# HPLC Analysis, antimicrobial and antioxidant activities of *Daphne cneorum* L.

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*Abstract*

The present study describes *in vitro* antimicrobial and antioxidant activities of methanol extracts obtained from the leaves and twigs of the plant *Daphne cneorum* L. The antimicrobial activity of these extracts was tested against human pathogenic microorganisms using a minimum inhibitory concentration (MIC) values. Total phenolics and flavonoids, as well as the antioxidant activity of the extracts were determined. The two tested extracts showed good antimicrobial and antioxidant activities. The results of a high performance liquid chromatographic (HPLC) method showed that 7,8-dihydroxycoumarin is one of the most abundant secondary metabolite in the tested extracts. The results of this study clearly indicated that the extracts of *D. cneorum* could be used as a potential source of natural antioxidant and antimicrobial agents.

*Keywords*: *Daphne cneorum*; HPLC; daphnetin; antimicrobial activity; antioxidant activity.

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The genus *Daphne* (Thymelaeaceae), including about 70 species, is distributed in Europe, Asia, North Africa and Australia. Species *Daphne cneorum* distributed in West, Central and East Europe, Mediterranean region, South–West Asia. In Serbia, *D. cneorum* is located on limestone mountains Rtanj and Suva Planina and serpentinite Raška region [1,2].

*D. cneorum* is an evergreen shrub with long, slender, smooth branches. The leaves of *D. cneorum* are rounded sitting, leathery, stiff, naked, young bright green and then dark green [2].

Previous phytochemicals studies have primarily included species of the genus *Daphne* populated Asia. Only some sort of settlement of Europe and the Middle East, such as *Daphne mezereum*, *Daphne oleides*, *Daphne gnidioides* and *Daphne laureola*, were the subject of phytochemical research. These studies indicate that genus *Daphne* contains a large number of classes of secondary metabolites, dominated by coumarins, flavonoids, lignans, diterpenes and steroids [3–7].

Species of the genus *Daphne* are used in natural medicine as a diuretic, laxative, an anticoagulant, in the treatment against skin diseases, toothache, and malaria. Previous studies of individual species of the genus Daphne indicate their potential broad application in medicine [7–11].

The aim of this study was to provide information on antimicrobial and antioxidant activities of methanol extracts of the leaves and twigs of *D. cneorum* L.

## MATERIAL AND METHODS

### Chemicals used

All chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steinheim, Germany) and Alfa Aesar (Karlsruhe, Germany). 7,8-Dihydroxycoumarin (Sigma-Aldrich) was used as a standard for HPLC analysis.

### Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV–Vis spectrophotometer MA9523-Spekol 211 (Iskra, Horjul, Slovenia).

### Plant material

The plant material was collected from Mt. Suva Planina in Serbia during May 2008. A voucher specimen 5496 has been deposited at the Herbarium Moesiacum Niš in the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Serbia.

### Preparation of the plant extracts

The air-dried plant *Daphne cneorum* (40 g) was broken into small 2–6 mm pieces by a cylindrical crusher,

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and extracted with methanol (180 ml) using a Soxhlet apparatus. The mixture was filtered through filter paper (Whatman, No. 1) and evaporated. The residue (5.8 g) was stored in a dark glass bottle for further processing. The extracts were used for chemical and antimicrobial analysis.

**Determination of total phenolic content**

Total phenols were estimated according to the Folin–Ciocalteu method [12]. The extract was diluted to the concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7.5%). After 15 min at 45 °C, the absorbance was measured at 765 nm using a spectrophotometer against a blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract), and the values are presented as means of triplicate analyses.

**Determination of flavonoid content**

Total flavonoids were determined according to known procedure [13]. A total of 0.5 ml of 2% aluminum chloride ($AlCl_3$) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 h of staying at room temperature, the absorbance was measured at 415 nm in a spectrophotometer against the blank sample. Total flavonoids were determined as rutin equivalents (mg RU/g dry extract), and the values are presented as means of triplicate analyses.

**HPLC Analysis**

High-performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1200 Series HPLC instrument with: C18 column (C18; 25 cm×4.6 mm, 10 m) and UV spectophotometric detector as described previously in the literature [14]. Acetonitrile–water––phosphoric acid (90:10:0.1, v/v/v) was used as solvent system. Acetonitrile was of HPLC grade, and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The sample injection volume was 10 µl. The flow rate was 1.0 ml/min. 7,8-Dihydroxycoumarin was identified on the base of its retention time and absorption spectrum (200–400 nm) and by comparison of these results with those of standard.

**Test microorganisms**

The antimicrobial activity of the plant extracts were tested *in vitro* against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The fungi were cultured on potato-glucose agar for 7 days at room temperature of 20 °C under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

**Minimum inhibitory concentration (MIC)**

Minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined by microdilution method in 96 multi-well microtiter plates [15]. All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µl stock solutions of extract (in methanol, 200 µl/ml) and cirsimarin (in 10% DMSO, 2 mg/ml) were pipetted into the first row of the plate. 50 µl of Mueller–Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of extract) was added to the other wells. A volume of 50 µl from the first test wells was pipetted into the second well of each microtiter line, and then 50 µl of scalar dilution was transferred from the second to the twelfth well. 10 µl of resazurin indicator solution (prepared by dissolution of a 270 mg tablet in 40 ml of sterile distilled water) and 30 µl of nutrient broth were added to each well. Finally, 10 µl of bacterial suspension ($10^6$ CFU/ml) and yeast spore suspension ($3\times10^4$ CFU /ml) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, where as ketoconazole was used as control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

**Antioxidant activity**

*Determination of total antioxidant capacity.* The total antioxidant activity of the *D. cneorum* extract was evaluated by the phosphomolybdenum method [16]. The assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds and subsequent for-

mation of a green phosphate/Mo (V) complex at pH below 7. A total of 0.3 ml of sample extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a spectrophotometer against the blank after cooling to room temperature. Methanol (0.3 ml) in place of extract was used as the blank. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry extract.

*Determination of DPPH free radical scavenging activity*. The method used by Takao *et al.* [17] was adapted with suitable modifications from Kumarasamy *et al.* [18]. DPPH (2,2-diphenyl-1-picrylhydrazyl) (8 mg) was dissolved in methanol (100 ml) to obtain a concentration of 80 μg/ml. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extract. Solutions (2 ml each) were then mixed with DPPH (2 ml) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in methanol to make the stock solution with the same concentration (1 mg/ml). Control sample was prepared containing the same volume without test compounds or reference antioxidants. 95% Methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated using Eq. (1), where *Ac* is the absorbance of control solution and *As* is the absorbance of the sample solution:

$$\text{Inhibition} = 100(Ac - As)/Ac \quad (1)$$

The $IC_{50}$ value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as μg/ml through a sigmoidal dose-response curve.

*Determination of inhibitory activity against lipid peroxidation.* Antioxidant activity was determined by the thiocyanate method [19]. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extracts, and 0.5 ml of each solution was added to linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 ml 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 ml with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 72 h, a 0.1 ml aliquot of the reaction solution was mixed with 4.7 ml of ethanol (75%), 0.1 ml $FeCl_2$ (20 mM) and 0.1 ml ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using Eq. (1), where *Ac* is the absorbance of control solution and As is the absorbance of the sample solution:

*Determination of hydroxyl radical scavenging activity.* The ability of *Daphne cneorum* to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according to the method described by Hinneburg *et al.* [20]. The reaction mixture contained 100 μl of extract dissolved in water, 500 μl of 5.6 mM 2-deoxy-D-ribose in $KH_2PO_4$–NaOH buffer (50 mM, pH 7.4), 200 μl of premixed 100 μM $FeCl_3$ and 104 mM EDTA (1:1, v/v) solution, 100 μl of 1.0 mM $H_2O_2$ and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50 °C for 30 min. Thereafter, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (*Ac*) and of the sample (*As*), where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

### Statistical analysis

The results are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using Student's *t*-test and one way analysis of variance. Multiple comparisons of means were performed by LSD (least significant difference) test. A probability value of 0.05 was considered significant. All computations were made by employing the statistical software (SPSS, version 11.0). $IC_{50}$ values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve.

## RESULTS AND DISCUSSION

### HPLC Analysis

Figure 1 shows HPLC chromatograms of the methanol extract of the leaves of *Daphne cneorum* at wavelength detection of 254 and 320 nm, respectively. Acetonitrile–water–phosphoric acid (90:10:0.1, v/v/v) was used as the mobile phase for HPLC analysis because it provides excellent separation of peaks in chromatograms. As can be seen from the chromatogram, one of the most abundant metabolites in the methanol extract of the leaves of *D. cneorum* is 7,8-dihydroxy-

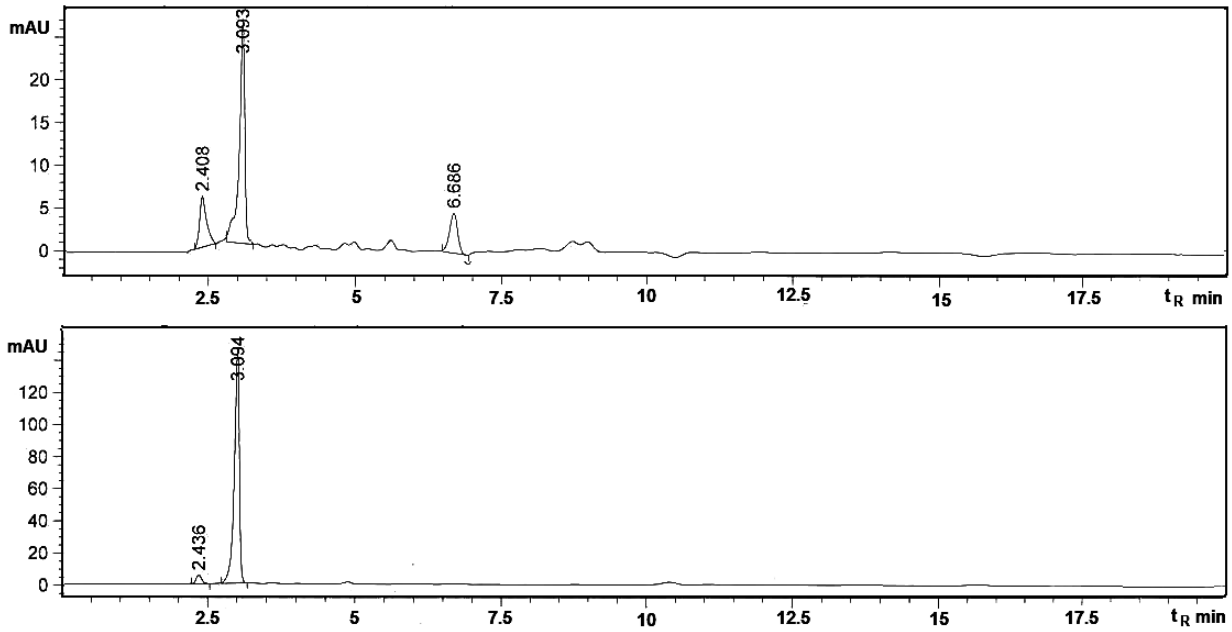


*Figure 1. HPLC Chromatograms of the methanol extract of the leaves of Daphne cneorum at wavelength detection at 254 and 320 nm, respectively.*

coumarin with retention time $t_R$ = 3.09 min. Its presence was confirmed by comparing of the retention time and UV spectrum with the standard. This compound has previously been found in some Daphne species and is known under the name daphnetin [21].

Figure 2 shows the HPLC chromatograms of the methanol extract of twigs of *D. cneorum* from which it could be seen that daphnetin ($t_R$ = 3.09 min) is one of the major secondary metabolite, as well as in the case of the leaves extract. The HPLC chromatogram and UV spectrum of standard (with absorbance maxima: 204, 267 and 325 nm) are shown in Figure 3.

**Antimicrobial activity**

The results presented in Table 1 reveal antimicrobial activity of the methanol extracts of *D. cneorum* within the concentration range from 15.62 to 62.50 μg/ml. The highest susceptibility to the methanol extract of *D. cneorum* branches among the bacteria tested was exhibited by *P. vulgaris* ATCC 13315 (MIC = 15.62

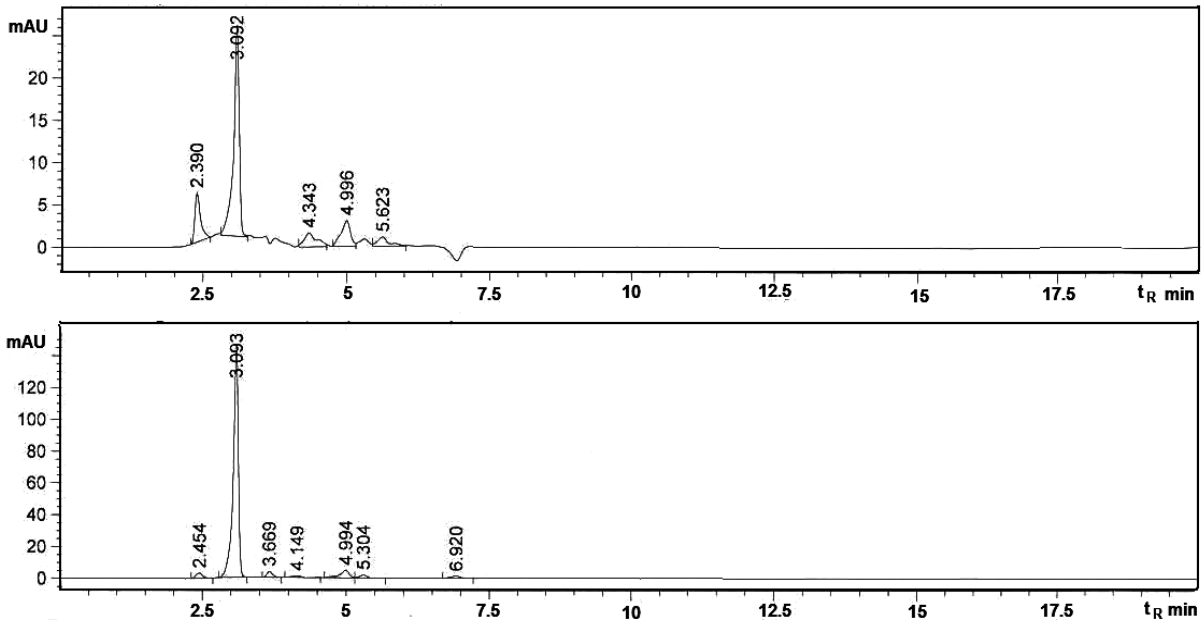


*Figure 2. HPLC Chromatograms of the methanol extract of twigs of Daphne cneorum at wavelength detection at 254 and 320 nm, respectively.*

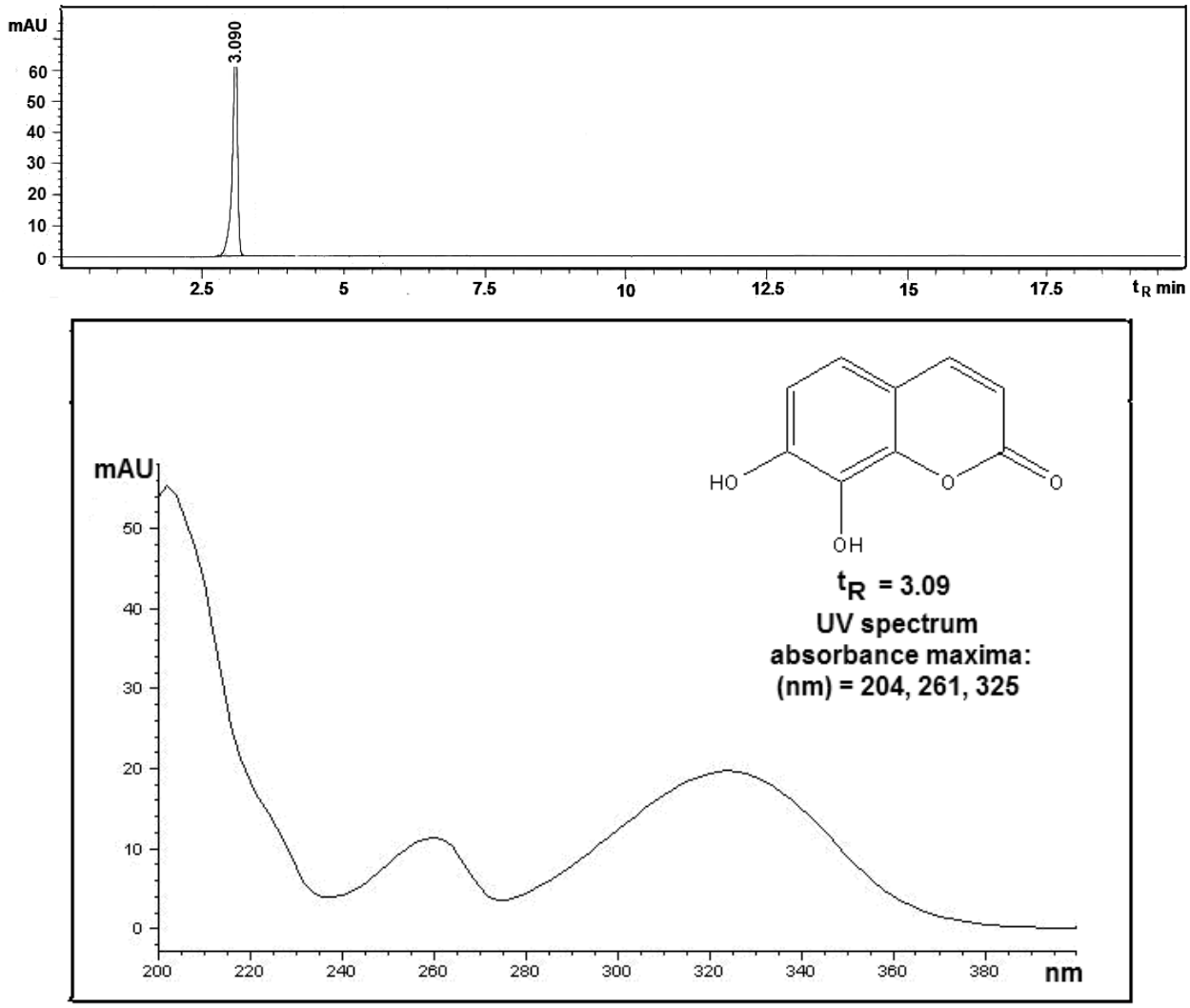


*Figure 3. HPLC Chromatogram and UV spectrum of daphnetin ($t_R$ = 3.09 min).*

μg/ml), followed by strains of *B. subtilis* ATCC 6633 and *K. pneumoniae* ATCC 13883 (MIC = 31.25 μg/ml) and *P. mirabilis* ATCC 14153*, S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (MIC = 62.5 μg/ml). Among the fungi, *C. albicans* ATCC 10231 (MIC =31.25 μg/ml) showed the highest susceptibility. On the other hand, *A. niger* ATCC 16404 (MIC = 62.5 μg/ml) demonstrated the lowest susceptibility.

The highest susceptibility to the methanol extract of *D. cneorum* newspaper among the bacteria tested was exhibited by *B. subtilis* ATCC 6633 (MIC = 15.62 μg/ml), followed by strains of *S. aureus* ATCC 25923*, E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 (MIC = = 31.25 μg/ml) and *P. mirabilis* ATCC 14153, *P. vulgaris* ATCC 13315 (MIC = 62.5 μg/ml). Among the fungi, *C. albicans* ATCC 10231 (MIC = 15.62 μg/ml) showed the

*Table 1. Minimum inhibitory concentrations, μg/ml, of the methanol extract of Daphne cneorum*

| Microorganism | Methanol extract of leaves | Methanol extract of twigs | Amracin | Ketokonazol |
|---|---|---|---|---|
| *Staphylococcus aureus ATCC 25923* | 62.5 | 31.25 | 0.98 | Not tested |
| *Klebsiella pneumoniae ATCC 13883* | 31.25 | 31.25 | 0.49 | Not tested |
| *Escherichia coli ATCC 25922* | 62.5 | 31.25 | 0.98 | Not tested |
| *Proteus vulgaris ATCC 13315* | 15.62 | 6.,5 | 1.95 | Not tested |
| *Proteus mirabilis ATCC 14153* | 62.5 | 6.5 | 1.95 | Not tested |
| *Bacillus subtilis ATCC 6633* | 31.25 | 15.62 | 0.24 | Not tested |
| *Candida albicans ATCC 10231* | 31.25 | 15.62 | 0.98 | Not tested |
| *Aspergillus niger ATCC 16404* | 62.5 | 62.5 | Not tested | 0.98 |

highest, while *A. niger* ATCC 16404 (MIC = 62.5 μg/ml) exhibited the lowest susceptibility.

**Antioxidant activity**

Phenolic compounds and flavonoids have been reported to be associated with antioxidant action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [22]. One of the more prominent properties of flavonoids is their excellent radical scavenging ability. It is also a valuable aspect for therapeutic and prophylactic applications of flavonoids [23]. The results on total phenolic, flavonoid and total antioxidant capacity of the methanol extracts of leaves and twigs of *D. cneorum* are given in Table 2. Total phenolic contents were determined and amounted to 76.45±0.79 and 69.67±0.85 mg GA/g, for leaves and twigs, respectively. The contents of flavonoids were determined and amounted to 24.67±0.35 and 34.23± ±0.89mg RU/g, for leaves and twigs, respectively.

Antioxidant activity of the methanol extracts was evaluated using the DPPH and hydroxy radical scavenging, lipid peroxidation and metal chelating assays. The results (Table 3) of the antioxidant activity were compared with control antioxidants, ascorbic acid, gallic acid, *α*-tocopherol and BHT. The results showed that *D. cneorum* extracts possess antioxidant activity, with total antioxidant capacity of 70.55±0.32 and 70.98±0.35 μg AA/g for leaves and twigs, respectively, and $IC_{50}$ values of 22.79±0.94 and 24.57±0.95 μg/ml for DPPH free radical scavenging activity, 37.17±1.2 and 35.24± ±0.55 μg/ml for inhibitory activity against lipid peroxidation, 96.56±0.89 and 96.45±0.79 μg/ml for hydroxyl radical scavenging activity and 42.79±0.98 and 44.57± ±1.35 μg/ml for metal chelating activity, for leaves and twigs, respectively.

**CONCLUSION**

Insensitive research for new, unexplored, plant antioxidant sources is very significant and can bring new natural products in pharmaceutical and food industry for their every day battle with reactive oxygen species. Finding new antioxidant sources could be important for health benefits considering many diseases that reactive oxygen species induce in biological systems. Discovering a natural source of antioxidants could also be significant for artificial toxic antioxidants replacement in food industry. The results of this study clearly indicated that methanol plant extracts are good scavengers of synthetic DPPH radicals, indicating that they could be used as antioxidant products. Also, they all possess reductive capabilities. The reductive capacities of the investigated extracts are lower than those of the investigated standard antioxidant compounds, so they could be considered primarily as antioxidants and then as products with reductive capabilities. All these characteristics uphold the use of supplements made this day in everyday free radical damage protection and prevention.

The methanol extracts of the leaves and twigs of *D. cneorum* exhibited good antimicrobial and antioxidant activities. HPLC–UV analysis showed that these two extracts contained 7,8-dihydroxycoumarin as one of the most abundant secondary metabolites. The activity of these extracts is probably derived from a coumarin derivative, a compound that has previously been found to possess various types of biological activity [24]. The results provided evidence that the studied plant might indeed be a potential source of natural antioxidant and antimicrobial agents.

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*Table 2. Total phenolics, flavonoids and total antioxidant capacity of the methanol extracts of Daphne cneorum*

| Extract | Total phenolics, mg GA/g | Flavonoids, mg RU/g | Total antioxidant capacity, μg AA/g |
|---|---|---|---|
| Methanol extract of leaves | 76.45±0.79 | 24.67±0.35 | 70.55±0.32 |
| Methanol extract of twigs | 69.67±0.85 | 34.23±0.89 | 70.98±0.35 |

*Table 3. Antioxidant activity ( $IC_{50}$ / μg $ml^{-1}$)of the Daphne cneorum extracts tested and standards*

| Extract and standard | DPPH scavenging activity | Inhibitory activity against lipid peroxidation | Metal chelating activity | Hydroxyl radical scavenging activity |
|---|---|---|---|---|
| Methanol extract of leaves | 22.79±0.94 | 37.17±1.23 | 42.79±0.98 | 96.56±0.89 |
| Methanol extract of twigs | 24.57±0.95 | 35.24±0.55 | 44.57±1.35 | 96.45±0.79 |
| Gallic acid | 3.79±0.69 | 255.43±11.68 | Not tested | 59.14±1.10 |
| Ascorbic acid | 6.05±0.34 | > 1000 | Not tested | 160.55±2.31 |
| BHT | 15.61±1.26 | 1.00±0.23 | Not tested | 33.92±0.79 |
| *α*-Tocopherol | Not tested | 0.48±0.05 | Not tested | Not tested |

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## IZVOD

### HPLC ANALIZA, ANTIMIKROBNA I ANTIOKSIDANTNA AKTIVNOST *Daphne cneorum* L.

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Ovaj rad prikazuje *in vitro* istraživanje antimikrobne i antioksidantne aktivnosti metanolskih ekstrakata lišća i grančica biljne vrste *Daphne cneorum* L. Antimikrobna aktivnost ovih ekstrakata testirana je na patogenim mikroorganizmima i određene su vrednosti minimalne inhibitorne koncentracije (MIC). Takođe je određen ukupan sadržaj fenola i flavonoida, kao i antioksidantna svojstva ekstrakata. Testirani ekstrakti ispoljili su dobru antimikrobnu i antioksidantnu aktivnost. Rezultati analize visokoefikasnom tečnom hromatografijom (HPLC) pokazali su da je 7,8-dihidroksikumarin jedan od najzastupljenijih sekundarnih metabolita u oba testirana ekstrakta ove vrste. Rezulatati ove studije jasno ukazuju da ekstrakati biljke *D. cneorum* mogu biti korišćeni kao potencijali izvori prirodnih antioksidanasa i antimikrobnih agensa.

*Ključne reči*: *Daphne cneorum* • HPLC • Dafnetin • Antioksidantna aktivnost • Antimikrobna aktivnost