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Quantitative Analysis of Biologically Active Polyphenols in Evening Primrose (*Oenothera paradoxa*) Seeds Aqueous Extracts

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The qualitative analysis of the aqueous extract showed the presence of polyphenolics belonging to different chemical groups: flavan-3-ol derivatives (catechin and procyanidin B_3), gallic acid derivatives (gallic acid, ethyl gallate and pentagalloylglucose) and depsides (ellagic acid). The content of total phenolic and procyanidin in extracts depended on the duration of seeds storage and varied from 469 to 388 mg/g and from 436 to 372 mg/g, respectively. The HPLC quantitative analysis of the three main compounds showed that their content varied from 3.70 to 6.18 mg/g for gallic acid, from 23.43 to 18.91 mg/g for (+)-catechin and from 12.50 to 8.02 mg/g for pentagalloylglucose. During seeds storage, the quantity of pentagalloylglucose in extracts decreased by about 30%, while gallic acid content increase was about 30%. The quantity of (+)-catechin has changed less significantly. The scavenging potential of the extracts determined with the DPPH method correlated with the higher concentration of pentagalloylglucose.

INTRODUCTION

Oenothera sp. (Oenotheraceae) are native to Central and South America, where they have been widely used by American Indians: root tea for obesity, bowel pains; poultice root for piles; rubbed root on muscles to give athletes strength [Foster & Duke, 2000]. Today, *Oenothera sp.* are also cultivated in Europe for the production of seeds as a source of oil, the main source of γ-linolenic acid (GLA). The principal species cultivated are *O. biennis* which yields an oil containing 7–9% GLA, *O. acerviphilla nova* (~15%) and *O. paradoxa* (~14%) [Evans, 2009]. Evening primrose oil (EPO) is used in the formulation of cosmetic products, dietary supplements, and more specifically for the treatment of atopic eczema, premenstrual syndrome, rheumatoid arthritis and diabetic neuropathy [Evans, 2009; Bruneton, 1999].

Companies manufacturing evening primrose oil may produce 50 tones of defatted seeds each year, on average, and therefore there is a growing need for utilization of this material. That is why, in the last few years, there has been a growing interest in the evening primrose due to its polyphenolics content. The plant contains flavonoids, phenolic acids and hydrolysable tannins (ellagitannins and gallotannins). Two macrocyclic ellagitanins: oenothein A and oenothein B were first isolated from *O. erythrosepala* and *O. biennis* and are also present in many species of the *Oenotheraceae* family [Hatano *et al*., 1989; Yoshida *et al.*, 1995]. A large number of flavonoids have been found in *Oenothera* sp., including myricetin, quercetin and kaempferol glycosides, as well as a biflavonoid of mirycetin, namely speciin [Howard *et al.*, 1972; Zinsmeister *et al.*, 1977; Marzouk *et al*., 2009]. The phenolic acids present in these species are mainly gallic acid together with ethyl- and metyl- esters, protocatechuic, caffeic and ellagic [Zinsmeister & Bartl, 1971; Zadernowski *et al*., 2002; Schmidt *et al*., 2003]. Fractionation of the seed extracts, obtained from defatted pulp, showed the presence of hydrolysable and condensed tannins. Gallic acid, (+)-catechin, (-)-epicatechin and a tetrameric procyanidin gallate were isolated as pure compounds from *O. biennis* seeds [Wettasinghe *et al.,* 2002a; Lu *et al.,* 1995]. In our previous study we have also identified (-)-epicatechin, (-)-epicatechin gallate, ellagic acid, caffeic acid, quercetin, penta-*O*-galloylo-β--D-glucose (PGG) and procyanidins from *O. paradoxa* seeds [Kiss *et al.,* 2008].

Pharmacological investigations have revealed that *Oenothera* (*O. biennis* and *O. paradoxa*) defatted seed extracts demonstrate antioxidant and iron (II) chelating activity [Shahidi *et al*., 1997; Bałasińska & Troszyńska, 1998; Amarowicz *et al.*, 1999; Wettasinghe *et al.,* 1999, 2002b], and the inhibition of vasopeptidase activity [Kiss *et al.,* 2008]. In the first case, gallic acid and (+)-catechin were the active constituent, whereas in the second case, penta-*O*-galloylo-β-D-glucose showed the strongest activity [Wettasinghe *et al.,* 2002a; Kiss *et al*., 2008]. Phenolic fraction purified from defatted seeds of *O. biennis* and *O. paradoxa* promoted selective apoptosis of human bone marrow-derived cell lines and human skin melanoma cells with a higher activity than against normal

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cells. Analysis of the fractions has shown the presence of gallic acid and penta-*O*-galloyl-β-D-glucose [Pellegrina *et al.,* 2005; Jaszewska *et al.,* 2009]. The mechanism of action of these extracts was attributed to the increasing activity of superoxide dismutase, the increase in intracellular oxygen reactive species levels, and decreased levels of GSH and ATP. However, no activation of caspase-3 was observed [Arimura *et al.,* 2003, 2004; Jaszewska *et al*., 2009]. Penta-*O*-galloyl-β-D-glucose, one of the main constituents of *O. paradoxa* seed extract, itself demonstrated significant antiproliferative activity against several cancer cell lines: prostate, breast, lung and liver, and displayed a number of biological activities related to inflammation [Zhang *et al.,* 2009].

In this study we identified the main compounds present in aqueous extracts obtained from defatted seeds of *O. paradoxa.* Secondly, we analysed the effect of storage of defatted seeds of *Oenothera paradoxa* on aqueous extracts active polyphenols quantity. We also compared the antioxidant activity of those extracts in relation to the presence of the most active compounds.

MATERIALS AND METHODS

Chemicals

Gallic and ellagic acids were purchased from ChromaDex (Santa Ana, CA), (+)-catechin from Carl Roth (Karlsruhe, Germany). Penta-*O*-galloyl-β-D-glucose, procyanidin B_3 and ethyl gallate were isolated as previously described [Kiss *et al.*, 2008; Granica, 2009]. Their structures were confirmed by MS, ^{1}H and ^{13}C NMR. All substances used were of >95% purity (HPLC). Methanol, butanol, acetic acid were from POCh (Gliwice, Poland). Acetonitrile was purchased from Merck KgaA (Daremstadt, Germany). 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Sigma (Sigma–Aldrich Chemie Inc., Steinheim, Germany). The Folin-Ciocalteu reagent was purchased from POCh (Gliwice, Poland). All solvents were of analytical or HPLC grade.

Plant material

Dried aqueous extracts of defatted seeds of *Oenothera paradoxa* were obtained from Agropharm S.A. (Tuszyn, Poland). The seeds were obtained from crops cultivated according to the GACP guidelines (EMEA/HMPWP/31/99 Rev. 3 of 2002). The defatted seeds were stored at an average temperature of 14°C and a humidity of 50% from November 2007 till July 2009, extracts were prepared every 6 months: in December 2007, July 2008, December 2008 and July 2009.

Total phenolic content

The total phenolic content was determined with the Folin-Ciocalteu reagent using gallic acid as the standard. Accurately weighed 0.002 g of extract was dissolved in 10 mL of water, then 0.5 mL of extract was mixed with 0.5 mL of the reagent and 10 mL of 10% (w/v) Na_2CO_3 solution and then filled up with water to 50 mL. The mixtures were incubated at room temperature in the dark for 30 min. The absorbance was measured spectrophotometrically at 765 nm on a 160A UV-VIS spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Total proanthocyanidin content

The total proanthocyanidin content was determined according to the 4th European Pharmacopoeia as cyanidin chloride. Accurately weighed 0.2 g of each extract were dissolved in 30 mL of methanol-water (7:3) (v/v) and heated under the reflux for 80 min with 15 mL of 25% HCl. After cooling, each extract was filtered through a narrow-pore filter paper and filled up with methanol-water (7:3) to 250 mL. Next, 50 mL of each solution was evaporated to about 3 mL and transferred to a separating funnel with 15 mL of water. The solutions were then extracted with butanol (3x15 mL, each). The organic layers were transferred to volumetric flasks and filled up with butanol to 100 mL. The absorbance was measured spectrophotometrically at 545 nm on a 160A UV-VIS Spectrophotometer (Shimadzu Corp., Kyoto, Japan).

High-Performance Liquid Chromatography (HPLC) analysis

The phenolics of extracts were analysed with HPLC method at a room temperature on an LC-10AT system consisting of two high-pressure mixing pumps and a diode array detector (SPD-M10A) (Shimadzu Corp., Kyoto, Japan). Separation was performed on a Luna C-18, 25x4.6 mm, 5 μm column (Phenomenex, Torrance, CA). The eluent was (A) 2.5% CH_3COOH (v/v) and (B) CH_3CN+2.5% CH_3COOH (80:20) (v/v). A gradient solvent system was used: 7–20% B (45'); 20–40% B (70'); 40–100% B (75'); 100% B (80'). The flow rate was 1 mL/min and the injection volume was 20 μL. UV spectra were recorded in the range of 200–400 nm, chromatograms were acquired at 254 nm for ellagic acid and 280 nm for other polyphenols.

Samples and standards were dissolved in MeOH + 2.5% CH_3COOH (1:1) (v/v). Quantitative determination was carried out using calibration curves from standard solutions in the concentration range of 5–125 μg/mL ($y=61424x+36311$; $R^2=1$; Rt=5.3) for gallic acid, 31.25–500 μg/mL ($y=142121x-35276$; $R^2=0.999$; Rt=17.9) for (+)-catechin and 15.625–250 μg/mL ($y=28845x-51203$; $R^2=0.9999$; Rt=55.2) for pentagalloylglucose.

Scavenging of DPPH radical

Determination of scavenging activity was performed using DPPH radical. In brief, 50 μL of extracts, dissolved in water, at concentrations of 10, 20, 50 μg/mL or pentagalloylglucose, dissolved in methanol-water (1:1 w/v), at concentrations of 1 to 50 μm/L were mixed with 150 μL of a methanolic solution of DPPH (200 mmol/L) in a 96 well plate. After 20 min the absorbance at 518 nm was measured in a BioTek microplate reader (Highland Park, USA) at a room temperature. The percent of scavenging activity was calculated in comparison to the control without test extracts/compound.

Statistical analysis

For quantitative analysis, the determinations were performed in triplicates, standard deviations (SD) were calculated. The precision of performed analysis, expressed as a correlation coefficient CV%, did not exceed 4% for each analysed compound. For analysis DPPH radical scavenging, the determinations were performed in triplicates in tree in-

dependent experiments, standard deviations (SD) were calculated. Statistical significance of differences between means was established by one way ANOVA. *P* values below 0.05 were considered statistically significant. All analyses were performed using Statistica 8.

RESULTS AND DISCUSSION

The qualitative analysis of the aqueous extract showed the presence of polyphenolics belonging to different chemical groups: flavan-3-ol derivatives (catechin and procyanidin B_3), gallic acid derivatives (gallic acid, ethyl gallate and pentagalloylglucose) and depsides (ellagic acid) (Figure 1). The R_t and λmax of the compounds analysed with HPLC-DAD are summarized in Table 1. The dominating compounds in *O. paradoxa* seed extract were gallic acid, (+)-catechin and pentagalloylglucose. In *O. biennis* seeds extract Wettasinghe *et al.* [2002a] showed that gallic acid and (+)-catechin were the main compounds responsible for its antioxidative activity. Additionally, Aitani *et al.* [2003] detected the presence of pentagalloylglucose, procyanidin B_3 and procyanidin B_1 in an ethanolic extract from defatted seeds of *O. biennis*. The contents of total phenolics and procyanidins in the extracts during seeds storage varied from 469 to 388 mg/g and from 436 to 372 mg/g, respectively. The polyphenolics content of 414 mg/g and 458 mg/g in aqueous extracts of *O. biennis* [Peschel *et al.,* 2007] was comparable to that determined in our study. In contrast, Bałasińska & Troszyńska [1998], found as little as 171 mg/g of polyphenolics in aqueous extract and as much as 580 mg/g of polyphenolics in 70% acetone extract of *O. paradoxa*. This discrepancy with our results may be due to the different source of seeds and different method of extracts preparation. Evening primrose seed extract obtained from *O. paradoxa* is a better source of polyphenols when compared with black currant seed extract (22–180 mg/g), sesame seed extract (14–65 mg/g) and grapevine leaves extracts (~250 mg/g), and similar to that of borage seed extract (~400 mg/g) and green tea extracts (~450 mg/g) [Wettasinghe *et al*., 2002b; Peschel *et al.,* 2007; Amarowicz *et al*., 2008].

TABLE 1. The R_t and λmax of analysed compounds by HPLC-DAD.

Compounds	R_t (min)	λmax (nm)
1. Gallic acid	5.3	273
2. Procyanidin B_3	12.9	236, 279
3. (+)-Catechin	17.9	236, 279
4. Ethyl gallate	35.8	281
5. Ellagic acid	49.13	254; 366
6. Pentagalloylglucose	55.2	281

The HPLC quantitative analysis of the three main compounds showed that their contents varied from 3.70 to 6.18 for gallic acid, from 23.43 to 18.91 for (+)-catechin and from 12.50 to 8.02 for pentagalloylglucose (Figure 3). In *O. biennis* defatted seeds extract the quantity of gallic acid was similar and reached 4.13 mg/g, while that of (+)-cathechin was higher and reached 101 mg/g [Wettasinghe *et al.,* 2002a]. In turn, Aitani *et al.* [2003] detected in ethanolic extract from defatted seeds of *O. biennis* 27 mg/g of pentagalloylglucose, 31 mg/g of gallic acid and 34 mg/g of (+)-cathechin.

During seeds storage the quantity of total polyphenolics in their extracts decreased by about 100 mg/g and that of total procyanidins by about 50 mg/g (Figure 2). What seems to be of importance is that the decrease in quantity of pentagalloylglucose was about 30%, while the gallic acid content increase was about 30%. The quantity of (+)-catechin has changed less significantly (Figure 3). In order to determine if these changes in the polyphenolics content affect the extract activity, we performed DPPH radical scavenging study on each of those extracts. All extracts showed a significant scavenging activity at the concentration of 50–10 μg/mL (Figure 4).

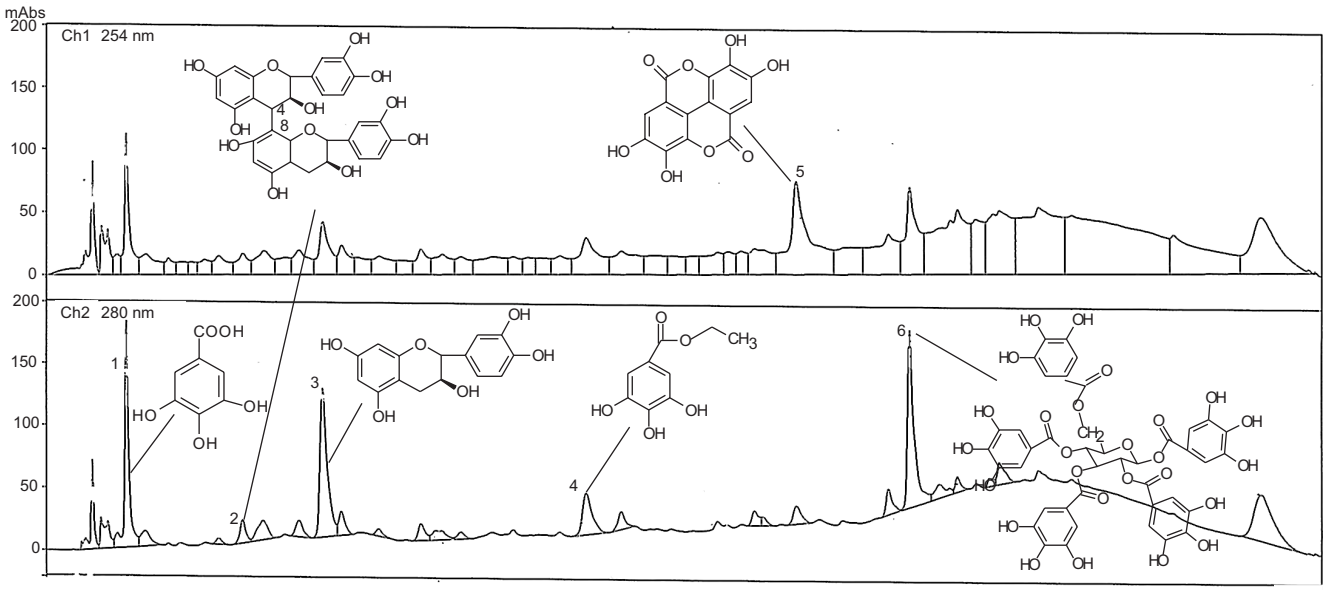

FIGURE 1. HPLC chromatograms of the extract (December 2007). **1**- Gallic acid, **2**- Procyanidin B_3, **3**- (+)-Catechin, **4**- Ethyl gallate, **5**- Ellagic acid, **6**- Pentagalloylglucose.

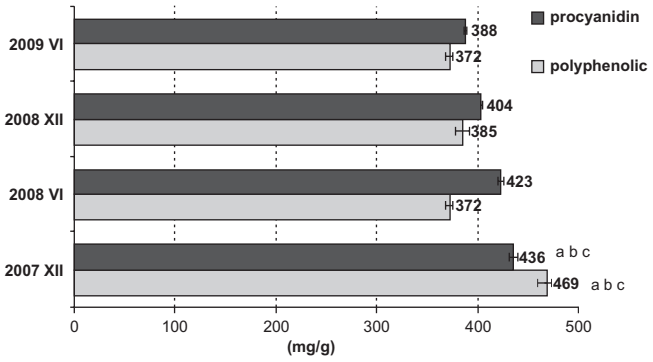

FIGURE 2. Content of total phenolics and procyanidins in extracts.
Data were expressed as mean ± SD; n=3, assayed in duplicate. Statistically significant difference between: a – June 2008, b – December 2008, c – June 2009, $p<0.05$.

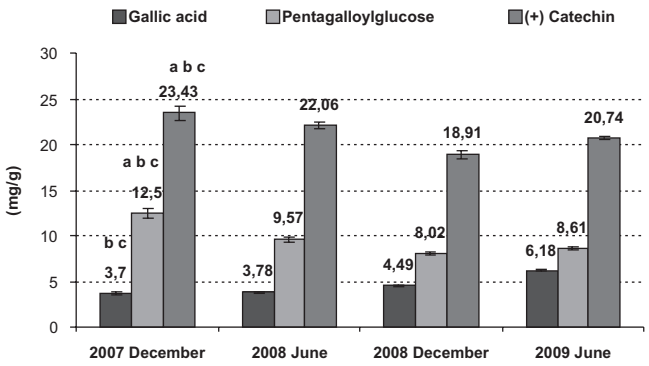

FIGURE 3. Content of gallic acid, pentagalloylglucose and (+)-catechin in extracts.
Data were expressed as mean ± SD; n=3, assayed in duplicate. Statistically significant difference between: a – June 2008, b – December 2008, c – June 2009, $p<0.05$.

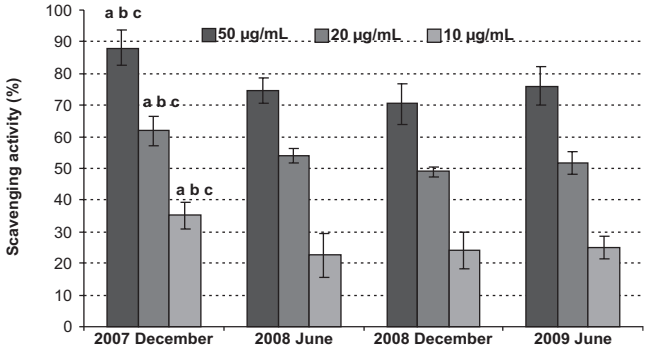

FIGURE 4. Scavenging effect of extracts on 2, 2-diphenyl-1-picrylhydrazyl radical.
Data were expressed as mean ± SD; n=3, assayed in triplicate. Statistically significant difference between: a – June 2008, b – December 2008, c – June 2009, $p<0.05$.

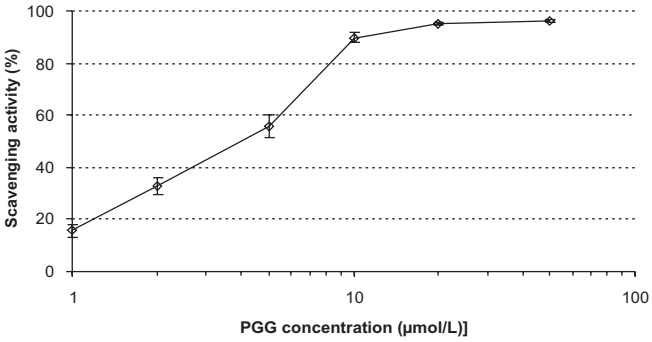

FIGURE 5. Scavenging effect of pentagalloylglucose (PGG) on 2, 2-diphenyl-1-picrylhydrazyl radical.
Data were expressed as mean ± SD; n=3, assayed in triplicate.

The scavenging potential of *O. paradoxa* seed extract in comparison with *O. biennis* seed extracts was quite the same. Peschel *et al.* [2007] found the DPPH scavenging activity of two extracts of *O. biennis* seed at 74% and 65% at the concentration of 10 μg/mL, while Amarowicz *et al*. [2000] reported a similar activity at the concentration of 100 μg/mL. During storage the scavenging potential of the seed extracts decreased, as is shown in Figure 4. The significance of the difference between the first extract from December 2007 and each extract prepared afterwards (June 2008, December 2008, June 2009) was determined using ANOVA. In each case the scavenging potential of the extract from December 2007 was significantly ($p=0.01$) higher. Interestingly, this correlated with the higher concentration of pentagalloylglucose. The compound itself showed a strong scavenging activity (Figure 5) with $IC_{50}= 3.9\pm0.7$ μmol/L. This result is comparable with that of $IC_{50}\sim 7$ μmol/L obtained by Piao *et al.* [2009] for the pentagalloylglucose isolated from *Elaeocarpus sylvestris* var. *ellipticus*.

CONCLUSION

Our findings show that the aqueous extract from *O. paradoxa* defatted seeds is a rich source of polyphenolics. The presence of pentagalloylglucose seems to be of importance for the antioxidant activity of extracts and therefore quality assessment of extracts obtained from evening primrose should be based on the quantitative determination of this compound.

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