

LIPID PEROXIDATION INHIBITION STUDY OF FLOWER EXTRACT AND TWO COUMARINS ISOLATED FROM *DAPHNE MEZEREUM* L.

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The medicinal importance of the genus *Daphne* L. is related to the richness in the expansive range of different classes of natural products and bioactive phytochemicals, such as coumarins, flavonoids, lignans and different classes of terpenes. The current study reports on the lipid peroxidation effect of diethyl-ether macerate of *Daphne mezereum* L. flowers and of two coumarins we have isolated from the aqueous subfraction of the crude diethyl-ether extract. All three tested samples, *D. mezereum* flowers extract (IC₅₀ = 25.1 ± 2.9 mM) and isolated coumarins: umbelliferone (IC₅₀ = 7.1 ± 2.6 mM) and herniarin (IC₅₀ = 19.0 ± 1.3 mM), exhibited notable antioxidative potential in lipid peroxidation assay. None of the samples, however, had an inhibitory effect as pronounced as standardly applied antioxidants Trolox (IC₅₀ = 22 ± 6 μM), caffeic acid (IC₅₀ = 15 ± 3 μM) and quercetin (IC₅₀ = 23 ± 6 μM). Taken altogether, the results of our studies bring forward new data regarding the antioxidant activities of *D. mezereum* species.

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Key words: *Daphne mezereum*, coumarin isolation, umbelliferone, herniarin, lipid peroxidation

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Introduction

Named after the water nymph in Greek myth that was turned into a laurel tree, encompassing 95 species of flowering shrubs, *Daphne* L. is the most diverse genus of the Thymelaeaceae family. The genus is native to certain regions of sub-tropical Asia but is also distributed in Europe and North Africa. To this day, 17 species of this genus have been evidenced in Europe's Flora (1). *Daphne mezereum* L. is among plants that grow mostly in Europe and Asia and one of seven *Daphne* species native to Serbia (2). The decorative, strongly scented flowers are produced in early spring on the bare stems before

the leaves appear. The fruit is a bright red berry, poisonous to humans, but despite striking toxicity, because of its desirable horticultural characteristics, *D. mezereum* has become one of the most popular perennial flowering shrubs (3).

As was confirmed in earlier studies, *Daphne* plants are a rich source of pharmacologically important molecules, indicating broad potential use in medicine (4–12). The genus has a long history in traditional medicine as a remedy for the treatment of rheumatism, ulcers, and treatments for aches, inflammation, and abortifacient (13, 14). Previous phytochemical studies of the genus report a large number of classes of bioactive secondary metabolites, dominated by coumarins, flavonoids, lignans, diterpenes and steroids (5, 6, 9, 11, 12).

Daphne mezereum was already subjected to phytochemical research (4, 15–22). The extracts and essential oil of *D. mezereum* demonstrated several biological properties; the plant is suspected to have an immune-stimulating effect, the water-alcohol extract has antileukemic activity on P-388 lymphocytic cells in mice (4), while pure compound mezerein, isolated from *D. mezereum*, shows an inhibitory effect against P-388 cells and L-1210 type of leukemia in mice in the 50 μg dosage (23). Interestingly, only one paper analyzed *D. mezereum* flowers in an analysis related to the floral fragrance chemistry (20).

Despite numerous data on the compositional analysis of *Daphne* species from Serbia (*Daphne*

alpina subsp. *alpina* (24), *Daphne cneorum* L. (25), *Daphne blagayana* L. Freyer (26, 27) and *Daphne malyana* Blečić (28)), with antioxidant capacity determined and correlated to coumarins, phenolic acids and flavonoids, a similar investigation was never published for *D. mezereum* (except the congress announcement we have reported in 2022 (29)). Therefore, we decided to provide information on the antioxidant effects of extract obtained from flowers by maceration with diethyl-ether. As part of our investigation, we isolated two simple coumarins from *D. mezereum* and tested their antioxidant properties in the lipid peroxidation assay.

Material and Methods

Chemicals

All inorganic and organic reagents were of analytical grade, obtained from commercial sources (unless specified otherwise, all chemicals were purchased from Merck (Darmstadt, Germany)). Phospholipids (Phospholipon® 90 – PL90) were obtained by courtesy of Phospholipid GMBH, Cologne, Germany. Analytical thin-layer chromatography was carried out on precoated TLC sheets ALUGRAM® Xtra SIL G/UV₂₅₄ (Macherey-Nagel). Preparative column chromatography was carried out on Silica Gel 60 (70–230 mesh).

Spectrophotometric measurements were performed using Thermo Scientific Evolution 60 Spectrophotometer (Fisher Scientific, UK). HPLC analysis was performed using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a diode array (DAD) detector and NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer.

Plant material and extraction procedure

Aerial parts of *D. mezereum* were collected in the flowering phase at Devojački grob, Suva Planina in June 2007. Taxonomic identification was performed by Professor B. Zlatković (Faculty of Science and Mathematics, University of Niš). Fresh samples were subjected to exhaustive macerations (800 mL, 3 × 48 h) with diethyl-ether (DE) as a solvent. The resultant solution was evaporated to dryness under reduced pressure below 40 °C, to give 3.1 g of DE extract. Extraction yield, expressed in % of used plant material, was 0.5%. The extract was purged with nitrogen and kept at -20 °C, under nitrogen atmosphere, until the final use.

Isolation of simple coumarins

Dry DE extract was subfractionated according to the modified procedures given by Komissarenko et al., 1994 (30) and Ness et al., 1996 (31) with hot water, filtrated and re-extracted with chloroform. Upon drying, chloroform extract was

evaporated to give 1.2 g, or 0.2% if expressed in % of used plant material. Subfractionated extract, i.e., water fraction was fractionated further with column chromatography first on a silica gel column (1.2 x 20 cm) and further chromatographed on Sephadex LH-20 column (2.5 cm x 150 cm) and eluted successively with deionized water (50 mL), aqueous ethanol (20%, 40%, 70% and 90% ethanol, 50 mL for each) and aqueous acetone (50% and 90% acetone, 50 mL for each) at room temperature (32). Coumarins umbelliferone and herniarin were eluted in different fractions, yielding 124.2 mg (extraction yield 0.02%) and 54.8 mg (extraction yield 0.009%), respectively. The composition of the extract and isolated coumarins were analyzed with HPLC-DAD and confirmed by recording ¹H and ¹³C NMR. For this purpose, samples were either diluted in methanol HPLC grade, filtered through 0.45 µm PTFE filters and subjected to HPLC analyses or dissolved in 1 mL DMSO-d₆, and 0.7 ml of the solution was transferred into a 5-mm Wilmad, 528-TR-7 NMR tube.

NMR analysis

¹H NMR and ¹³C NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer at 300 MHz and 75 MHz, respectively. All NMR spectra were recorded at 298 K in DMSO-d₆ (isotopic enrichment 99.95%) solution. Chemical shifts (δ) were given as parts per million (ppm), relative to tetramethylsilane (TMS) as an internal standard with multiplicity reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constants (*J*) are shown in hertz (Hz); number and assignment of protons. The experimental error in the measured ¹H-¹H coupling constants was ± 0.5 Hz.

HPLC analysis

HPLC analysis was performed using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a diode array detector. The test sample solutions were prepared in methanol diluted up to 100 ppm. The analyses were carried out on a reverse phase Purospher STAR RP-18e column (125 mm x 3 mm, 3.0 µm, Merck, KGaA, 64271 Darmstadt, Germany) by maintaining column temperature at 30 °C. Mobile phase A was a trifluoroacetic acid (0.1%) solution which was prepared by dissolving 1.0 mL of trifluoroacetic acid in 1000 mL of water, and methanol was used as mobile phase B. The injection volume was 10 µL and the flow rate was 0.5 mL/min. The wavelength was fixed at 254 nm. For crude DE extract (**A**) the data were acquired by using a gradient elution system: 0–10min, 50% A, increasing the ratio of phase B to 90% and decreasing phase A to 10%; 10–12 min, holding 10% A and 90% B phase; 12–13 min decreasing of B phase to 50% and increasing A phase to

50%; 13–15 min, holding 50% A and 50% B phase. For subfractionated extract (**B**) and coumarins (**C** and **D**) isolated therefrom, data were acquired by using gradient elution system: 0–18 min, 80% A, increasing the ratio of phase B to 90% and decreasing phase A to 10%; 18–20 min, holding 10% A and 90% B phase; 20–21 min decreasing of B phase to 20% and increasing A phase to 80%; 21–22 min, holding 20% A and 80% B phase.

Lipid peroxidation inhibition by thiobarbituric acid-malondialdehyde assay

Lipid peroxidation (LP) is a free radical-mediated chain reaction that once initiated results in the oxidative degradation of polyunsaturated lipids. The final product of lipid peroxidation is malondialdehyde (MDA), a short-chain aldehyde, which is a biochemical marker of cell membrane oxidative damage (33).

Lipid peroxidation and LP inhibition in the presence of the tested compounds were measured by thiobarbituric acid-malondialdehyde (TBA-MDA) assay according to the procedure given by Lazarević *et al.*, 2020 (34).

The absorbance of MDA-(TBA)² adduct in the supernatant, read at 530 nm, was used to calculate the inhibition percentage of LP using the following equation:

$$\text{LP inhibition (\%)} = 100 \times (\text{Ac}-\text{As})/(\text{Ac}-\text{Ab})$$

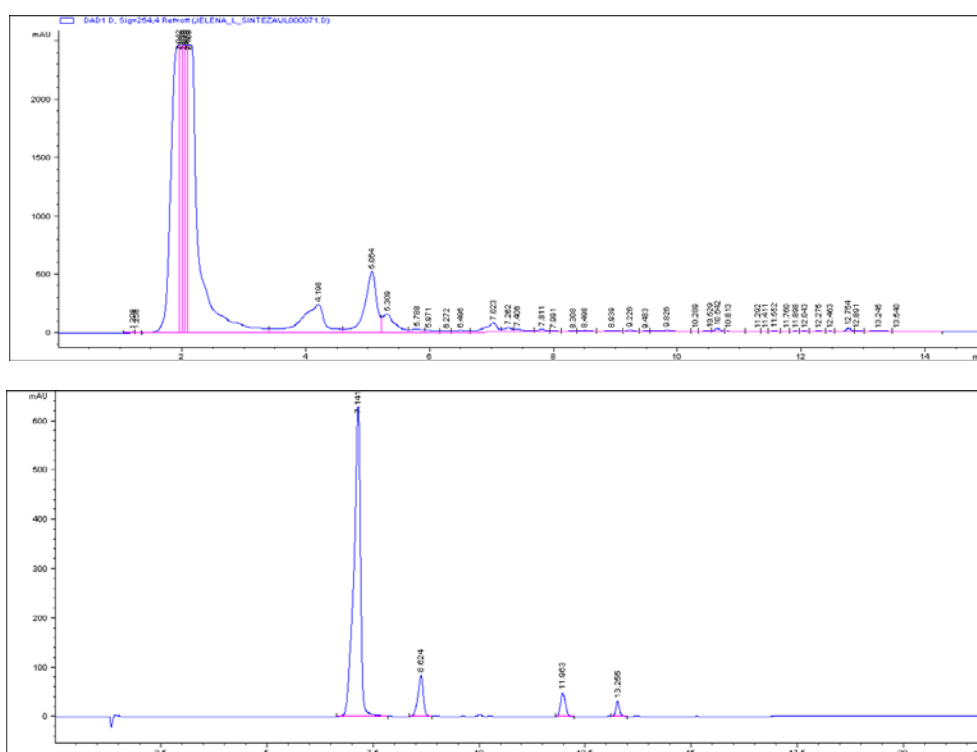
Ac - the absorbance of control (PL90 in methanol treated with the AAPH and TBA solution), As - the absorbance of samples (tested extract/compounds dissolved in PL90 solution, afterwards treated with the AAPH and TBA

solution) and Ab - the absorbance of blank (PL90 in methanol, not treated with AAPH, but with TBA solution).

Samples were evaluated for LP-inhibitory activity, and only those showing inhibition greater than 50% at 500 μM were investigated further in a broader concentration range to allow calculation of IC₅₀ values. The same type of experiment was done by using frequently used antioxidants as standards: caffeic acid, Trolox or quercetin. The standards were evaluated for LP-inhibitory activity at concentrations of 50 μM (caffeic acid) and 80 μM (quercetin and Trolox) in the final reaction mixture. All experiments were performed in triplicate.

Results and discussion

HPLC chromatograms of crude DE extract (Figure 1.A.), subfractionated water extract (1.B.) and of coumarins isolated from *D. mezereum*: umbelliferone (1.C.) and herniarin (1.D.), at wavelength detection of 254 nm, are presented in Figure 1. Unfortunately, a mixture of several co-eluting compounds, representing the most abundant compound from the chromatogram, was not detected. On the other hand, the presence of two simple coumarins umbelliferone and herniarin was confirmed by the comparison of the retention time and UV spectrum with the standards. Coumarins are among the important constituents of *Daphne* species and have been previously reported on many occasions (5, 10, 13, 18, 22, 25–27).



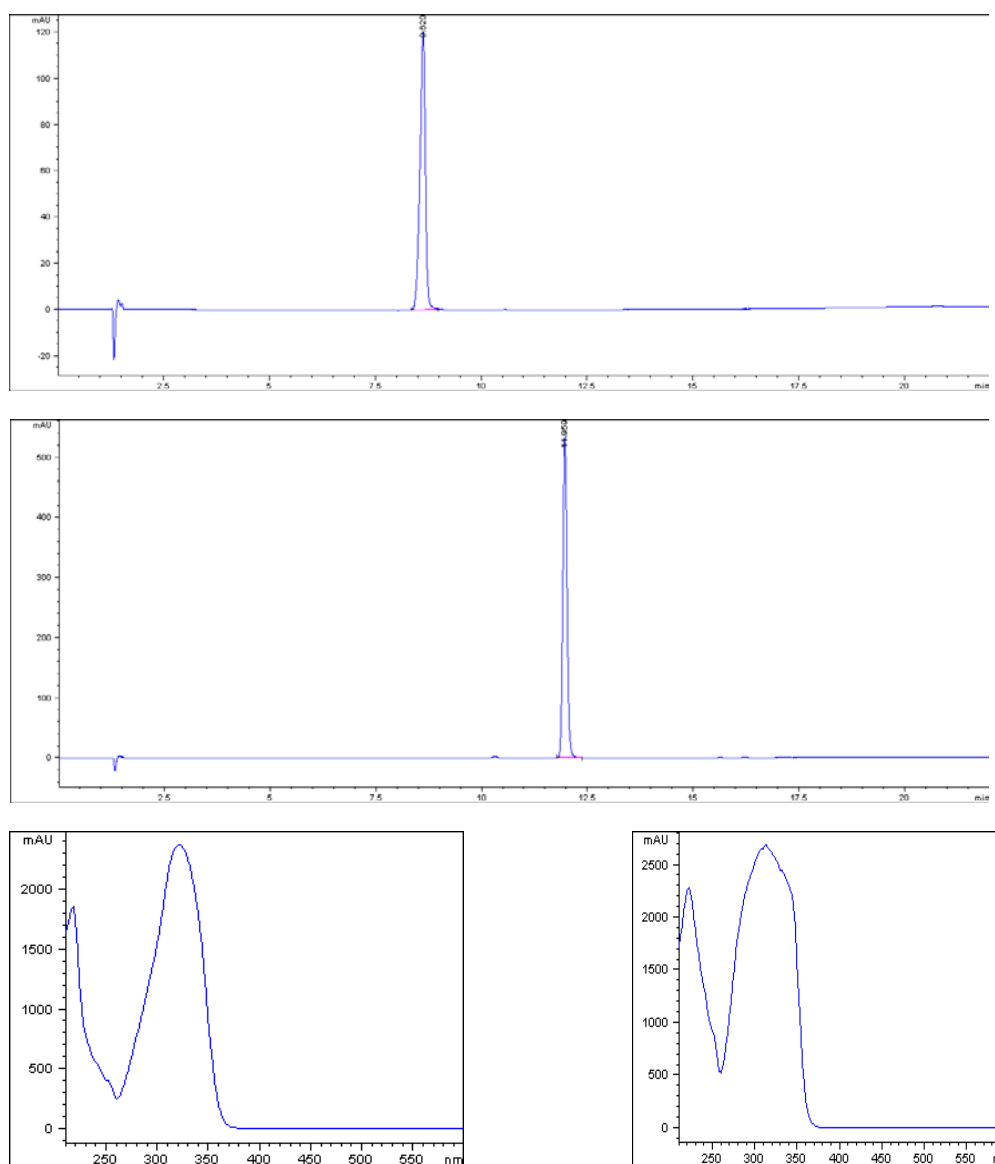


Figure 1. HPLC chromatograms of crude DE extract (**A**), subfractionated water extract (**B**) and of coumarins isolated from *D. mezereum*: umbelliferone (**C**) and herniarin (**D**) and UV and UV spectra of umbelliferone ($t_R = 8.62$ min) and herniarin ($t_R = 11.96$ min)

The composition of the extract was analyzed and confirmed with HPLC-DAD, by the comparison of the retention time and UV spectrum with the coumarin standards, and by recording ^1H and ^{13}C NMR for the isolated compounds. The obtained spectral data have confirmed the identity of the isolated coumarins. These data coincide well with the previous reports (35, 36) and fully assigned ^1H and ^{13}C spectra are presented in Figure 2.

Figure 2. ^1H and ^{13}C NMR spectra of umbelliferone (A) and herniarin (B)

Analytical properties of both isolated coumarins are given as follows:

Umbelliferone (7-Hydroxy-2H-chromen-2-one): White amorphous solid, $\text{C}_9\text{H}_6\text{O}_3$ ($M = 162.14$), HPLC purity $\geq 99\%$, ^1H NMR (DMSO- d_6 ,

300.13 MHz, δ , ppm): 10.59 (s, 1H, O-H), 7.95 (d, $J = 9.16$ Hz, 1H, C-H), 7.54 (d, $J = 8.53$ Hz, 1H, Ar-H), 6.81 (dd, $J = 8.47, 2.32$ Hz, 1H, Ar-H), 6.73 (d, $J = 2.38$ Hz, 1H, Ar-H), 6.22 (d, $J = 9.41$ Hz, 1H, C-H). ^{13}C NMR (DMSO- d_6 , 75 MHz, δ , ppm): 161.2, 160.4, 155.5, 144.5, 129.7, 113.1, 111.4, 111.2, 102.0.

Herniarin (7-Methoxy-2H-1-benzopyran-2-one): White amorphous solid, $\text{C}_{10}\text{H}_8\text{O}_3$ ($M = 176.17$), HPLC purity $\geq 99\%$, ^1H NMR (DMSO- d_6 , 300.13 MHz, δ , ppm): 7.99 (d, $J = 9.54$ Hz, 1H, C-H), 7.63 (d, $J = 8.66$ Hz, 1H, Ar-H), 7.0-6.93 (m, 2H, Ar-H), 6.3 (d, $J = 9.54$ Hz, 1H, =C-H), 3.87 (s, 3H, =C-H). ^{13}C NMR (DMSO- d_6 , 75 MHz, δ , ppm): 162.4, 160.3, 155.4, 144.3, 129.4, 112.4, 112.3, 112.3, 100.6, 55.9.

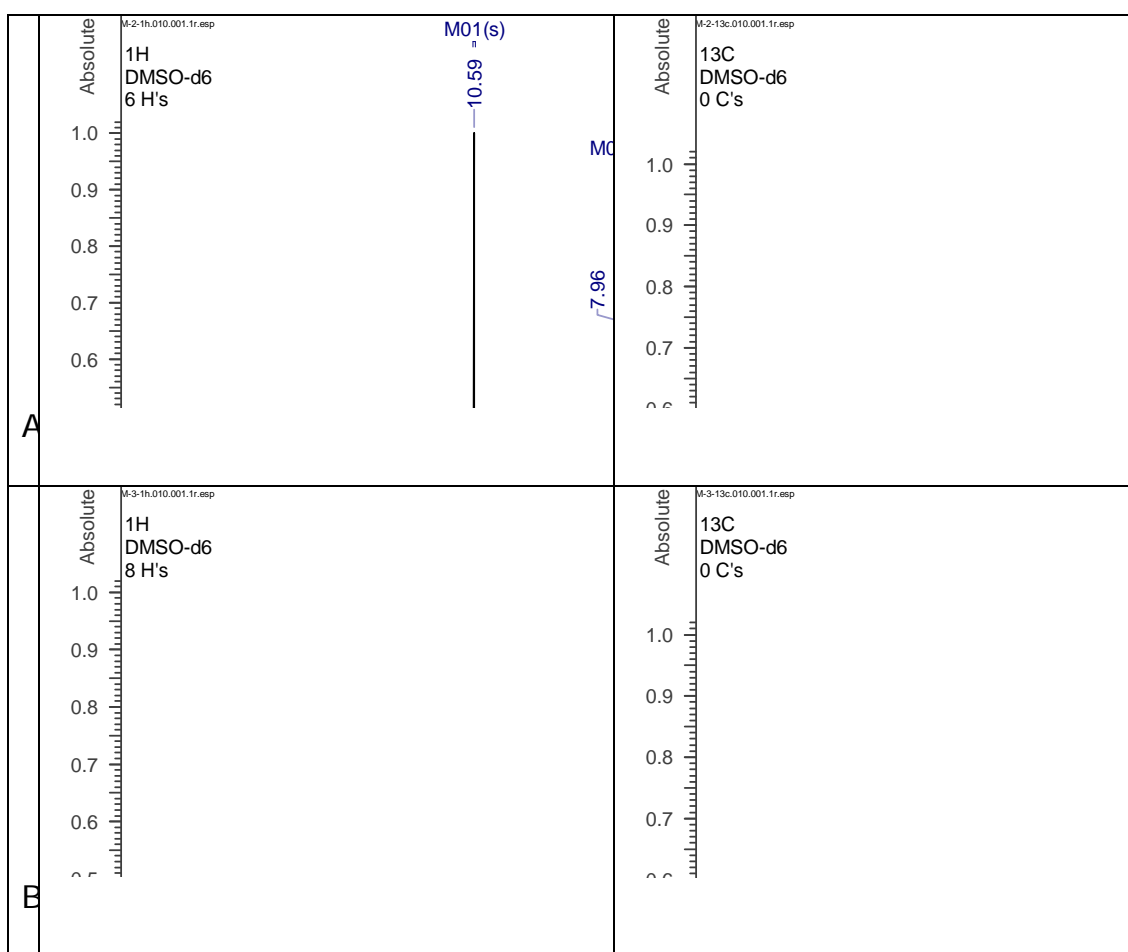


Figure 2. ^1H and ^{13}C NMR spectra of umbelliferone (A) and herniarin (B)

The lipid peroxidation inhibition effect of the tested samples: diethyl-ether macerate of *D. mezereum* flowers and isolated coumarins: umbelliferone and herniarin, measured using the method based on MDA-TBA assay, was notable. After performing experiments, the obtained results were plotted, IC_{50} values were calculated and reported in Table 1. The obtained results indicated that all samples [*D. mezereum* flowers DE ($\text{IC}_{50} = 25.1 \pm 2.9$ mM) and isolated umbelliferone ($\text{IC}_{50} = 7.1 \pm 2.6$ mM) and herniarin ($\text{IC}_{50} = 19.0 \pm 1.3$ mM)] exhibited significant potential in LP assay.

However, none of the tested samples were as effective as standardly applied antioxidants Trolox ($\text{IC}_{50} = 22 \pm 6$ μM), caffeic acid ($\text{IC}_{50} = 15 \pm 3$ μM) and quercetin ($\text{IC}_{50} = 23 \pm 6$ μM). Preliminary results have shown that *D. mezereum* DE extract, by inhibiting the LP process, has antioxidant properties and that this effect can be partially attributed to the presence of simple coumarins umbelliferone and herniarin, whose antioxidant effects have been investigated and reported in a number of studies (37, 38).

Table 1. Lipid peroxidation inhibition effects of the three tested samples (IC_{50} values given in mM) and the selected antioxidants (IC_{50} values given in μM)

Compound	LP inhibition IC_{50} (mM) \pm SD	Compound	LP inhibition IC_{50} (μM) \pm SD
<i>Daphne mezereum</i> flowers' DE	25.1 \pm 2.9	Trolox	22 \pm 6
umbelliferone	7.1 \pm 2.6	caffeic acid	15 \pm 3
herniarin	19.0 \pm 1.3	quercetin	23 \pm 6

Conclusion

Epidemiological studies link dietary intake of coumarin-based compounds with beneficial health effects, mainly due to their antioxidant activity. Exhibiting antioxidant activity by the inhibition of lipid peroxidation, studied coumarins represent such compounds. Additional phytochemical and pharmacological evaluations are needed before shedding further light on the potential application of *D. mezereum*.

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ISPITIVANJE LIPIDNE PEROKSIDACIJE EKSTRAKTA CVETA I DVA KUMARINA IZOLOVANA IZ BILJKE *DAPHNE MEZEREUM* L.

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Fitohemijskim ispitivanjem vrsta *Daphne* L. potvrđeno je prisustvo aktivnih sastojaka koji po svojoj strukturi pripadaju kumarinima, flavonoidima, lignanima i terpenima. U ovom radu ispitan je efekat inhibicije lipidne peroksidacije etarskog ekstrakta cveta *Daphne mezereum* L. i kumarina koji su postupkom frakcionisanja izolovani iz ekstrakta. Sva tri testirana uzorka – sirov etarski ekstrakt cvetova *D. mezereum* ($IC_{50} = 25,1 \text{ mM} \pm 2,9 \text{ mM}$) i izolovani jednostavni kumarini, umbeliferon ($IC_{50} = 7,1 \text{ mM} \pm 2,6 \text{ mM}$) i hernijarin ($IC_{50} = 19,0 \text{ mM} \pm 1,3 \text{ mM}$) – pokazala su dobar antioksidativni potencijal. Efikasnost ekstrakta i ispitivanih jedinjenja upoređivana je sa standardno primenjivanim antioksidansima: trolksom ($IC_{50} = 22 \text{ mM} \pm 6 \text{ mM}$), kafenom kiselinom ($IC_{50} = 15 \text{ mM} \pm 3 \text{ mM}$) i kvercetinom ($IC_{50} = 23 \text{ mM} \pm 6 \text{ mM}$). Rezultati ove studije doprinose razumevanju fitohemijske karakterizacije vrste *D. mezereum*, ukazujući na to da se ekstrakt pomenute biljke može koristiti kao izvor prirodnih antioksidanasa.

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Ključne reči: *Daphne mezereum*, izolovanje kumarina, umbeliferon, hernijarin, lipidna peroksidacija

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