radical, and singlet oxygen may cause various diseases such as cancer, inflammation, atherogenesis, as well as food deterioration. Natural antioxidants may be effective in eliminating these forms of oxygen. Many studies have dealt with the antioxidant activity of evening primrose meals. As mentioned above, the compounds that possess the antioxidant properties of evening primrose are polyphenols, including also flavonoids and tannins. Evening primrose meals have a high content of phenolic compounds (86.2 g/kg, for comparison rapeseed meals contain 79.7 g/kg of phenolic compounds); they contain gallic acid, caffeic acid, syringic acid, ferulic acid, and others. Out of flavonoids, catechin and epicatechin are present (Schmidt et al. 1998, 2003b; Wettasinghe et al. 2002).

The aim of this research was to identify the compounds present in the ethanol and ethyl acetate fractions of evening primrose meals that are responsible for their antioxidant character. In the next article, attention will be given to the antioxidant and antimicrobial properties of these fractions (Daud et al. 2005).

**MATERIAL AND METHODS**

**Material.** Gallic acid (3,4,5-trihydroxybenzoic acid), 97%; caffeic acid (trans-3,4-dihydroxycinnamic acid), 97%; sinapic acid (trans-3,5-dimethoxy-4-hydroxycinnamic acid), 98%; cinnamic acid (trans-3-phenylacrylic acid), 97%; ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid); syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), 95%; vanillic acid (4-hydroxy-3-methoxybenzoic acid), 97%; HPLC grade methanol, and HPLC grade acetonitrile were purchased from Sigma-Aldrich ChemiGmbH (Deisenhofen, Germany). (+)-Catechin hydrate, 98%, was purchased from Fluka (Steinheim, Germany). Silicagel L 100/200 was purchased from Lachema (Brno, Czech Republic).

Seeds of evening primrose grown in the Czech Republic (in the south of Moravia) were extracted with supercritical carbon dioxide on the industrial scale in Flaveko (Pardubice, Czech Republic). Defatted seed meal was air-dried for 24 h and stored in vacuum packaged polyethylene pouches at –20°C until used.

**Preparation of ethanol and ethyl acetate extracts.** Evening primrose meal extracts were prepared as follows: meals (20 g) were mixed with a solvent (180 ml, 96% v/v aqueous ethanol), refluxed gradually for 30 min in a Soxhlet extractor and left aside to cool overnight at an ambient temperature. The suspension was filtered through a Büchner funnel and the residue was washed twice, each time with 100 ml of the same solvent. The filtrates were combined and the solvent was evaporated under a reduced pressure. The ethanol extract (5 g) was re-extracted with ethyl acetate (45 ml) under occasional shaking for 4 h at an ambient temperature in a conical flask. The mixture was filtered and the solvent was evaporated under a reduced pressure. Both extracts were transferred into a conical flask filled with argon and stored in a freezer at –20°C. During the extraction and sample preparation, the used laboratory glass was covered with aluminium foil to protect the extract against the light.

**Determination of total phenolics content.** Each of the extract was dissolved in methanol to obtain a concentration of 0.5 mg/mL. Folin-Denis reagent (0.5 ml) was added to the centrifuge tubes containing 0.5 ml of the extract. The contents were mixed and 1 ml of saturated solution of sodium carbonate was added into each tube. The volume was adjusted to 10 ml by the addition of deionised water and the contents were mixed vigorously. The tubes were allowed to stand at an ambient temperature for 25 min and then centrifuged for 5 min at 4000 g. The absorbance of the supernatants was measured at 725 nm, using a spectrophotometer Shimadzu UV 1601 (Japan). A blank sample without extracts was used (Swain & Hillis 1959; Xu & Diosady 1997). The contents of total phenolics in the extracts were determined using a standard curve prepared for (+)-catechin. The total phenolics extracted were expressed as mg of (+)-catechin equivalents per gram of extract. Evening primrose extracts produced a pink coloured complex with an acidic solution of vanillin, which indicated the presence of tannins. Therefore, (+)-catechin was used as the standard for the calibration curve.

**Fractionation of ethanol and ethyl acetate extracts.** The ethanol extract and the ethyl acetate re-extract (1 g) were dissolved in 5 ml of HPLC grade methanol and the solutions were applied to a chromatographic column (30 mm diameter × 450 mm height) filled with Silicagel L 100/200. The extract was eluted with methanol; the flow rate was 1 ml/min. Methanol fractions (10 ml each) were collected in the test tubes and their absorbance was measured at 280 nm, using a UV/VIS
spectrophotometer Shimadzu UV 1601 (Japan). Eluates were then pooled into fractions I–VI for the ethanol extract and fractions I–V for the ethyl acetate extract on the basis of UV absorbance data. The solvent was evaporated under a reduced pressure. Both extracts were transferred into a conical flask filled with argon and stored in the dark in a freezer at −20°C for 2 days (Amarowicz & Shahidi 1994; Naczk & Shahidi 2004).

Analysis of phenolic compounds by high performance liquid chromatography. Watrex HPLC chromatograph (Germany) equipped with a pump DeltaChrom SDS 030 (Watrex, Germany), UV-VIS spectrophotometric detector UVD 250 (Watrex, Germany), and a chromatographic station CSW 32 were used for the separation and identification of the compounds in the obtained fractions. The conditions of the measurement were as follows: 25 cm column (Reprosil 100, C18, 5 μ, 250 × 4 mm, Watrex, Germany), water-acetonitrile-methanol-acetic acid (79.5:18:2:0.5 v/v/v/v) as the mobile phase, 1 ml/min flow rate, 20 μl injection volume, 30°C temperature of the analytical column, the detector was preset at 280 nm (Amarowicz & Shahidi 1994).

Statistical analysis. The statistical analysis was carried out using the program Statgraphics Plus, Version 3.0 for Windows (Manugistic Inc., Rockville, USA). The significance of differences between the mean values was determined at the \( P = 0.05 \) level, using a one-way analysis of the variance and the \( t \)-test.

RESULTS AND DISCUSSION

Preparation of ethanol and ethyl acetate extracts

The yields of the extraction of evening primrose meals are presented in Table 1. The crude ethanol extract was of a dark red-brown greasy consistence, while the ethyl acetate re-extract was of the same colour, but of powdery consistence. The yield of the ethanol extraction was 6.8% and that of the ethyl acetate re-extraction was 18.1% (in relation to ethanol extract). The differences in the extraction yields obtained with various solvents may have been due to different solubilities and polarities of the extracted compounds as well as of the solvents. The compounds possessing the antioxidant character which are present in evening primrose meals are soluble in medium polar solvents (ethanol, ethyl acetate, and acetone).

Determination of the content of total phenolics

The contents of total phenolics in evening primrose meal extracts and their fractions were de-

Table 1. The extraction yields and the content of total phenolics (mg (+)catechin equivalents/g extract) in crude evening primrose meal extracts

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Yield of extraction (%)</th>
<th>Total phenolics content (mg (+)catechin equivalents/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>6.8 ± 1.1</td>
<td>248 ± 13</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>18.1 ± 0.8</td>
<td>171 ± 11</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of silica gel column chromatography of the ethanol extract – relative content of individual fractions (%) and the content of total phenolics (mg (+)catechin equivalents/g extract) in evening primrose meal ethanol fractions

<table>
<thead>
<tr>
<th>Ethanol fractions</th>
<th>Relative content (%)</th>
<th>Total phenolics content (mg (+)catechin equivalents/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20.5 ± 1.3</td>
<td>122 ± 9</td>
</tr>
<tr>
<td>II</td>
<td>24.4 ± 1.5</td>
<td>146 ± 12</td>
</tr>
<tr>
<td>III</td>
<td>16.7 ± 0.9</td>
<td>289 ± 17</td>
</tr>
<tr>
<td>IV</td>
<td>14.3 ± 0.9</td>
<td>314 ± 19</td>
</tr>
<tr>
<td>V</td>
<td>10.9 ± 0.4</td>
<td>202 ± 10</td>
</tr>
<tr>
<td>VI</td>
<td>11.1 ± 1.6</td>
<td>180 ± 9</td>
</tr>
</tbody>
</table>
determined by Folin-Denis reagent, the results are shown in Tables 1–3. Folin-Denis reagent detects all phenolic compounds (phenolic acids, flavonoids, tannins) present in the extract. The content of phenolics was expressed as mg (+)catechin equivalents per gram of the extract. In the ethyl acetate re-extract, the content of phenolics was 171 ± 11 mg catechin per gram of the extract, and in its fractions ranged from 86 ± 8 mg catechin per gram of the extract to 266 ± 15 mg catechin per gram of the extract. In the ethanol extract, the content of phenolics was 248 ± 13 mg catechin per gram of the extract, and in its fractions ranged from 122 ± 9 mg catechin per gram of the extract to 314 ± 19 mg catechin per gram of the extract. The content of total phenolics may vary because of the different polarity and solubility of both extracts, as well as of the different polarity of the compounds found in the fractions and the solvent used for the extraction. In the study of Wettasinghe et al. (2002), the content of total phenolics of the acetone extract of evening primrose meal fractions varied from 158 ± 10 mg to 445 ± 25 mg catechin per gram of the extract and the content of total phenolics in the crude extract was 304 mg catechin per gram of the extract. In another study, the content of total phenolics in evening primrose meal extracts was expressed as mg caffeic acid equivalents per gram of the extract. In the ethanol extract, the content of phenolics was 406 ± 25 mg caffeic acid per gram of the extract. In the ethyl acetate re-extract, the content of phenolics was 87 ± 12 mg caffeic acid per gram of the extract (Niklová et al. 2001).

### Fractionation of ethanol and ethyl acetate extracts from evening primrose meals

Both extracts (ethanol and ethyl acetate) were fractionated on Silicagel column using methanol.
as the mobile phase. Six and five major fractions, respectively, were obtained from ethanol and ethyl acetate extracts, on the basis of absorbance readings at 280 nm (Figures 1 and 2). Each fraction contained phenolic compounds which were detected by the reaction with Folin-Denis reagent that results in a characteristic blue colour. The relative content of each fraction and the total phenolics content of the fractions were calculated and summarised in Tables 1–3 as mentioned above.

In some studies, Sephadex LH 20 column packing was used (Schmidt et al. 2003b; Wettasinghe & Shahidi 2000; Amarowicz et al. 2007). The relative content of each fraction and the total phenolics content may have been affected according to the column packing and the mobile phase that was used.

(a) ethanol fraction III of evening primrose meals: 1 – gallic acid, 3 – catechin, 5 – syringic acid (2, 4 – unidentified compounds)

(b) ethanol fraction IV of evening primrose meals: 1 – gallic acid, 2 – catechin, 4 – syringic acid, 5 – ferulic acid (3 – unidentified compound)

(c) ethyl acetate fraction III of evening primrose meals: 2 – vanillic acid, 3 – caffeic acid (1, 4 – unidentified compounds)

(d) ethyl acetate fraction IV of evening primrose meals: 1 – gallic acid, 6 – syringic acid (2, 3, 4, 5 – unidentified compounds)

Figure 3. HPLC chromatography profile of particular fractions of evening primrose meal.
for the fractionation of the extract. In the study of Schmidt et al. (2003b), ethanol as the mobile phase was used. Different relative contents of the ethyl acetate re-extract fractions were obtained in this study, the highest relative content have been achieved in fractions I (43.0%) and II (22.6%).

**Content of individual phenolic compounds in the extracts and their fractions**

HPLC of evening primrose ethanol and ethyl acetate extracts and their fractions shown the presence of various phenolic compounds. For the identification of the phenolic compounds, eight standards were used. The presence of particular compounds is shown in Figure 3. The chromatograms revealed that the phenolic compounds found both in evening primrose ethanol extract and ethyl acetate re-extract were gallic acid and syringic acid. In the ethanol extract, catechin was also found, and in the 4th fraction ferulic acid. In the 3rd fraction of the ethyl acetate re-extract, vanillic and caffeic acids were found. The results correspond with those obtained by Wettasinghe et al. (2002) and with the study by Niklová et al. (2001). In the study by Wettasinghe and Shahidi (2002), gallic acid, catechin, and epicatechin were indicated as three major compounds of evening primrose meal. Niklová et al. (2001) referred that caffeic acid, gallic acid, and catechin were reported as major phenolics of evening primrose meal.

**CONCLUSION**

The aim of this paper was to identify low molecular weight phenolic compounds present in the crude ethanol extract and in the ethyl acetate re-extract from evening primrose meal. According to the results obtained by HPLC measurements, evening primrose meals contain catechin, gallic acid, caffeic acid, syringic acid, and ferulic acid as major phenolics. Thus, the industrial evening primrose meals are a rich source of antioxidants, for which there may be a great number of applications (in cosmetics, food, pharmaceuticals, etc.). In a following paper, the antioxidant activity of the addition of particular fractions into vegetable oils will be discussed.

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