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POLYPHENOL COMPOSITION OF EXTRACT FROM AERIAL PARTS OF *CIRCAEA LUTETIANA* L. AND ITS ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY IN VITRO

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The chemical composition and bioactivity of a water/methanol extract prepared from aerial parts of *Circaea lutetiana* were determined. HPLC-DAD-MS^3 analysis revealed the presence of 14 different compounds comprising phenolic acids, ellagitannins and flavonoids. Antioxidant assays showed the extract's strong scavenging activity towards DPPH (SC_{50} 33.1±3.1 μg/ml), O_2^- (SC50 4.0±2.3 μg/ml) and H_2O_2 (SC_{50} below 2 μg/ml). Enzyme-based studies revealed that *Circaea lutetiana* extract inhibits the activity of hyaluronidase (IC_{50} 13.3±2.4 μg/ml) and lipoxygenase (IC_{50} 44.7±1.4 μg/ml). These results support some traditional uses of *Circaea lutetiana*.

Key words: *Circaea lutetiana*, enchanter's nightshade, lipoxygenase, hyaluronidase, antioxidant activity.

INTRODUCTION

Circaea lutetiana L., enchanter's nightshade, is a perennial plant of the Oenotheraceae family. It is widespread throughout North America, Europe and Asia. Usually it grows in forests in deep shade on moist soil (Boufford, 1982). The herb of *C. lutetiana* has been used in Chinese folk medicine in the treatment of colic, dysuria and dysmenorrhea (Kim and Kingston, 1996). Native Americans have used infusions of enchanter's nightshade as a remedy for wounds and as a wash on injured parts of the body (Moerman, 1998). Investigations of the chemical composition of *Circaea* have shown flavonoids to be the main group of compounds. Among the major constituents, *C*-glycosides of apigenin such as vitexin, isovitexin, isoorientin, vicenin-1 and vicenin-2 have been found. Apart from *C*-glycosides, *O*-glycosylated flavones and flavonols including apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside, quercetin 3-*O*-glucoside (isoquercitrin) and kaempferol 3-*O*-glucoside (astragalin) have also been detected (Boufford et al., 1978; Averett and Raven, 1984; Averett and Boufford, 1985). The herb of *Circaea* contains other constituents besides flavonoids, such as galloyl- and octylglucose derivatives as well as icariside B_2 (rare caprylic alcohol) (Kim and Kingston, 1996). Recent work of ours has shown the aerial parts of *Circaea lutetiana* to be a rich source of the macrocyclic ellagitannin oenothein B, which is characteristic for the whole Oenotheraceae family, and that the main flavonoid compounds in this plant material consist of glycosides of apigenin, among which some rare *C*,*O*-glycosides occur in high quantities (Granica et al., 2012; Granica and Kiss, 2013). The presence of ellagic acid derivatives as well as ellagic acid itself has also been confirmed in *C. lutetiana* (Granica and Kiss, 2013). In the literature there are no studies of the bioactivity of extracts prepared from enchanter's nightshade, though there are quite extensive studies of the bioactivities, especially the antioxidant and anti-inflammatory properties, of other medicinal plants of the Oenotheraceae family (Shikov et al., 2006; Zaugg et al., 2006; Wojdyło et al., 2007; Hevesi et al., 2009; Márquez-Flores et al., 2009; Kiss et al., 2010, 2011, 2012; Kiss and Naruszewicz, 2012). In this study we examined the anti-inflammatory and antioxidant activity of standardized extract from the herb of *Circaea lutetiana*.

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MATERIAL AND METHODS

CHEMICALS

NBT (nitroblue tetrazolium), xanthine, xanthine oxidase, H_2O_2, HRPO (horseradish peroxidase), LOX (lipoxygenase from soybean), LA (linoleic acid), DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), indomethacin, ascorbic acid (vitamin C), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) and PVP (poly-(vinyl-polypyrrolidone)) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium tetrahydridoborate ($NaBH_4$), 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). Heparin was purchased from Polfa Warszawa (Warsaw, Poland). Phosphate-buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Deionized water was produced with a Millipore Simplicity System (Bedford, MA, USA). Oenothein B for quantitative analysis had previously been isolated in our laboratory.

PLANT MATERIAL

Aerial parts of *Circaea lutetiana* L. were collected from natural stations in Bremen (Germany) during blooming in August 2011. Plant material was authenticated by Dr. Anna Kiss based on morphological characters and was confirmed by Dr. Wojciech Szypula (Department of Biology and Pharmaceutical Botany, Medical University of Warsaw). Voucher specimen (no. CL082011) are deposited in the Plant Collection, Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Poland.

PREPARATION OF EXTRACT

Dried aerial parts of *Circaea lutetiana* L. (100 g) were extracted with methanol/water (50:50, v/v; 3 × 1 l, reflux, 5 h). The extracts were concentrated in a vacuum to eliminate the methanol and then freeze-dried to obtain dry residue (15 g).

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

HPLC-DAD-MS^3 CONDITIONS

HPLC analysis of *Circaea lutetiana* extract was carried out on a reversed-phase Zorbax SB-C_{18}, 150 × 2.1 mm, 1.9 μm column (Agilent, California, 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, flow rate 0.2 ml/min. The column was equilibrated for 10 min between injections. The sample of lyophilized extract (~5 mg) was dissolved in 1 ml of the mixture of phase A and MeOH (50:50, v/v) and then the solution was filtered through a 0.45 μm Chromafil membrane (Macherey-Nagel, Duren, Germany). The volume of 3 μl was injected to the HPLC column. UV spectra were recorded over a range of 200–450 nm, and chromatograms were acquired at 254, 280 and 350 nm. The 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 ions most abundant at the time. Detection of neutral losses was set for the sugar moieties characteristic for glycoside fragmentation (132, 146, 162 and 176 *amu*). In case of detection of one of the neutral loss masses, MS^3 fragmentation was performed in order to obtain the fragmentation spectrum of the aglycon moiety. Nebulizer pressure was 40 psi, drying gas flow 9 l/min, drying temperature 300°C and capillary voltage 4.5 kV. The analysis was carried out using scans from *m/z* 200 to 2,200.

TOTAL POLYPHENOL CONTENT

Total polyphenol content was determined using Folin-Ciocalteau reagent following the method described previously (Wojdyło et al., 2007): 2 ml of extract (1mg/ml) was added to 30 ml deionized water and mixed with Folin-Ciocalteau reagent (2 ml); then the reaction was diluted with 10 ml 10% sodium carbonate (Na_2CO_3). The absorbance of the resulting blue color was measured at 765 nm after 30 min. Gallic acid (GA) was the standard. The results are expressed as percentages of polyphenols in the prepared extract calculated from the calibration curve developed for gallic acid. Three independent samples were analyzed in triplicate.

TOTAL TANNIN CONTENT

Total tannin content was determined as described by Makkar (2000). The method is based on precipitation of tannins by poly-(vinyl-polypyrrolidone) (PVPP) and measurement of the quantity of polyphenols left in the supernatant: 200 mg PVPP, 2.0 ml H_2O and 2 ml extract (1 mg/ml) were mixed and kept for 15 min at 4°C, mixed again and centrifuged (3000 g, 10 min), and then the polyphenol content was determined as described above. The difference between total polyphenol content and polyphenol content after tannin precipitation gave the total tannin content in the analyzed extract. The results are expressed as percentages of tannins in the prepared extract. Three independent samples were analyzed in triplicate.

HYALURONIDASE ASSAY

Inhibition of hyaluronidase by the extract was determined by a turbidimetric method according to USP XXII-NF XVII (1990; 644–645, United States Pharmacopeia Convention, Inc., Rockville, MD), which was modified for 96-well microtiter plates as described previously (Piwowarski et al., 2011). Changes in turbidity at 600 nm were measured with a microplate reader (BioTek). Both extracts were tested across a concentration range of 10–50 μg/ml. For the investigated extract three independent experiments were carried out in duplicate. Heparin solution was the positive control in all the experiments. The activity of the tested extract was calculated as hyaluronidase enzyme activity percentage ($\%_{activity}$) 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} is the absorbance of solution without enzyme, Ab_{HYAL} is the absorbance of solution without the tested extract (negative control), and Ab_W is the absorbance of solution with the tested extract.

LIPOXYGENASE ASSAY

Inhibition of lipoxygenase by the extract was determined by a spectrophotometric method adjusted for 96-well microtiter plates. To begin, 100 μl of the tested extract solution was added to potassium phosphate buffer (pH 8.5), then 50 μl LOX solution (167 U/ml) in potassium phosphate buffer (pH 8.5) was added, and finally 50 μl linoleic acid (LA) solution (134 μM) was added and the changes in absorbance at 234 nm were measured at 30 s intervals for 25 min. The extract was tested across a 2–50 μg/ml concentration range. Indomethacin was the positive control. The activity of the tested extract was calculated as LOX enzyme activity percentage ($\%_{activity}$) in relation to maximum activity (negative control) after in all cases subtracting the extract's 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 is the absorbance of solution with the tested extract, Ab_b is the absorbance of solution without the enzyme, and Ab_0 is the absorbance of solution without the tested extract (negative control). The calculations used the absorbance values at the 10^{th} min of the reaction.

DPPH SCAVENGING ASSAY

The ability to scavenge the DPPH free radical was monitored according to a previously described method (Choi et al., 2002), with our modifications. DPPH solution (0.3 mM) was prepared in 95% ethanol. Lyophilized extract was 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 final concentrations of 2–50 μg/ml in the assay mixture. 100 μl DPPH solution and 100 μl of the tested extract at the different concentrations were added to a 96–well plate. Then the samples were incubated at room temperature for 30 min. After 30 min, absorbance at 518 nm (Ab) was measured with a microplate reader (BioTek). Vitamin C was the positive control. The absorbance values were converted to antioxidant activity percentages according to Eq. 3:

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

where Ab_t is the absorbance of DPPH solution with the tested extract, Ab_0 is the absorbance of DPPH solution with the addition of ethanol:water (1:1, v/v), and Ab_b is the absorbance of the tested extract solution with the addition of 95% ethanol.

O_2- SCAVENGING CAPACITY ASSAY

The xanthine/xanthine oxidase system with nitrobluetetrazolium (NBT) reduction was used to determine superoxide anion scavenging capacity (Choi et al., 2002). Lyophilized extract was dissolved in (Ca^{2+})-free PBS buffer to obtain stock solutions (1 mg/ml). The final concentrations of the tested extract in the reaction mixture were in the 2–50 μg/ml range. 50 μl of the tested extract in PBS, 50 μl xanthine oxidase (0.1 mU in PBS) and 100 μl 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 at 5 min intervals for 30 min with a microplate reader (BioTek). Vitamin C was the positive control. The absorbance values (Ab) were converted to O_2- production percentages using Eq. 4:

$$\%_{production} = \left(\frac{Ab_t - Ab_b}{Ab_0 - Ab_{0b}}\right) \times 100\% \quad (4)$$

where Ab_t is the absorbance of solution with the tested extract, Ab_b is the absorbance of solution without enzyme, Ab_0 is the absorbance of solution without the tested extract, and Ab_{0b} is the absorbance of solution without tested extract and without enzyme. The calculations used absorbance values at 30 min.

To eliminate the possibility of direct interaction between the tested extract and xanthine oxidase we monitored uric acid production by the enzyme at 295 nm. For uric acid production the procedure described above was used, except that the substrate solution did not contain NBT (only 0.4 mM xanthine).

TABLE 1. Uv-Vis and MS data of compounds detected in enchanter's nightshade extract by HPLC

	Compound	Retention time [min]	UV [nm]	$[M-H]^-$ *m/z*	MS^2 ions	MS^3 ions	$[M+H]^+$	MS^2 ions	MS^3 ions
1	unknown compound	2.8	212, 261	405	387, 301, 191b, 179, 128, 111	–	407	389, 301, 215b	–
2	gallic acid*	4.3	271	169	125b	–	171	160, 153, 127b, 112	–
3	caffeic acid pentoside	11.5	241, 300sh, 325	311	243, 179, 149b, 135	–	335[b]	317, 185b, 173	–
4	oenothein B*	16.9	220, 265	**783**[a], 1568	765b, 665, 447, 273	–	**804**[c], 1592[b]	728b, 634, 453	–
5	unknown ellagitannin	18.4	215, 265	783	765, 613, 481b, 301	–	**807**[b]	767, 623, 453b, 315	–
6	unknown ellagitannin	21.0	265	783	-	–	804[c]	-	–
7	vicenin-1*	29.8	269, 350	563	545, 473, 443b, 425, 383, 353	–	565, 587[b]	547b, 529, 499, 481, 445	–
8	isovitexin 2'-*O*-glucoside*	32.7	261, 350	593	473, 413b, 311, 293	–	595	**433b**, 415, 367, 337, 313, 283	415, 397, 367b, 337, 313
9	isovitexin 2'-*O*-arabinoside*	34.3	267, 350	563	443, 413b, 341, 311, 293	–	565	**433b**, 415, 367, 337	415, 397, 379, 367b, 337, 313
10	isovitexin*	34.8	slope	431	413, 341, 311b	–	433	415, 397, 379, 367b, 337, 313, 283	–
11	apigenin di-*C*-pentoside	35.2	258, 347	533	473, 443b, 425, 383, 353	–	535	517, 499b, 481, 469, 445, 409	–
12	4'-*O*-methylellagic acid xyloside*	41.4	255, 265	447	327, **315b**, 284, 255, 227	300b	449	317b, 287	–
13	apigenin *O*-rhamnohexoside	42.4	265, 347	577	413, 269b	–	579	**433**, 271b	415, 367, 271b
14	4'-*O*-methylellagic acid rhamnoside*	43.6	253, 264	461	**315b**	300b	485[b]	454, 339, 323b, 309	–

[a] $[M-2H]^{2-}$; [b] $[M+Na]^+$; [c] $[M+K]^{2+}$; *– comparisons with chemical standard were made;
sh – shoulder; b – base peak (most intense ion in recorded spectrum); in bold – ion subjected to MS^3 fragmentation

H_2O_2 SCAVENGING ASSAY

Hydrogen peroxide scavenging activity was determined using the horseradish peroxidase (HRPO) method as previously described (O'Dowd et al., 2004), with slight modifications. Lyophilized extract was dissolved in (Ca^{2+})-free PBS buffer to obtain stock solutions (1 mg/ml). The final concentrations of the tested extracts in the reaction mixture were in the 2–50 μg/ml range. 50 μl of the tested extract in PBS, 50 μl horseradish peroxidase (5 mU in PBS) and 50 μl 0.03% H_2O_2 solution in PBS were added to a 96-well plate. Immediately after adding 50 μl luminol solution in PBS (2 mM), chemiluminescence was measured with a microplate reader at 2 min intervals for 40 min. With vitamin C as the positive control, scavenging activity was calculated as the H_2O_2 production percentage at maximum chemiluminescence according to Eq. 5:

$$\%_{production} = \left(\frac{CL_t - CL_b}{CL_0 - CL_{0b}}\right) \times 100\%$$

where CL_t is the chemiluminescence of solution with the tested extract, CL_b is the chemiluminescence of

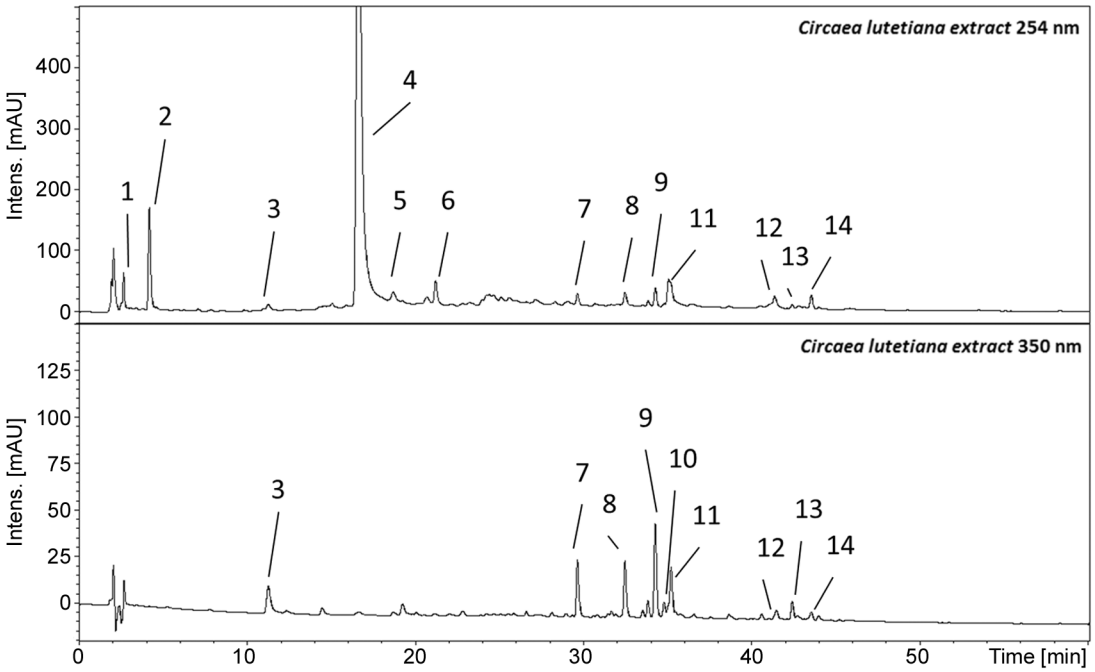

Fig 1. Chromatograms of *Circaea lutetiana* extract acquired at 254 and 350 nm.

solution without enzyme, CL_0 is the chemiluminescence of solution without the tested extract, and CL_{0b} is the chemiluminescence of solution without the tested extract and without enzyme.

STATISTICAL ANALYSIS

The results are expressed as means ± SEM of the indicated number of experiments. The IC_{50} values of the tested compounds were calculated based on concentration-inhibition curves. The statistical significance of differences between means and control was determined by ANOVA with Dunnett's post hoc test. P values below 0.05 were considered statistically significant. All analyses used Statistica 8.

RESULTS AND DISCUSSION

The chemical composition of *Circaea lutetiana* extract was evaluated by the HPLC-DAD-MS^3 method. We detected 14 constituents consisting of two phenolic acids (2, 3), two ellagic acid derivatives (12, 14), six flavonoids (7–11, 13), three ellagitannins (4–6) and one undetermined chemical (1). Ten of the detected compounds were fully or partially identified. Figure 1 shows chromatograms of the extract acquired at 254 and 350 nm, and Table 1 presents the UV-Vis and MS data of the detected compounds. A previous HPLC study showed that macrocyclic ellagitannin is the dominant constituent in *C. lutetiana* herb, forming 27.64% of the dry material (Granica et al., 2012). The present study confirmed this: total tannin content in the extract was 30.94±0.81% and total polyphenol content was 35.08±1.81%; this means that other phenolics such as flavonoids or phenolic acids are minor constituents, amounting to ~4%.

The antioxidant activity of the extract was evaluated using three noncellular assays: scavenging activity towards artificial radical (DPPH) and two reactive oxygen species occurring in living organisms (superoxide anion $O_2^{\cdot-}$ and hydrogen peroxide generated in vitro). In the assays with DPPH, *C. lutetiana* extract exhibited strong scavenging activity in a concentration-dependent manner, with SC_{50} at 33.1±3.1 μg/ml (Fig. 2), significantly higher than for vitamin C (3.9±0.2 μg/ml).

Circaea lutetiana extract in the 2–50 μg/ml concentration range scavenged $O_2^{\cdot-}$ in a concentration-dependent manner (Fig. 3). SC_{50} at 4.0±2.3 μg/ml shows that the extract exhibits strong scavenging activity towards the superoxide anion but is less active than vitamin C (1.3±0.6 μg/ml). We also evaluated the inhibitory activity of *Circaea lutetiana* extract towards the enzyme used in the experiment. The data show that over the whole concentration range the extract did not inhibit xanthine oxidase activity (Fig. 3). In the hydrogen peroxide scavenging assay the extract presented very strong scavenging activity, SC_{50} below 2 μg/ml, stronger than vitamin C (1.3±0.6 μg/ml) (Fig. 4).

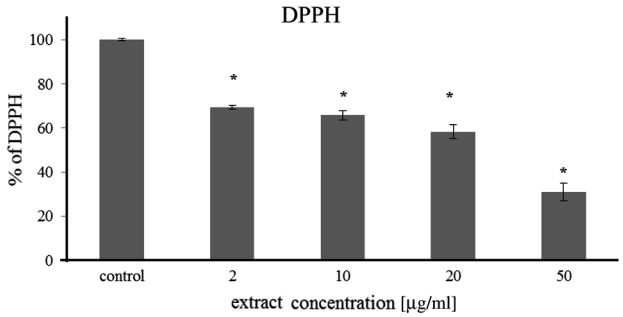

Fig. 2. Effect of 2–50 µg/ml *Circaea lutetiana* extract on percentage of DPPH artificial radical. Values are means ± SEM of at least three independent assays done in triplicate. Data are percentages of DPPH remaining in the sample after incubation with extract. Asterisked values differ significantly from the control at $p < 0.05$.

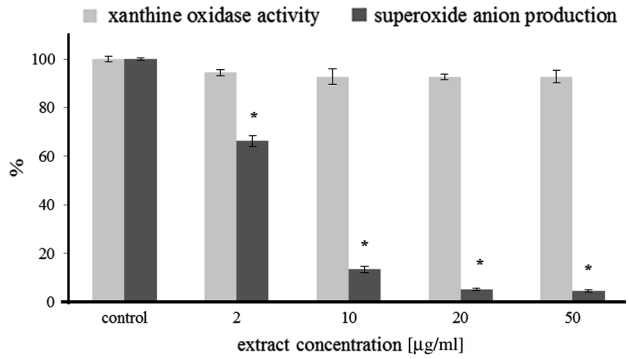

Fig. 3. Effect of 2–50 µg/ml *Circaea lutetiana* extract on superoxide anion production and xanthine oxidase activity. Values are means ± SEM of at least three independent assays done in triplicate. Asterisked values differ significantly from the control at $p < 0.05$.

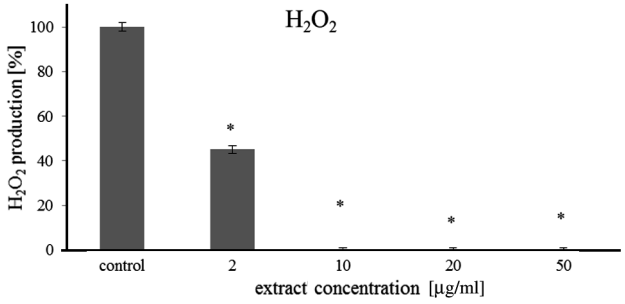

Fig. 4. Effect of 2–50 µg/ml *Circaea lutetiana* extract on hydrogen peroxide production (H_2O_2). Values are means ± SEM of at least three independent assays done in triplicate. Asterisked values differ significantly from the control at $p < 0.05$.

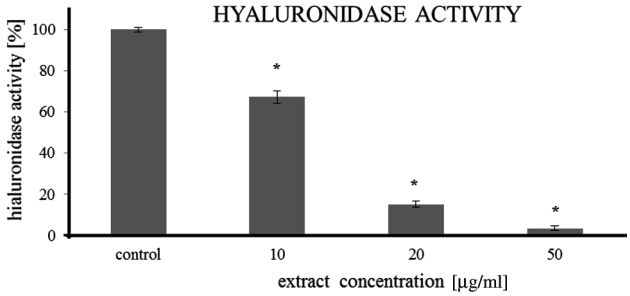

Fig. 5. Inhibitory effect (%) of 2–50 µg/ml *Circaea lutetiana* extract on hyaluronidase activity. Values are means ± SEM of at least three independent assays done in triplicate. Asterisked values differ significantly from the control at $p < 0.05$.

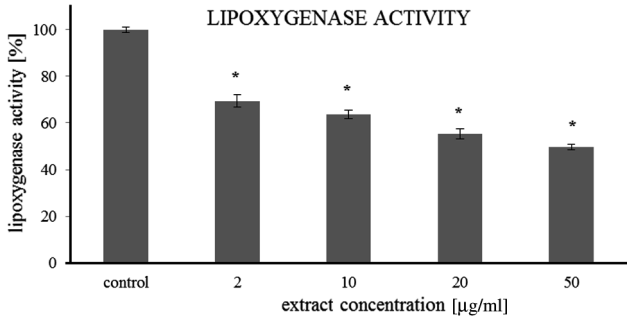

Fig. 6. Inhibitory effect (%) of 2–50 µg/ml *Circaea lutetiana* extract on lipoxygenase activity. Values are means ± SEM of at least three independent assays done in triplicate. Asterisked values differ significantly from the control at $p < 0.05$.

We evaluated the anti-inflammatory activity of *Circaea lutetiana* extract using hyaluronidase and lipoxygenase inhibition models. The extract significantly reduced hyaluronidase activity in a concentration-dependent manner (Fig. 5). The IC_{50} value was 13.3±2.4 µg/ml, indicating that *C. lutetiana* extract is a strong hyaluronidase inhibitor, similar to other tannin-rich extracts investigated by Piwowarski (unpubl. data), who determined the IC_{50} values for extracts from *Lythri herba* (8.1±0.8 µg/ml) and *Gei urbani radix* (12.9±1.1 µg/ml), which were the most active extracts reported from his experiments. The IC_{50} value for hyaluronidase was also lower than for the positive control (heparin; 62.1±7.5 µg/ml), showing that the investigated extract is a stronger hyaluronidase inhibitor. *C. lutetiana* extract also inhibited the activity of lipoxygenase in a concentration-dependent manner (Fig. 6). The IC_{50} value for LOX was 44.7±1.4 µg/ml, indicating that *C. lutetiana* extract possesses moderate LOX inhibitory activity as compared with plant extracts investigated by Akula and Odhav (2008) but is a significantly stronger LOX inhibitor than the positive control, indomethacin (83.9±7.7 µg/ml).

Oenothein B was determined to be the major constituent in the investigated extract. Previous

studies demonstrated that oenothein B possesses strong anti-inflammatory and antioxidant activity (Kiss et al., 2011), suggesting that this compound is responsible for the *Circaea lutetiana* extract bioactivities observed in this study. Other constituents such as flavonoids or phenolic acids are less likely to have a significant influence on the extract's bioactivity as their content is much lower than that of oenothein B, although they also may exhibit anti-inflammatory and antioxidant activities (Furuta et al., 2004; Shie et al., 2010; Zhou and Lv, 2012).

This is the first report on the biological activities of extracts prepared from aerial parts of *C. lutetiana*. Our results indicate that this plant-derived material is an anti-inflammatory agent and support the traditional use of enchanter's nightshade infusion as an external treatment for skin conditions and wounds.

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