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Chemical Composition, Antioxidative and Anti-Inflammatory Activity of Extracts Prepared from Aerial Parts of *Oenothera biennis* L. and *Oenothera paradoxa* Hudziok Obtained after Seeds Cultivation

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ABSTRACT: In the present study we investigated the chemical composition of extracts prepared from aerial parts of *Oenothera paradoxa* Hudziok and *Oenothera biennis* L. and their antioxidative and anti-inflammatory activities. Ultra high pressure liquid chromatography (UHPLC)-DAD-MS/MS studies showed that both extracts contain a wide variety of polyphenols (39 identified constituents) among which macrocyclic ellagitannin turned out to be the main constituent. During the *in vitro* studies, using noncellular models, both extracts scavenged reactive oxygen species (ROS) in a concentration-dependent manner, and the lowest SC_{50} values were obtained for O_2^- and H_2O_2. Both extracts inhibited ROS production by stimulated human neutrophils. The stronger activity in the case of formyl-met-leu-phenylalanine stimulation suggests that both extracts may act through the receptor-dependent pathway. *O. paradoxa* extract and *O. biennis* extract exhibited anti-inflammatory activity by the inhibition of hyaluronidase and lipoxygenase in a concentration-dependent manner. The stronger activity of *O.biennis* extract toward lipoxygenase may be explained by its higher oenothein B content.

KEYWORDS: *evening primrose, Oenothera paradoxa, Oenothera biennis, hyaluronidase, polyphenols, reactive oxygen species, antioxidants, neutrophils, lipoxygenase*

INTRODUCTION

Oenothera biennis L. and *Oenothera paradoxa* Hudziok are two biennial herbaceous plants commonly known as evening primroses. *O. biennis* is widely spread throughout Europe, North America, and Asia. *O. paradoxa* has been identified as a stable hybrid naturally occurring in Central and Western Europe.[1] Both species are cultivated by pharmaceutical companies for their seeds, which containing oil rich in unsaturated fatty acids. The oil has been shown to have several beneficial effects on human health including antidiabetic, anti-inflammatory, and antipremenstrual activity.[2] After seed harvesting, cultivators obtain ground aerial parts of evening primrose which are then used as a fertilizer. There are no studies that indicate other possible applications of these leftovers in pharmacy or medicine. Some studies have focused on another evening primrose byproduct — defatted seeds obtained after oil pressing. It has been proven that defatted seed and their extracts are a rich source of bioactive polyphenols and may also have beneficial health effects as antioxidative and anti-inflammatory agents.[3−11]

There is no detailed information on the chemical composition and bioactivities of extracts prepared from aerial parts of *O. paradoxa*. Reports concerning the *O. biennis* herb are limited.[12−14] On the other hand, quite extensive studies have been carried out on other *Oenothera* species, which have showed that aerial parts contain flavonoids, phenolic acids, and tannins.[15−18]

During the past several years there has been growing interest in reactive oxygen species (ROS) playing a vital role in the pathogenesis of several human diseases such as arteriosclerosis, diabetes, chronic inflammation, neurodegenerative disorders, and some types of cancer.[19] It has also been proven that ROS are involved in the deterioration of food products.[20,21] Thus, preparations from *Oenothera* might be of interest as a source of antioxidants that could modulate the biological response.

Hyaluronidase is one of the enzymes responsible for the degradation of the extracellular matrix associated with inflammatory response. Lipoxygenase (LOX) is a pro-inflammatory enzyme that catalyzes the conversion of arachidonic acid into leukotrienes that play a crucial role in inflammatory response in humans and other mammalians. It has been shown that plant extracts can significantly inhibit the activity of both enzymes.[22−26]

Therefore, the aim of the present study was to investigate and compare the chemical composition of extracts prepared from aerial parts of two popularly cultivated species of *Oenothera* using ultra high pressure liquid chromatography (UHPLC) coupled with DAD detector and ion-trap mass detector. Then the antioxidant activity of extracts for an array of DPPH and ROS, such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) was evaluated in a noncellular system, as well as in a cellular screening model with human neutrophil oxidative burst. Finally,

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the antihyaluronidase and antilipoxygenase *in vitro* assays were carried in order to establish inhibitory activity of extracts.

MATERIALS AND METHODS

Materials. Luminol, 4β-phorbol-12β-myristate-α13-acetate (PMA), formyl-met-leu-phenylalanine (f-MLP), nitrobluetetrazolium (NBT), xanthine, xanthine oxidase, H_2O_2, horseradish peroxidase (HRPO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), lipoxygenase from soybean (LOX), linoleic acid (LA), and DTNB 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Lucigenin was purchased from Carl Roth (Karlsruhe, Germany). NaOCl, sodium tetrahydridoborate ($NaBH_4$), ethylenediaminetetraacetic acid (EDTA), Folin−Ciocalteau reagent, KH_2PO_4, K_2HPO_4, methanol for extraction and acetonitrile (MeCN) gradient grade for UHPLC were purchased from POCH (Gliwice, Poland). Deionized water was produced with Millipore Simplicity System (Bedford, MA, USA).

Phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Hanks' balanced salt solution (HBSS) and RPMI 1640 medium were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Oenothein B for quantitative analysis had prevously been isolated in our laboratory.

Plant Material. The aerial parts of *O. biennis* L. were collected from their natural state in Rzeszów (Poland) in August 2010. *O. paradoxa* Hudziok herb was supplied by Firma Zielarska − Lewandowski (Kruszyn, Poland). Aerial parts were dried in shade at room temperature. Plant material was authenticated by Prof. Krzysztof Rostański from the Department of Systematic Botany, University of Silesia, Katowice, Poland (*O. biennis)* and Dr. Anna Kiss (*O. paradoxa*) based on morphological characters.[1] Voucher specimens (No. OB082010, OP 072010) were deposited in the Plant Collection of the Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Poland.

Preparation of Plant Extracts. Cut and grounded samples of both plants (50 g) were extracted with 50% methanol (v/v) in a boiling water bath (3 × 500 mL). Afterward, filtration methanol was evaporated under the reduced pressure, and the water residue was lyophilized to give crude extracts of *O. biennis* (OBE) and *O. paradoxa* (OPE) (8.7 and 8.0 g, respectively).

UHPLC-DAD-MS^3 Analysis. The UHPLC-DAD-MS analysis was performed using an UHPLC-3000 RS system (Dionex, Germany) equipped with a dual low-pressure gradient pump, an autosampler, a column compartment, a diode array detector, and an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Germany).

UHPLC analyses of *O. paradoxa* and *O. biennis* extracts were carried out on a reversed-phase Zorbax SB-C_{18}, 150 × 2.1 mm, 1.9 μm column (Agilent, CA, USA). Column temperature was 25 °C. The mobile phase (A) was water/acetonitrile/formic acid (95:5:0.1, v/v/v), and the mobile phase (B) was acetonitrile/formic acid (100:0.1, v/v/). A linear gradient system was used: 0−60 min. 1−26% B. The flow rate was 0.2 mL/min. The column was equilibrated for 10 min between injections. UV spectra were recorded over a range of 200−600 nm, and chromatograms were acquired at 254 nm, 280 and 350 nm. LC eluate was introduced directly into the ESI interface without splitting. Compounds were analyzed in negative and positive ion mode. MS^2 fragmentation was obtained for the two most abundant ions at the time. The detection of neutral loses was set for the sugars and galloyl moieties characteristic for glycosides fragmentation (162, 132, 176, 152). In the case in which one of the neutral loss masses was detected MS^3 fragmentation was performed in order to obtain the fragmentation spectrum of the aglycon moiety. The nebulizer pressure was 40 psi; dry gas flow 9 L/min; dry temperature 300 °C; and capillary voltage 4.5 kV. Analysis was carried out using scan from m/z 200 to 2200.

Total Polyphenol Content. Total polyphenol content was determined using the Folin−Ciocalteau reagent following the method described previously by Lowry.[27] The extracts were oxidized with the Folin-Ciocalteau reagent, and the reaction was neutralized with sodium carbonate. Absorbance of the resulting blue color was measured at 765 nm after 30 min. Gallic acid (GA) was used as the standard. The results were expressed as a percentage of polyphenols in prepared extracts. For both extracts, three independent samples in triplicate were analyzed.

Quantification of OeB in Prepared Extracts. The percentage content of oenothein B in both extracts was established based on the validated UHPLC method developed in the Department of Pharmacognosy and Molecular Basis of Phytotherapy, which has been previously reported.[28] For both extracts, three independent samples in triplicate were analyzed.

Hyaluronidase Assay. Inhibition of hyaluronidase by extracts was determined by turbidimetric method according to USP XXII-NF XVII (1990) 644−645, United States Pharmacopeia Convention, Inc., Rockville, MD, which was modified to 96-well microtiter plates as described previously.[25] Changes in turbidity at 600 nm were measured by a microplate reader (BioTek). Both extracts were tested in a concentration range of 10−50 μg/mL. For investigated extract three independent experiments were carried out in duplicate. Heparin solution was used as a positive control in all the experiments. The activity of tested extract was calculated as enzyme activity percentage ($\%_{activity}$) of hyaluronidase as shown in eq 1.

$$\%_{activity} = \left(\frac{(Ab_{HA} - Ab_{HYAL}) - (Ab_W - Ab_{HYAL})}{(Ab_{HA} - Ab_{HYAL})} \right) \times 100\% \quad (1)$$

where Ab_{HA} − absorbance of solution without enzyme, Ab_{HYAL} − absorbance of solution without tested extract (negative control), Ab_W − absorbance of solution with tested extract.

Lipoxygenase Assay. Inhibition of lipoxygenase by extracts was determined by a spectrophotometric method adjusted to a 96-well microtiter plates. The protocol started by adding 100 μL of tested extracts solution in potassium phosphate buffer (pH = 8.5), then 50 μL of LOX solution (167 U/mL) in potassium phosphate buffer (pH = 8.5) was added, finally 50 μL of LA solution (134 μM) was added, and changes in absorbance at 234 nm were measured over a period of 25 min with 30 s intervals. Both extracts were tested in the concentration range of 2−50 μg/mL. Indomethacin was used as a positive control. The activity of tested extracts was calculated as enzyme activity percentage ($\%_{activity}$) of LOX in relation to maximum activity (negative control) after subtracting in all cases extracts' absorbance at 234 nm as shown in eq 2:

$$\%_{activity} = \left(\frac{Ab_t - Ab_b}{Ab_0} \right) \times 100\% \quad (2)$$

where Ab_t − absorbance of solution with tested extracts, Ab_b − absorbance of solution without enzyme, Ab_0 − absorbance of solution without tested extracts (negative control). For the calculation the values of absorbance in the 10th minute of reaction were used.

DPPH Scavenging Capacity Assay. The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a previously described method, with our modifications.[29] DPPH solution (0.3 mM) was prepared in 95% ethanol. Lyophilized extracts were dissolved in a mixture of ethanol and water (1:1, v/v) to obtain stock solutions (1 mg/mL). Then each stock solution was diluted to obtain a final concentration in the assay mixture in a range of 2−50 μg/mL. 100 μL of DPPH solution and 100 μL of tested extracts of different concentrations were added to a 96-well plate. Then samples were incubated at room temperature for 30 min. After 30 min, absorbance at 518 nm (Ab) was measured in a microplate reader (BioTek). The absorbance values were converted to the percentage of antioxidant activity according to eq 3.

$$\%_{DPPH} = \left(\frac{Ab_t - Ab_b}{Ab_0} \right) \times 100\% \quad (3)$$

where Ab_t − absorbance of DPPH solution with tested extracts, Ab_0 − absorbance of DPPH solution with addition of ethanol/water (1:1, v/

v), Ab_b − absorbance of tested extracts solution with addition of 95% ethanol.

O_2^- Scavenging Capacity Assay. The system xanthine/xanthine oxidase, with nitrobluetetrazolium (NBT) reduction, was used to determine superoxide anion scavenging capacity.[29] Lyophilized extracts were dissolved in a (Ca^{2+})-free PBS buffer to obtain stock solutions (1 mg/mL). The final concentrations of tested extracts in the reaction mixture were in the range of 2−50 μg/mL. 50 μL of tested extract in PBS, 50 μL of xanthine oxidase (0.1 mU in PBS), and 100 μL of 0.4 mM xanthine and 0.24 mM NBT solution in PBS were added to a 96-well plate. The reduction of NBT to corresponding formazan was measured at 560 nm over a 30 min period at intervals of 5 min in a microplate reader (BioTek). The absorbance (Ab) values were converted into the percentage of production of O_2^- using eq 4.

$$\%_{\text{production}} = \left(\frac{Ab_t - Ab_b}{Ab_0 - Ab_{ob}}\right) \times 100\% \quad (4)$$

where Ab_t − absorbance of solution with tested extracts, Ab_b − absorbance of solution without enzyme, Ab_0 − absorbance of solution without tested extracts, Ab_{0b} − absorbance of solution without tested extracts and without enzyme. The values of absorbance after 30 min were used for all the calculations.

In order to eliminate the possibility of direct interaction of tested extracts with xanthine oxidase we monitored the uric acid production by the enzyme at 295 nm. In the case of uric acid production we used the procedure described above. The only difference was that the substrate solution did not contain NBT (only 0.4 mM xanthine).

H_2O_2 Scavenging Capacity Assay. Hydrogen peroxide scavenging was performed with horseradish peroxidase (HRPO) method as previously described,[30] with small modifications. Lyophilized extracts were dissolved in the (Ca^{2+})-free PBS buffer to obtain stock solutions (1 mg/mL). The final concentration of tested extracts in the reaction mixture was in the range of 2−50 μg/mL. 50 μL of tested extract in PBS, 50 μL of horseradish (5 mU in PBS), and 50 μL of 0.03% H_2O_2 solution in PBS were added to a 96-well plate. Chemiluminescence was measured in a microplate reader right after the addition of 50 μL of luminol solution in PBS (2 mM) over a 40 min period at intervals of 2 min. The scavenging activity was calculated as a percentage of production of H_2O_2 in the maximum of chemiluminescence according to eq 5.

$$\%_{\text{production}} = \left(\frac{CL_t - CL_b}{CL_0 - CL_{ob}}\right) \times 100\% \quad (5)$$

where CL_t − chemiluminescence of solution with tested extracts, CL_b − chemiluminescence of solution without enzyme, CL_0 − chemiluminescence of solution without tested extracts, CL_{0b} − chemiluminescence of solution without tested extracts and without enzyme.

HOCl Scavenging Capacity Assay. The hypochlorous acid (HOCl)-scavenging effect of tested compounds was determined, using 5-thio-2-nitrobenzoic acid (TNB) as a reductant oxidized by HOCl into 5,5-dithiobis-(2-nitrobenzoic acid) by modifying a previously described method.[31] Lyophilized extracts were dissolved in the (Ca^{2+})-free PBS buffer to obtain stock solutions (1 mg/mL). The final concentration of tested extracts in reaction mixture were in the range of 2−50 μg/mL. 50 μL of tested extract in PBS and 150 μL of NaClO solution (0.45% in PBS) were added to a 96-well plate. The reaction mixture was incubated in the dark at room temperature for 5 min. Next, 50 μL of 0.1 mM TNB solution in PBS was added. The changes in absorbance at 412 nm were measured in a microplate reader. The absorbance (Ab) values were converted into the percentage of HOCl production using eq 6.

$$\%_{\text{production}} = \left(\frac{Ab_t - Ab_b}{Ab_0 - Ab_{100}}\right) \times 100\% \quad (6)$$

where Ab_t − absorbance of solution with tested extracts, Ab_b − absorbance of solution without TNB, Ab_{100} − absorbance of solution without tested extracts, Ab_0 − absorbance of solution without tested extracts and NaClO solution

Isolation of Human Neutrophils. Peripheral venous blood was collected from healthy human donors (20−35 years old) at the Warsaw Blood Donation Center. Donors all declared that they were nonsmokers and did not take any medications. They were confirmed to be healthy and all tests carried out showed values within normal range. Neutrophils (polymorphonuclear cells; PMNs) were isolated using a standard method by dextran sedimentation and centrifugation in a Ficoll Hypaque gradient. The purity of neutrophils preparation was over 97%. Afterward, isolation cells were suspended in (Ca^{2+})-free HBSS and were maintained at 4 °C before use.[32]

Evaluation of ROS Production by Human Neutrophils. The ROS production by f-MLP- or by PMA- stimulated neutrophils was determined using luminol- or lucigenin-dependent chemiluminescence. The concentrations of extracts used in the experiment ranged from 2 to 50 μg/mL. Following isolation, cells were resuspended in HBSS. Cell suspension (3.5×10^5) was incubated with 50 μL of the samples with tested compounds in the proper concentration and luminol (20 mM) or lucigenin (0.4 mM) in a 96-well plate. ROS production was initiated by the addition of f-MLP (0.1 μg/mL) or PMA (1 μg/mL) to obtain a 200 μL/well. Changes in chemiluminescence were measured over a 40 min period at intervals of 2 min in a microplate reader. Background chemiluminescence produced by nonstimulated cells was also determined. As a positive control, vitamin C was used. The percentage of ROS production was calculated in comparison to the control without inhibitors, taking into account chemiluminescence emission inhibited by tested compounds, as per eq 7:

$$\%_{\text{production}} = \left(\frac{CL_0 - CL_c}{CL_0}\right) \times 100\% \quad (7)$$

where CL_0 is chemiluminescence without tested compounds and CL_c is chemiluminescence with addition of the tested compounds.

Cytotoxicity Assay. Plant extracts cytotoxicity for neutrophils and monocytes was tested by MTT colorimetric assay. A cell suspension of PMN (5×10^5 in RPMI 1640 medium) with 50 μL of tested extracts in the concentration range of 2−100 μg/mL was incubated in a 96-well plate for 1 h at 37 °C and then MTT (1 mg/mL) was added and incubated at 37 °C for an additional 1 h. The insoluble formazan product was dissolved in 200 μL 0.04 M HCl in isopropanol and measured spectrophotometrically at 570 nm using a microplate reader.

Statistical Analysis. The results were expressed as a mean ± SEM of the indicated number of experiments. The IC_{50} values of tested compounds were calculated based on concentration- inhibition curves. The statistical significance of the differences between the means was established by one-way ANOVA with Bonferroni *posthoc* test or with nonparametric Kruskal−Wallis one-way analysis of variance. Statistical significance of differences between mean and control was established by one-way ANOVA with Dunnett's *posthoc* test. *P* values below 0.05 were considered as statistically significant. All analyses were performed using Statistica 8.

RESULTS

Phytochemical Characterization of OBE and OPE by UHPLC-DAD-MS^n. In this study, the comprehensive analysis of chemical composition of extracts from aerial parts of two popular *Oenothera* species was carried out for the first time. The total polyphenol content established by the Folin-Ciocalteu method was 343.1 ± 9.7 mg/g for OPE and 375.2 ± 9.8 mg/g for OBE. In both extracts, the main constituent occurring in high amounts was oenothein B^{11} with characteristic pseudo-molecular ions $[M - 2H]^{2-}$ and $[M - H]^-$ at *m*/*z* 783 and 1567 respectively.[33] The OeB content had been previously been established by a developed and validated method. OPE contained 83.2 ± 3.4 mg/g of OeB while the quantity of OeB in OBE turned out to be higher and was determined as 112.2 ±

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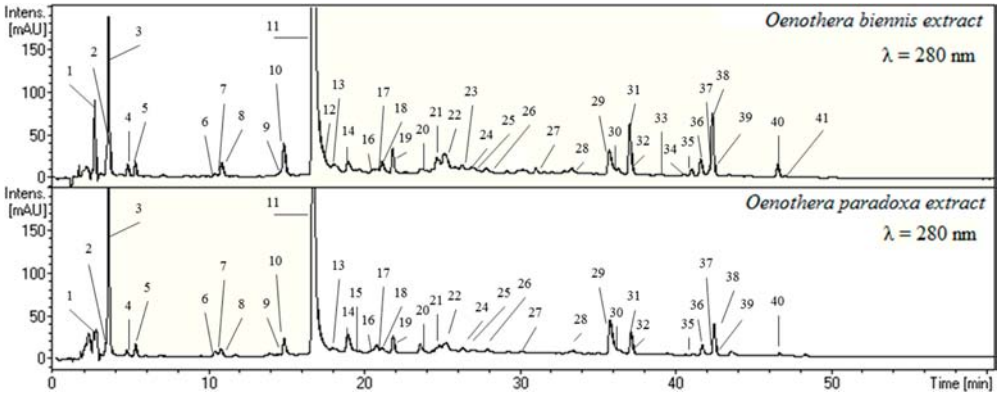

Figure 1. UHPLC UV−vis chromatogram of OPE and OBE acquired at 280 nm.

2.6 mg/g. The chromatograms acquired at 280 nm (Figure 1) showed that apart from OeB, both extracts contained a wide variety of other polyphenolic compounds including ellagitannins, flavonoids, phenolic acids, and their derivatives, as well as one flavan-3-ol.

Peaks **2**, **4**, and **7** with a single UV maximum at approximately 275 nm were identified as three different galloyl-HHDP-glucose isomers with $[M - H]^-$ at m/z 633 and fragmentation ions corresponding to galloyl cleavage $[M - H - \text{galloyl}]^-$ at m/z 481 and $[M - H - \text{galloyl-}H_2O\text{-hexose}]$ ion at m/z 301 proving the presence of ellagic acid moiety in the molecules of the analyzed chemicals.[34]

Peaks **1**, **3**, **5**, **10**, and **15** with a single UV maximum at approximately 270−280 nm were identified as gallic acid and its ester derivatives. Compound **3** with $[M - H]^-$ at m/z 169 followed by fragmentation to ion at m/z 125 was identified as gallic acid. Compounds **1**, **5**, and **15** were identified as galloyl- $[M - H]^-$ at m/z 331, digalloyl- $[M - H]^-$ at m/z 483 and tris-galloylglucose $[M - H]^-$ at m/z 635, respectively. The fragmentation path showed the presence of single or multiple galloyl moiety, which was detected as neutral losses of 152 amu per each moiety (Table 1).[35] Peak **10** was assigned as gallic acid methyl ester with $[M - H]^-$ at m/z 183 and MS^2 ion at m/z 125. Methyl gallate (**10**) should be considered as an artifact that is created during the extraction process that involved heating with 50% methanol.

Peaks **19** and **20** with a single UV maximum at 264−265 nm were ellagitanins. Compound **20** with $[M - H]^-$ at m/z 785 and MS^2 fragmentation ions at m/z 765, 633, 483, and 301 was identified as digalloyl-HHDP-glucose (pedunculagin II).[34] Compound **19** $[M - H]^-$ at m/z 783 and MS^2 fragmentation ions at m/z 765, 615, 275 undeniably has an ellagitannin structure but was not identified in this study.

Peaks **6**, **13**, **14**, **18**, **21**, and **25** with UV maximum at 325 nm were identified as different phenolic acids. Compound **6** and **14** with the same $[M - H]^-$ at m/z 353 were distinguished by MS^2 fragmentation as 3-caffeoylquinic (MS^2 most abundant ion at m/z 191) and 4- caffeoylquinic acid (MS^2 most abundant ion at m/z 173), respectively.[36] Compounds **18** and **21** showed the same $[M - H]^-$ at m/z 337 and were identified by MS^2 fragmentations as 4-*p*-coumaroylquinic (MS^2 most abundant ion at m/z 173) and 3-*p*-coumaroylquinic acid (MS^2 most abundant ion at m/z 163), respectively.[36] Compounds **13** and **25** with $[M - H]^-$ at m/z 367 and the main MS^2 ions at m/z 193 or at m/z 173 and 192 were identified as 3-*p*-feruloylquinic and 4-*p*-feruloylquinic acid, respectively.[36]

Peak **8** with UV maxima at 243 and 326 nm was identified as caffeic acid pentoside with $[M - H]^-$ at m/z 311 and MS^2 fragmentation ions at m/z 243, 179 corresponding to $[M - H - \text{pentose}]^-$ and 149.

Compound **9** with $[M - H]^-$ at m/z 387 probably has a phenolic acid structure, but its identification was not possible due to insufficient data.

Peak **12** with UV maximum at 277 nm was identified as catechin with $[M - H]^-$ ion at m/z 289 and MS^2 fragmentation ions at m/z 245 and 208.[35]

Peaks **22**, **23**, **24**, **26**, **28**, **29** and **39** with UV maxima at 253 and 366 nm were identified as ellagic acid and its derivatives. Compound **29** with $[M - H]^-$ ion at m/z 301 and MS^2 ions at m/z 253 and 258 was identified as ellagic acid.[37] Compound **24** with $[M - H]^-$ ion at m/z 463 and MS^2 ions $[M - H - \text{hexose}]^-$ at m/z 301 was identified as ellagic acid hexoside (neutral loss of 162 amu).[37] Compound **28** with $[M - H]^-$ ion at m/z 433 and MS^2 ions $[M - H - \text{pentose}]^-$ at m/z 301 was identified as ellagic acid pentoside (neutral loss of 132 amu).[37] Compounds **23** and **39** with $[M - H]^-$ ions at m/z 469 and 483, respectively were identified as valoneic acid dilacone and its methyl ester. Both chemicals presented MS^2 ion at m/z 301 corresponding to free ellagic acid. In the case of valoneic acid dilacone (**23**) the characteristic MS^2 fragmentation ion at m/z 425 was detected.[37] Valoneic acid dilactone methyl ester (**39**), the same as methyl gallate (**10**), may be considered as an artifact created during the extraction process. Nevertheless its occurrence in the extract still proves the presence of valoneic acid dilactone (**23**) in the raw material. Compounds **22** and **26** based on their UV−vis spectra are ellagic acid derivatives with $[M - H]^-$ at m/z 542 and 626 respectively. In addition both constituents had MS^2 spectra ion at m/z 301 corresponding to ellagic acid moiety.

Peaks **16**−**17**, **27**, **30**−**38**, and **40**−**41** with two UV maxima at approximately 250−260 and 355−365 nm were recognized as different flavonoids. Compound **27** with $[M - H]^-$ at m/z 493 and neutral loss of 176 amu leading to MS^2 ion at m/z 317 was identified as flavonol glucuronide. MS^3 ions at m/z 299, 271, 179, and 151 obtained for aglycone moiety together with comparison of retention time with the available standard confirmed that compound **27** is myricetin glucuronide. Compounds **16**−**17**, **30**−**33**, and **35**−**36** with fragmentation ion in MS^2 spectrum at m/z 301 were identified as quercetin *O*-glycosides. The structure assignment was carried out based on the monitoring of the neutral losses characteristic for hexose (−162 amu), pentose (−132 amu), or glucuronic acid cleavage and by MS^3 spectra obtained for constituents for which the

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Table 1. Retention Times, UV-Vis and MS^3 Data for OPE and OBE

	compounds	retention time [min]	UV [nm]	$[M - H]^-$ *m/z*	MS^2 ions	MS^3 ions	$[M + H]^+$	MS^2 ions	MS^3 ions
1	galloylglucose	2.7	270	331	**169**	125	355[b]	337, 193, 185	
2	galloyl-HHDP-glucose isomer	3.2	275	633	481, 301		635	617, 303, 277	
3	gallic acid[c]	3.5	270	169	125		171	153, 127	
4	galloyl-HHDP-glucose isomer	4.6	275	633	481, 301		657[b]	487, 337	
5	digalloylglucose	5.4	283	483	**331**, 313, 271, 169				
6	3-caffeoylquinic acid[c]	10.2	325	353	191, 173		377[b]	359, 215	
7	galloyl-HHDP-glucose isomer	10.6	275	633	481,301				
8	caffeic acid pentoside	10.9	243, 326	311	243, 179, 149		335[b]	185, 173	
9	unknown	14.5	254, 301	387	341, 179				
10	methyl gallate[c]	14.9	269	183	125		185	153	
11	oenothein B[c]	16.8	262	783[a], 1567					
12	catechin[c]	17.5	277	289	245, 208		291	273, 165, 139, 123	
13	3-*p*-feruloylquinic acid	18.3	322	367	193				
14	4-caffeoylquinic acid	19.0	324	353	173		377[b]	359, 215	
15	tris-galloyl-glucose	19.9	272	635	483, 465, 313, 421	423, 331, 313			
16	quercetin glucuronylhexoside	20.7	256, 355	639	**463**, 301	301	641	**465**, 303	303
17	quercetin dihexoside	21.2	256, 355	625	**463**, 301		627	**465**, 303	303
18	4-*p*-coumaroylquinic acid	21.3	315	337	173				
19	unknown	22.0	265	783	765, 615, 275				
20	digalloyl-HHDP-glucose	23.7	264	785	765, **633**, 483, 301	614, 402, 301, 188			
21	3-*p*-coumaroylquinic acid	24.6	310	337	163, 306				
22	ellagic acid derivative	25.2	253, 366	542	301				
23	valoneic acid dilactone[c]	26.4	253, 366	469	425, 301		471	303, 453	
24	ellagic acid hexoside	27.1	253, 366	463	301	255, 229, 213	463	303	286, 276, 205
25	4-*p*-feruloylquinic acid	27.3	slope	367	173, 192		391[b]	373, 217, 201	
26	ellagic acid derivative	27.9	253, 366	626	604, 301				
27	myricetin 3-*O*- glucuronide[c]	31.1	261, 356	493	**317**	299, 271, 179, 151	495	**319**	301, 273, 165
28	ellagic acid pentoside	33.6	253, 366	433	**301**	272, 258, 244	435	**303**	286, 276
29	ellagic acid[c]	35.8	253, 366	301	253, 258		303	285, 267, 230, 160	
30	quercetin 3-*O*-galactoside[c]	36.5	256, 355	463	**301**	271, 255, 179, 151	465	**303**	285, 257, 229
31	quercetin 3-*O*-glucuronide[c]	37.2	257, 354	477	**301**	257, 179, 151	479	**303**	285, 257, 229
32	quercetin 3-*O*-glucoside[c]	37.5	slope	463	**301**	271, 255, 179, 151	465	**303**	285, 257, 229
33	quercetin pentoside	39.3	258, 356	433	**301**	271, 255, 229, 179, 151	435	**303**	285, 257, 229
34	kaempferol 3-*O*-rhamnoglucoside[c]	40.7	264, 351	593	285		595	**449**, 287	287
35	quercetin 3-*O*-(2′-galloyl-glucuronide)[c]	41.0	265, 355	629	459, 327		631	**479**, 329, 303	303
36	quercetin pentoside	41.7	265, 355	433	**301**	273, 227, 179, 151	435	303	285, 247, 257, 229
37	kaempferol 3-*O*-glucoside[c]	42.3	slope	447	285		449	287	231, 213, 165
38	kaempferol 3-*O*-glucuronide[c]	42.6	265, 349	461	**285**	257, 229, 163, 151	463	**287**	259, 231, 213, 165
39	valoneic acid dilactone methyl ester[c]	43.5	253, 366	483	301		485	453, 302	
40	kaempferol 3-**O**-(2′-galloyl-glucuronide)[c]	46.6	264, 351	613	327		615	**463**, 329, 287	287
41	kaempferol pentoside	47.0	264, 351	417	285, 255		419	287	

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Table 1. continued

[a][M − 2H]$^{2-}$. [b][M + Na]$^{+}$. [c]Comparisons with chemical standard have been made.

intensity of aglycone ion signal was sufficient. As the MS/MS spectra are not sufficient for complete structure elucidation, comparisons of retention times with chemical standards were made. In the case of compound **35**, identified as quercetin 3-*O*-(2″-galloylglucoronide), MS spectra in positive mode were crucial as they revealed the presence of the galloyl group for which characteristic neutral loss of 152 amu was observed ([M + H-galloyl]$^{+}$ at *m*/*z* 479), followed by the cleavage of glucuronic acid (−176 amu) to obtain aglycone signal at *m*/*z* 303. Compounds **34**, **37**−**38**, and **40**−**41** for which MS^2 fragmentation ion at *m*/*z* 285 was obtained were identified as *O*-glycosidic derivatives of kaempferol. As in the case of quercetin derivatives the identification was carried basing on neutral loss monitoring and comparisons of retention time with chemical standard. Interestingly, compound **40** exhibited a similar fragmentation pattern as compound **35** and was identified as kaempferol 3-*O*-(2″-galloylglucoronide).[38,39] For full MS data and all the constituents identification, see Table 1.

Scavenging of ROS in Cell Free Systems. Both extracts examined in this study exhibited significant scavenging activity in a concentration-dependent manner (Figure 2A−C) especially toward superoxide anion and hydrogen peroxide On the other hand, the same extracts had rather low ability to scavenge HOCl and the artificial DPPH radical (SC_{50} value is given in Table 2). In the case of superoxide anion, the extracts did not directly affect the xanthine oxidase activity evaluated by the monitoring of uronic acid production (data not shown). Table 2 presents the SC_{50} values established in all the experimental models used and allows for comparison of both extracts' scavenging activity.

Effect on ROS production by neutrophils. Both extracts inhibited ROS generation in human neutrophils. The inhibitory effect was evidently stronger in the case of f-MLP used as a stimulating agent than in the case of PMA (Figure 3A,B). The values of IC_{50} for f-MLP were 3.7 ± 1.7 μg/mL (OPE) and 4.1 ± 2.2 μg/mL (OBE) and for PMA were 16.8 ± 2.4 μg/mL (OPE) and 14.9 ± 1.3 μg/mL (OBE). At the same time, the IC_{50} values for positive control vitamin C were 4.9 ± 2.0 μg/mL for PMA and 1.0 ± 0.4 μg/mL for f-MLP (not shown). Both tested extracts in the concentrations up to 100 μg/mL had no adverse effect on neutrophils (after 1 h incubation) viability (Figure 3C).

Effect on hyaluronidase activity. Both extracts significantly inhibited hyaluronidase activity (Figure 4). The inhibition of hyaluronidase activity at a concentration of 50 μg/mL was 97.3 ± 3.0% and 97.9 ± 1.7% for OPE and OBE, respectively. At the same time the IC_{50} value for positive control heparin was 62.1 ± 7.5 μg/mL (not shown).

Effect on lipoxygenase activity. Both extracts inhibited lipoxygenase activity. In the case of OBE the response was concentration dependent, while the statistical analysis for OPE could not fully confirm the dose-dependent response (Figure 5). OBE exhibited statistically significant stronger activity than OPE. The values of IC_{50} were over 50 μg/mL and 12.1 ± 1.6 μg/mL for OPE and OBE, respectively. At the same time the IC_{50} value for positive control indomethacin was 83.9 ± 7.7 μg/mL (not shown).

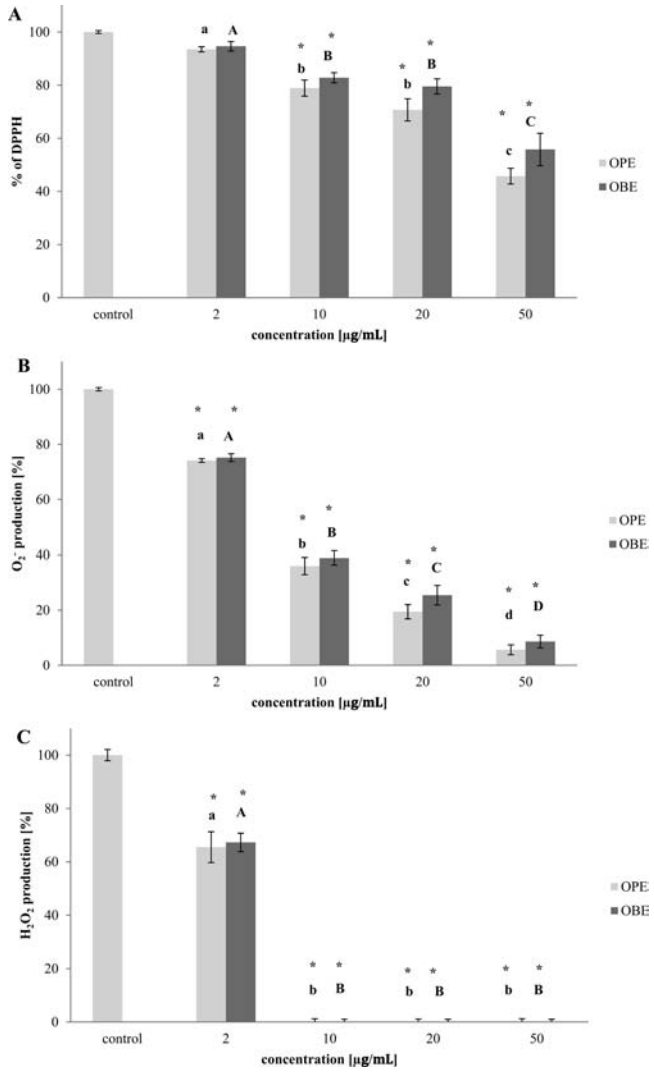

Figure 2. Free radical scavenging effects of OPE and OBE in the concentration range of 2−50 μg/mL in selected cell free systems. Effect on DPPH radical scavenging [%] (A). Effect on $\cdot O_2^-$ scavenging [%] (B). Effect on H_2O_2 scavenging [%] (C). Data were expressed as mean ± SEM; performed in at least three independent experiments, assayed in triplicate. Statistical significance of differences compared to control was calculated by one-way ANOVA with Dunnett's *posthoc* test, *$P < 0.05$. The statistically significant differences between the means for different concentrations of the same extract were analyzed by one-way ANOVA with Bonferroni *posthoc* test (panels A and B). In the case of panel C, differences were analyzed by nonparametric Kruskal−Wallis one-way analysis of variance. Different letters a−d (for OPE) or A−D (for OBE) indicate statistically significant differences between means, $P < 0.05$.

DISCUSSION

In the present study we investigated for the first time the phytochemical composition and bioactivity of extracts prepared from aerial parts of two popular *Oenothera* species that are commonly cultivated as a source of seeds for oil production.

Previous chemical studies on the composition of *O. paradoxa* were focused on extracts prepared from defatted seeds. It was proven that extracts from defatted seeds are a rich source of

Table 2. SC_{50} Values Established in Chosen Cell Free Experiments[a]

	SC_{50} values	
	OPE (μg/mL)	OBE (μg/mL)
	noncellular models	
$\cdot O_2^-$	5.9 ± 0.7	6.5 ± 0.7
H_2O_2	2.7 ± 0.6	2.8 ± 0.9
HOCl	>50	47.9 ± 1.8
DPPH	55.5 ± 2.9	58.2 ± 2.7

[a]Data were expressed as mean ± SEM; performed in at least three independent experiments, assayed in triplicate.

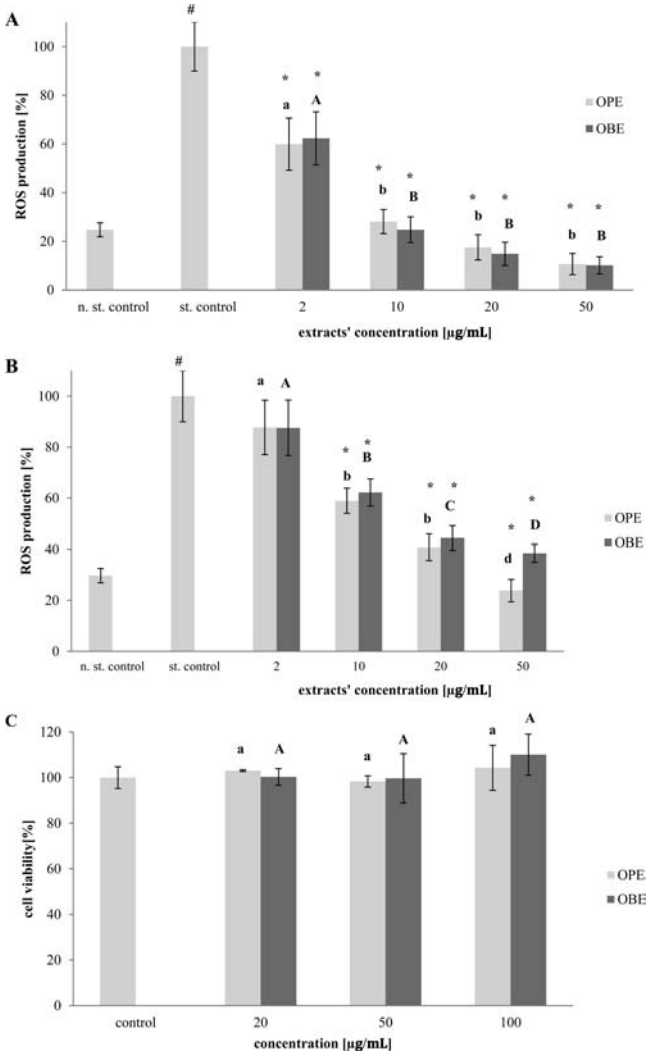

Figure 3. Inhibitory effects [%] of OPE and OBE in the concentration range of 2−50 μg/mL on ROS production by f-MLP (A) and PMA (B) stimulated neutrophils and effect on cell viability [%] in MTT assay (C). Data were expressed as mean ± SEM; performed in at least three independent experiments, assayed in triplicate. Experiments were performed using cells of different donors. Statistical significance of differences were calculated by one-way ANOVA with Dunnett's *posthoc* test, $^{\#}P < 0.001$ compared to not stimulated control; $^{*}P < 0.05$ compared to stimulated control. The statistically significant differences between the means for different concentrations of the same extract were analyzed by one-way ANOVA with Bonferroni *posthoc* test. Different letters a−d (for OPE) or A−D (for OBE) indicate statistically significant differences between means, $P < 0.05$.

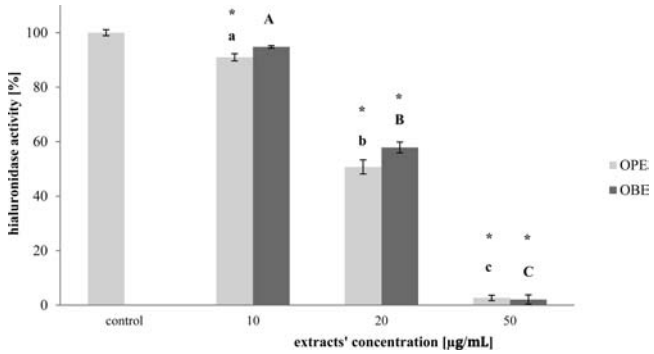

Figure 4. Inhibitory effect [%] of OPE and OBE in the concentration range of 2−50 μg/mL on hyaluronidase activity. Data were expressed as mean ± SEM; performed in at least three independent experiments, assayed in triplicate. Statistical significance of differences compared to control was calculated by one-way ANOVA with Dunnett's *posthoc* test, $^{*}P < 0.05$. The statistically significant differences between the means for different concentrations of the same extract were analyzed by one-way ANOVA with Bonferroni *posthoc* test. Different letters a-c (for OPE) or A−C (for OBE) indicate statistically significant differences between means, $P < 0.05$.

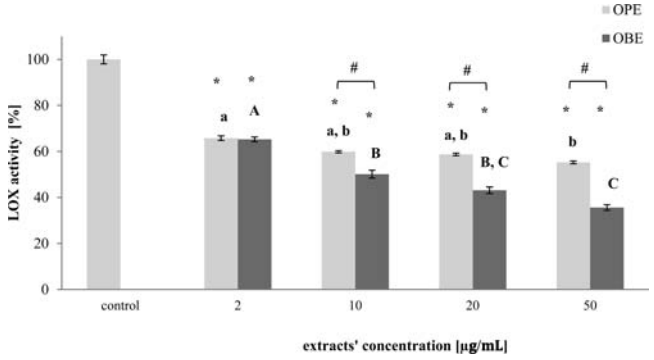

Figure 5. Inhibitory effect [%] of OPE and OBE in the concentration range of 2−50 μg/mL on lipoxydase activity. Data were expressed as mean ± SEM; performed in at least three independent experiments, assayed in triplicate. Statistical significance of differences compared to control was calculated by one-way ANOVA with Dunnett's *posthoc* test, $^{*}P < 0.05$. The statistically significant differences between the means for different concentrations of the same extract were analyzed by one-way ANOVA with Bonferroni *posthoc* test. Different letters a−b (for OPE) or A−C (for OBE) indicate statistically significant differences between means, $P < 0.05$. The statistically significant differences between the means for the same concentrations of different extracts were analyzed by one-way ANOVA with Bonferroni *posthoc* test, $^{\#}P < 0.05$.

polyphenols — mainly gallic acid and its esters, flavan-3-ol including catechin, procyanidin B_3, catechin gallate and other procyanidin oligomers as well as hydrolyzing tannins including pentagalloylglucose, tetragalloylglucose, trigalloylglucose, and hexahydroxydiphenyl (HHDP)-digalloylglucose.[3,9] There were no studies on the chemical composition of extracts form aerial parts of this plant. Using UHPLC coupled with DAD detector and ion-trap mass spectrometer we detected over 30 constituents comprising phenolic acids, tannins, flavan-3-ol and flavonoids (Figure 1, Table 1). Most of the compounds were detected for the first time in the investigated plant material. Among flavonoids, two dominating constituents were glucuronides of quercetin and kaempferol accompanied by their

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galloyl derivatives that seem to be rare and characteristic for different European *Oenothera* species.[40]

In the case of *O. biennis* most of the previous studies had also been focused on the chemical composition of defatted seeds extracts. Those studies showed similar *O. paradoxa* meal cake preparation containing mainly phenolic acids, flavan-3-ol derivatives, and some gallotanins.[3,9] Studies concerning the chemical composition of the *O. biennis* herb proved that its main constituent is macrocyclic ellagitannin oenothein B.[28,33] This observation was confirmed in the present study. Apart from OeB extracts from aerial parts were proven to contain some flavonoids mainly quercetin and kaempferol glycosides including quercetin and kaempferol glucuronides.[12,13,17] Our studies confirmed the presence of 14 flavonoids among which compounds **30**−**34** and **36**−**38** had already been reported,[41] and compounds **16**−**17**, **27**, **35**, and **40**−**41** were detected for the first time in the investigated plant material. In the case of *O. biennis,* we have also proven the presence of two rare galloylated derivatives of quercetin and kaempferol glucuronides (**35** and **40**).

The presence of myricetin glucuronide (**27**) as well as different phenolic acids (**6**, **13**, **14**, **18**, **21**, and **25**) were detected for the first time in both investigated plant material, which seems to be of great importance as those compounds possess interesting bioactivities.[42,43]

UHPLC studies showed no significant differences in the chemical composition of both extracts. This result may be connected with a close taxonomical relationship of both species.[1]

In the literature there are no reports concerning any biological activities of extracts form *O. biennis* and *O. paradoxa* herbs. All of the previous studies were focused on bioactivity of extracts of different polarity obtained from defatted seeds of both species.[3−7,9] In this study we determined for the first time the antioxidative and anti-inflammatory properties of OPE and OBE.

The results showed that both extracts have strong scavenging activity toward superoxide anion and hydrogen peroxide and rather moderate activity as far as HOCl and artificial DPPH radical are concerned (see Table 2). There were not statistically significant differences between extracts. However lower SC_{50} values for OPE toward O_2^- and H_2O_2 may be connected with higher total polyphenol content as it is well-known that polyphenols are the most popular group of compounds responsible for antioxidant activity of plant extracts.[44]

It has been shown that neutrophils are the main source of ROS during tissue injury, as well as in the induction and progression of inflammation. Excessive neutrophil activation in the human body contributes to the development of many chronic inflammatory diseases.[45] In our experiments we used human neutrophils stimulated with either f-MLP or PMA. Depending on the stimulator, the cell produces either O_2^-, H_2O_2, and HOCl (f-MLP) or only O_2^- and H_2O_2 (PMA). The differences can be explained by fact that the f-MLP stimulation leads to myeloperoxidase release that is responsible for the HOCl production from H_2O_2. In our study we established that OPE and OBE are significantly stronger inhibitors of ROS production after stimulation with f-MLP rather than after stimulation with PMA (IC_{50} values are 10-fold lower in the case of f-MLP). This effect may be connected with the mechanism of action of both extracts. In the case of f-MLP, ROS generation is induced by the activation of specific receptor while PMA is a direct kinase C activator. The lower IC_{50} in the experiments in which f-MLP was used as a stimulator may indicate that constituents of both extracts inhibit ROS production by a receptor-mediated pathway apart from the scavenging effect. As both extracts exhibit strong antioxidant activity in neutrophils' burst models their usage as external anti-inflammatory agents should be considered.

Hyaluronidase is an enzyme associated with degradation of hyaluronan, and in this way it decreases the integrity of the tissues during the progression of the inflammation. It has been shown that extracts prepared from different plant materials,[22,23] especially those containing high quantities of tannins,[24,25] have a significant ability to inhibit the activity of this enzyme. Both investigated extracts exhibited significant inhibitory activity at 50 μg/mL toward hyaluronidase in *in vitro* studies. OPE as well as OBE have stronger inhibitory activity than heparin, which is a known inhibitor of that enzyme. On the other hand, Piwowarski et al., who investigated tannin rich extracts, showed that some were potent hyaloronidase inhibitors at 10 μg/mL, while OPE and OBE had rather weak inhibitory activity at this concentration.[25] The strong antihyaluronidase activity of OPE and OBE at 50 μg/mL may be caused by the presence of high quantities of OeB in both preparations as this compound has been previously reported as a strong hyaluronidase inhibitor.[24]

It has been established that plant extracts may inhibit the activity of lipoxygenase decreasing the progression of inflammation in many diseases.[46] That is why we decided to evaluate the antilipoxygenase activity of the extracts investigated in the present study. Both extracts exhibited the antilipoxygenase activity. OBE activity turned out to be statistically stronger than in the case of OPE (IC_{50} values 12.1 ± 1.6 μg/mL and over 50 μg/mL respectively). Quantification of OeB in both extracts showed that OBE contained higher amounts of this compound. Since OeB has already been reported as a strong LOX inhibitor the differences between the investigated extracts may be at least in part assigned to the varying OeB content. Akula et al. tested 18 plant extracts as LOX inhibitors.[26] The IC_{50} values were in the range of 21.8−81.4 μg/mL. Our results obtained for OBE show that this extract may be considered as a strong LOX inhibitor with quite low IC_{50}, while OPE can be placed in the middle of the reported range.

In conclusion, the chemical composition of extracts prepared from aerial parts of *O. paradoxa* and *O. biennis,* as well as their antioxidative and anti-inflammatory properties may contribute to the establishment of future use of aerial parts of evening primrose that have been obtained after seed cultivation for medical purposes.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ROS, reactive oxygen species; OeB, oenothein B; OPE, *Oenothera paradoxa* extract; OBE, *Oenothera biennis* extract

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