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Lipid peroxidation inhibition study of flower extract and two coumarins isolated from *Daphne mezereum* L.

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Abstract

The medicinal importance of the plants belonging to the genus *Daphne* L. is proved by the richness in the large variety of different classes of natural products and valuable bioactive phytochemicals, such as coumarins, flavonoids, lignans and different classes of terpenes. In the present study, we have investigated 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 (IC_{50} = 25.1 ± 2.9 mM) and isolated coumarins: umbelliferone (IC_{50} = 7.1 ± 2.6 mM) and herniarin (IC_{50} = 19.0 ± 1.3 mM), exhibited notable antioxidative potential in LP assay. None of the samples however, had an inhibition effect as pronounced as standardly applied antioxidants Trolox (IC_{50} = 22 ± 6 μM), caffeic acid (IC_{50} = 15 ± 3 μM) and quercetin (IC_{50} = 23 ± 6 μM). Taken altogether, the results of our studies bring forward new data regarding the antioxidant activities of *D. mezereum* species.

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

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Ispitivanje lipidne peroksidacije etarskog ekstrakta cveta i iz ekstrakta izolovanih kumarina biljke *Daphne mezereum*

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Fitohemijskim istraživanjem *Daphne* vrsta je potvrđeno prisutvo 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* i iz ekstrakta, postupkom frakcionisanja, izolovanih kumarina. Sva tri testirana uzorka: sirov etarski ekstrakt cvetova *D. mezereum* (IC_{50} = 25,1 ± 2,9 mM) i izolovani jednostavni kumarini: umbeliferon (IC_{50} = 7,1 ± 2,6 mM) i hernijarin (IC_{50} = 19,0 ± 1,3 mM) ispoljili su dobar antioksidativni potencijal. Efikasnost ekstrakta i ispitivanih jedinjenja upoređivana je sa standardno primenjivanim antioksidansima troloksom (IC_{50} = 22 ± 6 mM), kafenom kiselinom (IC_{50} = 15 ± 3 mM) i kvercetinom (IC_{50} = 23 ± 6 mM). Rezultati ove studije doprinose razumevanju fitohemijske karakterizacije vrste *D. mezereum*, ukazujući da se ekstrakt biljke može koristiti kao potencijalni izvor prirodnih antioksidanasa.

Key words: Daphne mezereum, izolovanje kumarina, umbeliferon, herniarin, lipidna peroksidacija

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 Thymelaeaceae family. The genus is native to certain regions of sub-tropical Asia, but is also distributed in Europe and north Africa. Until now, in Europe's Flora, the presence of 17 species of this genus has been identified [1]. *Daphne mezereum* L. is among plants that grows 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, very poisonous for humans, but despite striking toxicity, because of its desirable horticultural characteristics, *D. mezereum* become one of the most popular perennial flowering shrubs [3].

As was confirmed in earlier studies, *Daphne* plants are rich source of pharmacologically important molecules, indicating broad potential use in medicine [4-12]. The genus is having a long history in traditional medicine as a remedy for the treatment of rheumatism, ulcers, treatments for aches, inflammation, and abortificient [13, 14]. Previous phytochemical studies shows that plants of the genus contain 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* exhibited several biological properties; the plant is suspected to have immune-stimulating effect, the water-alcohol extract has shown antileukemic activity on P-388 lymphocytic cells in mice [4], while pure compound mezerein, isolated from *D. mezereum*, shown a significant inhibitory effect against P-388 cells and L-1210 type of leukemia in mice in the 50μg dosage [23]. Interestingly, only one paper analyzed *D. mezereum* flowers in analysis related to 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, similar investigation was never published for *D. mezereum* (except the congress announcement we have reported in 2022 [29]). Therefore, the aim of our study was to provide information on antioxidant activities of extract obtained from flowers by maceration with diethyl-ether. Along with this investigation we are reporting data on two simple coumarins we have isolated from *D. mezereum* whose antioxidant properties we also tested in lipid peroxidation assay.

Experimental

Material and methods

Chemicals

All of the starting materials, standards and solvents were of analytical reagent grade, obtained from commercial sources and used without further purification (unless specified otherwise, all chemicals were purchased from Merck (Darmstadt, 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 Evolution 60 Thermo scientific 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 wild growing *Daphne mezereum* were collected in blossoming phase at Devojački grob, Suva Planina in June 2007. Taxonomic identification and authentication 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 sub-fractioned according to the modified procedures given in Komissarenko et al., 1994 [30] and in 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 × 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 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 was analyzed and confirmed with HPLC-DAD and ^{1}H and ^{13}C NMR. For this purpose, extracts 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-d^{6}, and 0.7 ml

of the solution was transferred into a 5-mm Wilmad, 528-TR-7 NMR tube.

NMR analysis

^{1}H NMR and ^{13}C 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) are given as parts per million (ppm), relative to tetramethylsilane (TMS) as 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 Darmtstadt, Germany) by maintaining column temperature at 30 °C. Mobile phase A was 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 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 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 the LP inhibition in the presence of the tested compounds, was measured by thiobarbituric acid-malondialdehyde (TBA-MDA) assay according to the procedure given in Lazarević et al., 2020 [34].

The absorbance of TBA-MDA complex in the supernatant, measured at 530 nm, was used to calculate the inhibition percentage of lipid peroxidation given by the equation:

Inhibition of lipid peroxidation (%) = 100 x (Ac-As)/(Ac-Ab)

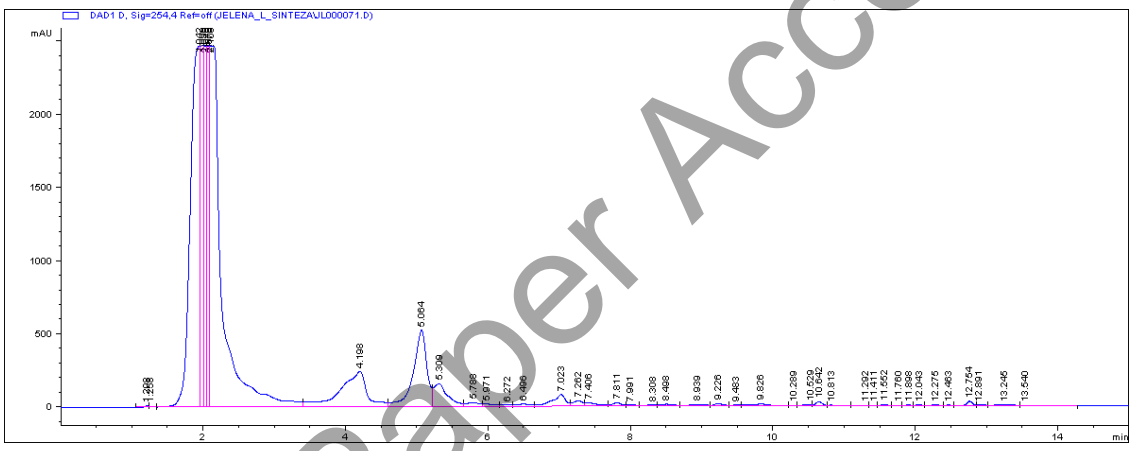
Ac - the absorbance of control (PL90 in methanol treated with the AAPH and TBA solution), As - the absorbance of samples (tested extract/ compounds dissolved in PL90 solution, afterwards treated with the AAPH and TBA solution) and Ab - the absorbance of blank (PL90 in methanol, not treated with AAPH, but with TBA solution).

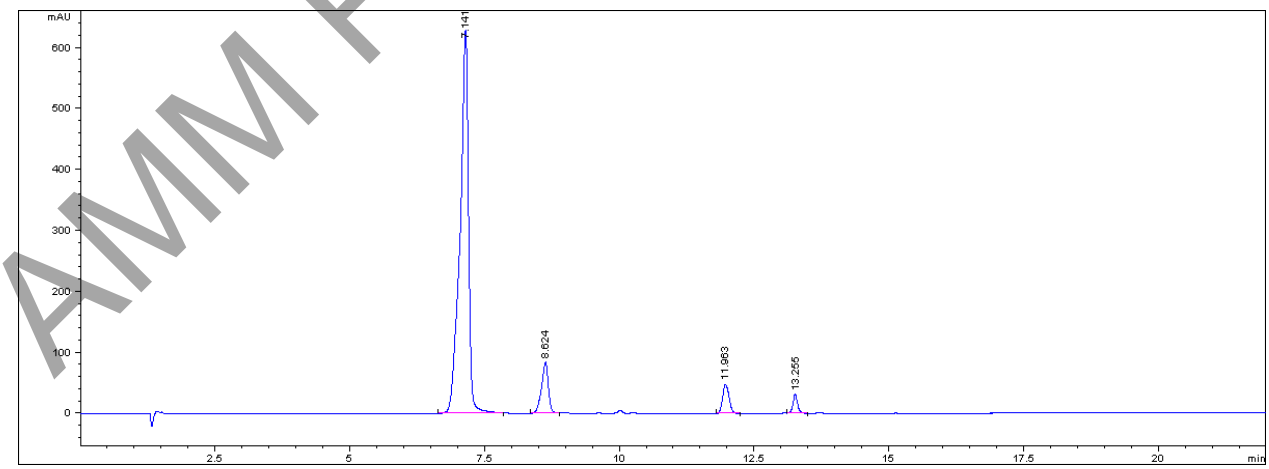
Samples were evaluated for LP-inhibitory activity, and only those showing inhibition greater than 50% at 500 μM were investigated further in a broader concentration range to allow calculation of IC_{50} values. The same type of analysis was done by using either Trolox, quercetin or caffeic acid (frequently used antioxidants) as standards. 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. The measurements were done 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, the most abundant

compound from the chromatogram not be detected since was a mixture of several coeluting compounds. 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 important constituents of *Daphne* species and have been previously reported on many occasions [5, 10, 13, 18, 22, 25-27].





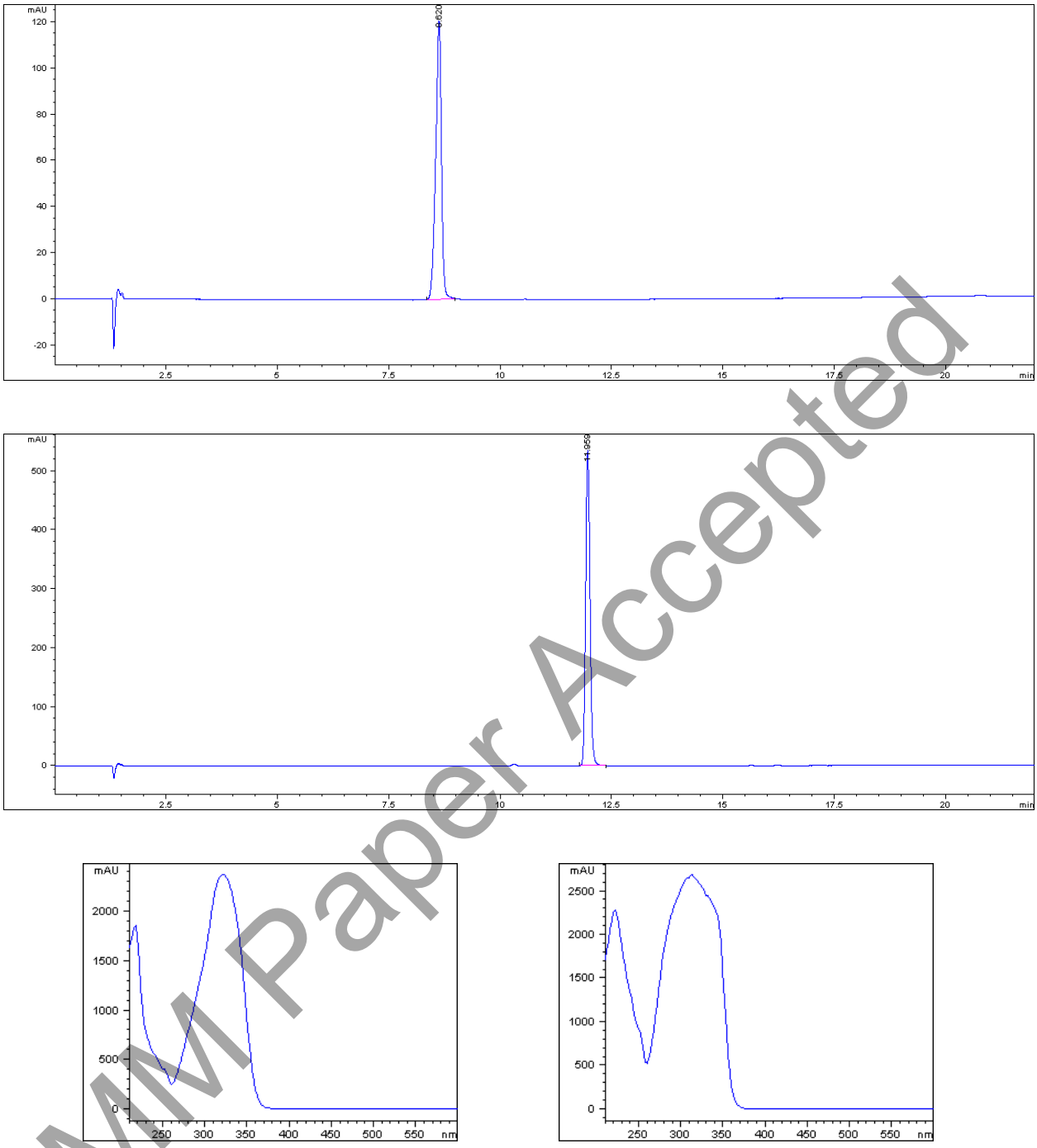


Figure 1. HPLC chromatograms of crude DE extract (***A***), subfractionated water extract (***B***) and of coumarins isolated from *D. mezereum*: umbelliferone (***C***) and herniarin (***D***) and UV and UV spectra of umbelliferone (t_R = 8.62 min) and herniarin (t_R = 11.96 min).

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 ^{1}H 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 ^{1}H and ^{13}C spectra are presented in Figure 2.

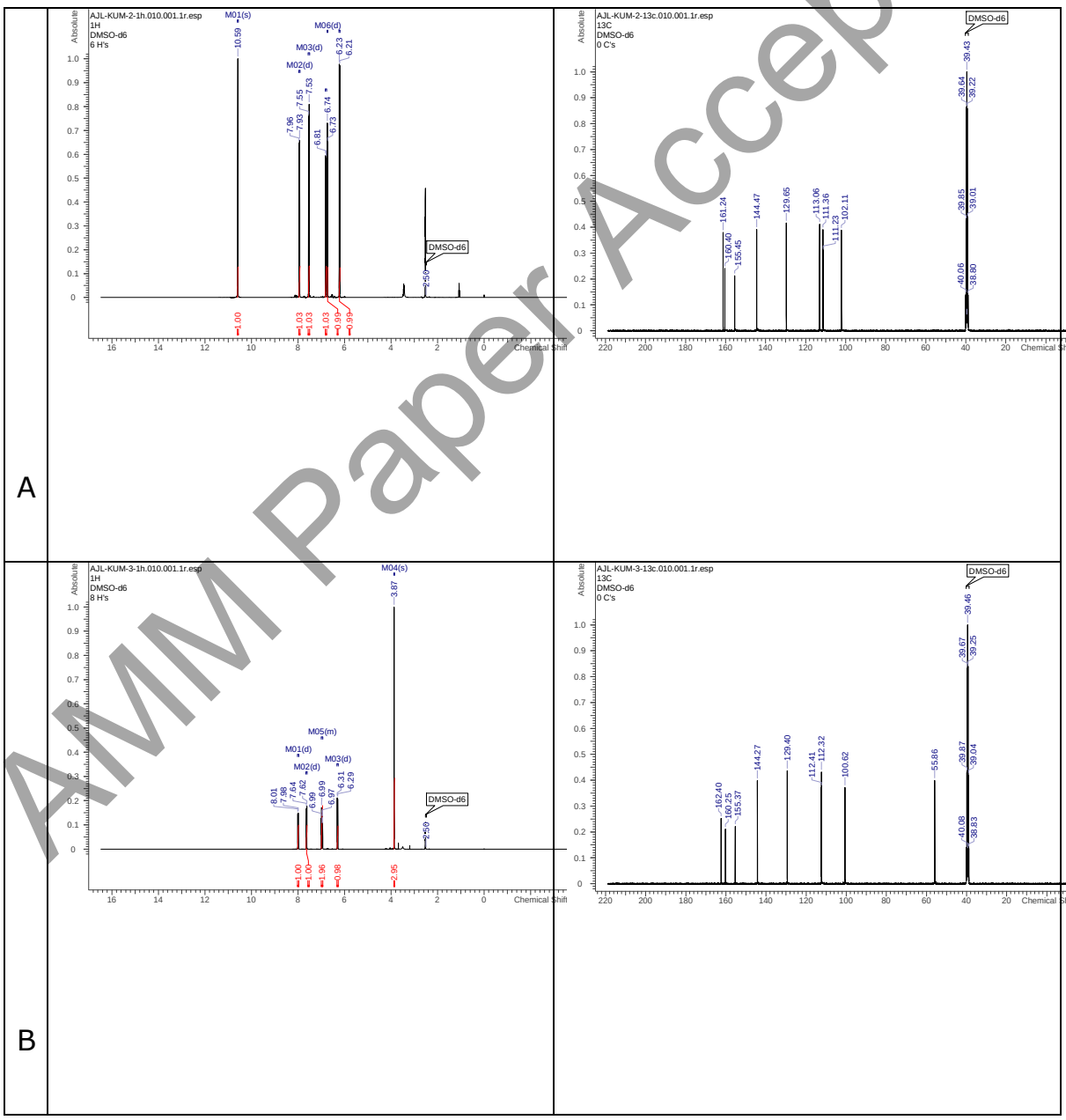


Figure 2. ^{1}H and ^{13}C NMR spectra of umbelliferone (A) and herniarin (B)

Analytical properties for both isolated coumarins are given as follows:

Umbelliferone (7-Hydroxy-2*H*-chromen-2-one): White amorphous solid, $C_9H_6O_3$ (M = 162.14), HPLC purity ≥ 99%, ^{1}H 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): 161.24 (C=O), 160.4 (C_{Ar}), 155.45 (C_{Ar}), 144.47 (C-$_H$), 129.65 (C_{Ar}), 113.06 (C-$_H$), 111.36 (C_{Ar}), 111.23 (C_{Ar}), 102.01 (C_{Ar}).

Herniarin (7-Methoxy-2*H*-1-benzopyran-2-one): White amorphous solid, $C_{10}H_8O_3$ (M = 176.17), HPLC purity ≥ 99%, ^{1}H 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): 162.40 (C=O), 160.25 (CAr), 155.37 (CAr), 144.27 (=C-H), 129.40 (CAr), 112.41 (=C-H), 112.28 (CAr),112.32 (CAr), 100.62 (CAr), 55.86 (C-H).

Lipid peroxidation inhibition effect of the tested samples: diethyl-ether macerate of *D. mezereum* flowers and isolated coumarins: umberlliferone and herniarin, measured using method based on MDA-TBA assay, was notable. After performing experiments, the obtained results were plotted, IC_{50} values were calculated and are reported in Table 1. The obtained results indicated that all samples [*D. mezereum*

flowers DE (IC_{50} = 25.1 ± 2.9 mM) and isolated umberlliferone (IC_{50} = 7.1 ± 2.6 mM) and herniarin (IC_{50} = 19.0 ± 1.3 mM)] exhibited significant potential in LP assay. However, none of the tested samples were as effective as standardly applied antioxidants Trolox (IC_{50} = 22 ± 6 μM), caffeic acid (IC_{50} = 15 ± 3 μM) and quercetin (IC_{50} = 23 ± 6 μM).

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

Compound	**LP inhibition IC_{50} (mM) ± SD**	**Compound**	**LP inhibition IC_{50} (μM) ± SD**
Daphne mezereum flowers' DE	25.1 ± 2.9	trolox	22 ± 6
umbelliferone	7.1 ± 2.6	caffeic acid	15 ± 3
herniarin	19.0 ± 1.3	quercetin	23 ± 6

Preliminary results have shown that *D. mezereum* DE extract, by inhibiting the LP process, have 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].

Conclusion

Epidemiological studies have correlated the ingestion of coumarin based compounds in the diet with beneficial health effect mainly due to their antioxidant activity. Exhibiting antioxidant activity by inhibiting lipid peroxidation, studied coumarins are representing such compounds. Further phytochemical and pharmacological evaluations are needed before shedding further light on the potential application of *D. mezereum*.

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