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Original Research Article

Phytochemical, Antioxidant and Antimicrobial Profiles of Extracts of *Daphne alpina* (Thymelaeaceae) L Leaf and Twig from Mt Kopaonik (Serbia)

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Abstract

Purpose: *To investigate the phytochemical composition, as well as antioxidant and antimicrobial activities of the leaf and twig extracts of Daphne alpina L. (Thymelaeaceae).*
Methods: *The dry chloroform and methanol extracts of the leaf and twigs of Daphne alpina were used for analysis. Total phenolic and flavonoid contents were determined by established procedures. Antioxidant potential was investigated by several methods. The antimicrobial properties of the extracts were obtained by microdilution method. High performance liquid chromatography (HPLC) was employed for the identification of the most abundant metabolites, present in D. alpina extracts.*
Results: *The total phenolics of the extracts ranged from 78.98 to 88.98 mg GA/g while total flavonoids were in the range 28.09 to 34.65 mg GA/g of fresh weight. HPLC analysis of the extracts showed the presence 4-hydroxybenzoic acid, 7,8-dihydroxycoumarine and 7-hydroxycoumarine. Total antioxidant capacity ranged from 69.71 µg AA/g for the methanol leaf extract to 73.55 µg AA/g for the chloroform twig extract. All the extracts showed DPPH radical scavenging activity (21.57 - 25.45 µg/mL), inhibitory activity against lipid peroxidation (26.79 - 35.24 µg/mL), ferrous ion chelating ability (21.57 - 45.45 µg/ml) and hydroxyl radical scavenging activity (87.98 - 98.86 µg/mL). Minimum inhibitory concentration (MIC) was in the range 15.62 - 125 µg/mL.*
Conclusion: *The extracts possess moderate antioxidant and antimicrobial activities due probably to the phenolic compounds in the extracts.*

Keywords: *Daphne alpina, Coumarines, 4-Hydroxybenzoic acid, Phenols, Flavonoids, Antimicrobial activity, Antioxidant activity*

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INTRODUCTION

Daphne alpina L is an erect, deciduous small, generally calcicolous shrub of the mountains of southern and central Europe [1]. The relict species *Daphne alpina* L belongs to the family Thymelaeaceae section Daphnanthes C.A. Mayer, subsection *Alpinae* [2]. The first compound to be isolated from *D. alpina* was coumarin daphnin (8-hydroxy-7-β-D-glucosyloxycoumarin) by Vauquelin 1812 [3]. So far, phytochemical analysis has been carried out on the species from the Far East, namely, *D. odora, D. tangutica, D. cannabina, D. genkwa, D. acuminata* and *D. papyracea*. Of the European species of the genus Daphne, the most numerous data are for the following species: *D.*

mezereum, D. oleoides, D. gnidium, D. laureola, D. cneorum and partially *D. malyana* [4].

Analysis of the chemical characteristics of this genus show that plants of the genus Daphne contains many classes of secondary metabolites, some of which are dominated coumarins, flavonoids, lignans, and steroids [5-11]. Daphne species is used for the treatment of skin diseases, malaria, toothache, and also in traditional medicine as a purgative, anticoagulant and diuretic. The bark and fruits of *D. mezerum* in Europe is used for treatment of ulcers, rheumatism and as a purgative. A tincture of *D. mezereum* is used to treat skin diseases such as herpes and dermatitis [7]. About 40 years ago, it was found that mezerein of the species *D. mezereum* has antileukemic activity in the P - 388 mouse lymphocytes [12]. Extracts of leaf and twigs of Daphne species showed antibacterial activity [9,10,13-15]. However, to date there is still no large number of studies that suggest a wider application of biologically active compounds of this genus in medicine [16]. Continuing our investigation of Daphne species, we relate here the results of the phytochemical analysis (total phenols, flavonoid content, HPLC-UV) of leaf and twigs of *Daphne alpina*.

EXPERIMENTAL

Plant materials

The mature leaf and twigs of *Daphne alpina* L was collected from Mt Kopaonik (Oštri krš-Gobelja), Serbia (UTM DN 97) in July 2007. A sample of the plant was deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, Serbia (HMN 5506).

Preparation of extracts

Dried and powdered leaf and twigs of *D. alpina* (2 x 100 g) were extracted in Soxhlet extractor separately with 500 mL chloroform and methanol for 6 h. The chloroform and acetone extracts were than filtered and dried in rotary vacuum evaporator at 45 ^{o}C: The dry extracts were used for chemical analysis as well as determination of antimicrobial and antioxidant activities.

HPLC (high performance liquid chromatography) analysis of *D. alpina* extracts

The chloroform and acetone extracts and standards (500 µL acetone) subjected to High performance liquid chromatography (HPLC) analysis (Agilant Technologies 1200 Series HPLC instrument). The column C18 (25 cm × 4.6 mm, length of column, 10 m) and UV spectrophotometric detector was used. As eluent, methanol-water-phosphoroc acid (85:15: 0.9, v/v/v) was used at a flow-rate of 1 ml/min and the sample injection volume of 10 µl. Phosphoric acid was of analytical grade. HPLC grade methanol (E.Merck, Darmstadt, Germany) was used for the HPLC analysis. Deionized water is purchased from Milli-Q academic water purification system (Milford, MA, USA). Standards used in the study (daphnetin, 4–hydroxybenzoic acid and umbeliferone) were purified from Sigma –Aldrich (USA).

Microorganisms used in evaluation antimicrobial activity

The antimicrobial effect of the extracts was determined *in vitro* toward the standard microbes strains, including bacteria such as *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC13883, *Proteus mirabilis* ATCC14153, *Proteus vulgaris* ATCC13315, *Bacillus subtilis* ATCC6633 and fungi *Aspergillus niger* ATCC16404 and *Candida albicans* ATCC10231). The bacteria were cultured on agar for 7 days at room temperature of 25 ºC under alternating light and dark conditions. They were recultured on a new agar substrate for another 5 days. Fungal strains were grown on potato-glucose agar at room condition involving temperature of 20 ºC, intermittently light and dark condition during for 7 days. Subculturing of the fungi is carried out for another 7 days using the new potato-glucose medium. The cultivation procedure of tested microorganisms was repeated four times to obtain pure culture. The microorganisms that were used were confirmed to Department of Microbiology, Institute Torlak, Belgrade, Serbia.

Measurement of minimum inhibitory concentration (MIC) of *D. alpina* extracts

MIC of the crude extracts was performed by microdilution method using the 96 multi-well microtiter plates [17]. All testing of bacterial and fungal cultures were performed in Mueller–Hinton broth and Sabouraud dextrose broth, respectively. In the first row of the plate was applied a volume of 100 µL methanol stock solutions of *D. alpina* extracts (200 µg/mL) and a solution of cirsimarin (2 mg/mL) in 10 % DMSO. In other wells was added 50 µL of Mueller Hinton or Sabouraud dextrose broth with addition a Tween 80 to the total concentration of 0.5 % (v/v) for analysis of extracts. From the first row test wells was transferred volume of 50 µL into the second row wells. Thereafter, from the second to

the twelfth well a volume of 50 µL of scalar dilution was transferred. Then, to each well was added 10 µL of indicator solution (prepared by dissolving resazurin in sterile distilled water) and 30 µL of nutrient broth. Finally, to each well was added 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL). Amracin (tetracycline hydrochloride) and ketoconazole were used as positive control for the test bacteria and yeast, respectively. The plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated. The plates were prepared in triplicate and incubated at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast. Mean MIC (n = 3) for the test extracts and standard drug were taken.

Determination of the total phenolic and flavonoid contents

The total phenolic content of the chloroform and acetone extracts were determined spectrophotometrically using the Folin-Ciocalteu method [18]. The absorbance was read at 765 nm using spectrophotometer. Total phenolic content was expressed as mg gallic acid equivalents per gram of crude extract (mg GA/g extract). Total flavonoid content of the tested extracts was determined following a previously reported method [19]. Total flavonoid content was expressed as mg rutin equivalents per gram of extract (mg RU/g extract).

Phytochemical screening of the tested extracts

Phytochemical screening of the tested extracts for the presence of coumarins and flavonoids, was carried out.

Test for coumarins: 300 mg of the extracts was covered with filter paper moistened with 1 M NaOH in a small test tube. The test tube was placed for few minutes in a boiling water bath and after removing the filter paper it was examined under UV light. Presence of coumarines was confirmed by yellow florescence. All tested extracts (CHLT, CHLL, METT and METL) gave positive test for coumarines.

Test for flavonoids: 50 mg of the extracts was suspended in 100 ml of distilled water to get the filtrate. 5 ml of dilute ammonia solution was added to 10 ml of filtrate followed by few drops of concentrated H_2SO_4. Yellow colouration indicated the presence of flavonoids. All tested extracts (CHLT, CHLL, METT and METL) gave positive test for flavonoids.

Determination of total antioxidant capacity

A spectrophotometric method, based on the reduction of Mo (VI) to Mo (V), applied to assessment the total antioxidant activity of D. alpina extracts [20]. The solution of tested extracts in methanol was cooled to room temperature and absorbance was measured at 695 nm against the blank. As a blank test was used methanol in the absence of extract. Ascorbic acid (AA) was used as the positive control and total antioxidant capacity was expressed as ascorbic acid equivalents (mg AA/g crude extract).

Determination of antioxidant activity by DPPH assay

Diphenyl - picryl-hydrazyl assay described by Takao with modifications from Kumarasamy was used to estimate DPPH radical scavenging ability of *D. alpina* extracts [21,22]. Stock solution of the reference antioxidants was prepared by dissolving the substance in methanol (in the same concentrations). For reference standards, gallic acid (GA), butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used while methanol was used as blank. The percentage inhibition of the DPPH radical was calculated as in Eq 1.

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

where Ac and As are the absorbance of control and test samples, respectively

The concentration of extract that reduces absorbance of DPPH by 50 % defined as the IC_{50} value. IC_{50} was calculated graphically from sigmoidal dose-response curve and expressed in µg/mL.

Peroxy radical scavenging activity

The thiocyanate method used to determine antioxidant activity of tested extracts [20]. As reference compounds were used gallic acid (GA), ascorbic acid (AA), butylated hydroxytoluene (BHT) and α-tocopherol. 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 to eliminate the solvent effect. The percent of inhibition of linoleic acid peroxidation was calculated using the Eq. 1.

Measurement of ferrous ion chelating effect

The ferrous ion chelating ability was determined by measuring the decrease in absorbance at 562 nm as a result of the direct reduction of Fe^{3+} to Fe^{2+} [23,24]. Increased absorbance of the reaction mixture indicates grater reduction capability. The percentage ferrous ion chelating ability of the extracts was calculated relative to the control and express by the following Eq.1.

Measurement of hydroxyl radical scavenging activity

Hydroxil radical scavenging activity of the chloroform and methanol extracts was determined following a previously reported method [25]. The absorbance of the solution at 532 nm was obtained to determine extent of oxidation of 2-deoxyribose. 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 presented values are mean values of three measurements.

Statistical analysis

All analyses were carried out in triplicates and the data presented as mean ± standard deviation (SD). IC_{50} values were obtained by nonlinear regression analysis from the sigmoidal dose-response inhibition curve. Statistical differences were analyzed using one-way ANOVA, followed by Tukey`s HSD post hoc comparison test; $p < 0.05$ was regarded as significant. The statistical software, SYSTAT 12 (Systat Software, Inc. 2007), was used for all computations.

RESULTS

Total phenolic and total flavonoid contents

Total phenolic and total flavonoid contents of the chloroform and methanol extracts are shown in Table 1. The total phenolic contents varied over the range 78.98-88.98 mgGA/g, among the tested extracts. The highest total phenolic contents (88.98 mgGA/g extract) was observed for METT. The otal flavonoid content ranged over 28.09-34.65 mg RU/g extract. The highest total flavonoid content was recorded for CHLT, while the lowest was for CHLL. The content of total phenolic and flavonoids of the chloroform and methanol extracts of *D. alpina* was statistical different, while recorded differences were not significant between CHLT/CHLL and METT/METL.

Chromatographic data for *D. alpina* extracts

The HPLC chromatograms for standards (daphnetin, 4–hydroxybenzoic acid and umbeliferone) and extracts of *D. alpina* are shown in Figures 1 - 3.

Table 1: Total phenolic and total flavonoid content and total antioxidant capacity of the chloroform and methanol extracts of *D. alpina*

***D. alpina* extract**	**Total phenolic content (mg GA/g)**	**Total flavonoids (mg RU/g)**	**Total antioxidant capacity (µg AA/g)**
CHLT	80.56 ± 0.35	34.65 ± 0.89	73.55 ± 1.02
CHLL	78.98 ± 0.67	28.09 ± 0.85	70.01 ± 0.54
METT	88.98 ± 1.05	31.45 ± 0.15	71.11 ± 1.12
METL	85.88 ± 0.97	32.65 ± 0.89	69.71 ± 0.54
	***P*-value**		
ANOVA	*	*	*
Tukey's HSD test			
CHLT/CHLL	n.s.	*	*
CHLT/METT	*	*	*
CHLT/METL	*	*	n.s.
CHLL/METT	*	*	n.s.
CHLL/METL	*	*	n.s.
METT/METL	*	n.s.	n.s.

Values are the mean ± SD. Data were analysed by analyses of variance (ANOVA) procedure and Tukey`s HSD post hoc comparison test (p < 0.05; n.s. not significant). CHLT-chloroform extract of twigs; CHLL- chloroform extract of leafs; METT-methanol extract of twigs; METL-methanol extract of leaf*

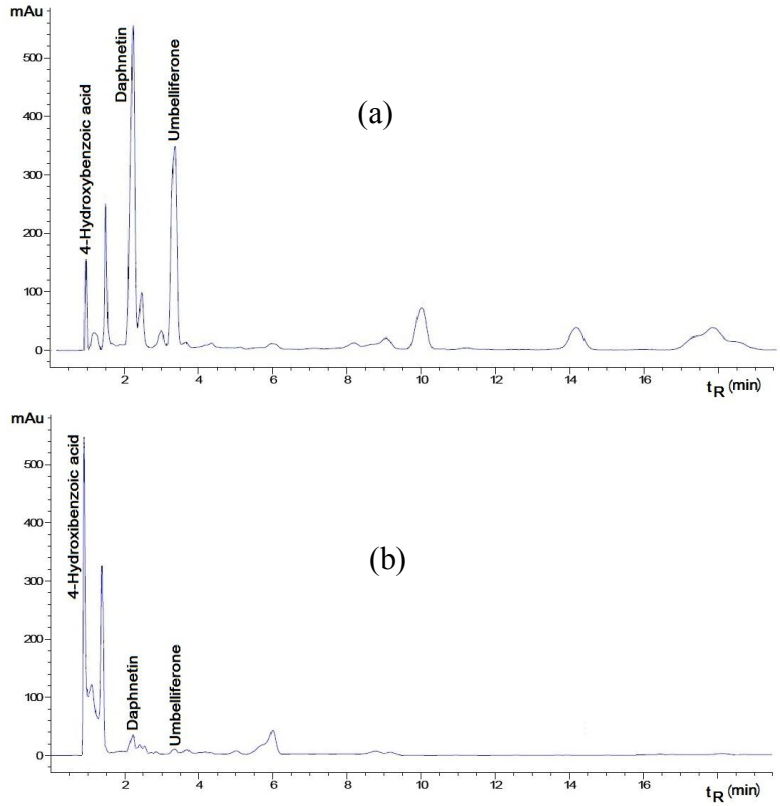

Figure 1: HPLC chromatogram of the *D. alpina* twigs at 325nm. *Note:* a) chloroform extract; b) methanol extract

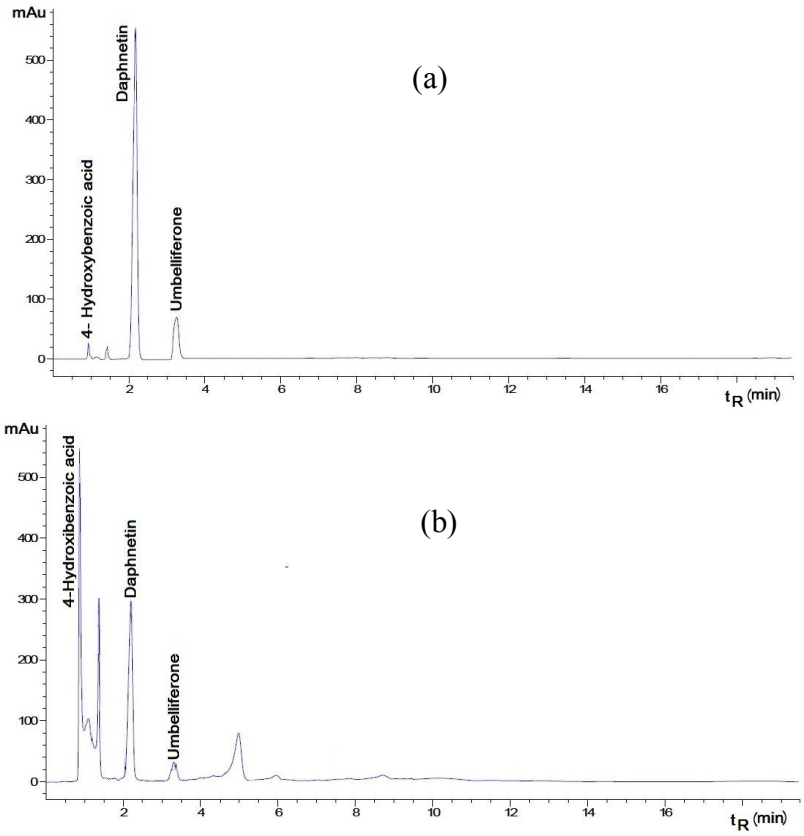

Figure 2: HPLC chromatogram of *D. alpina* leaf at 325nm
a) chloroform extract; b) methanol extract

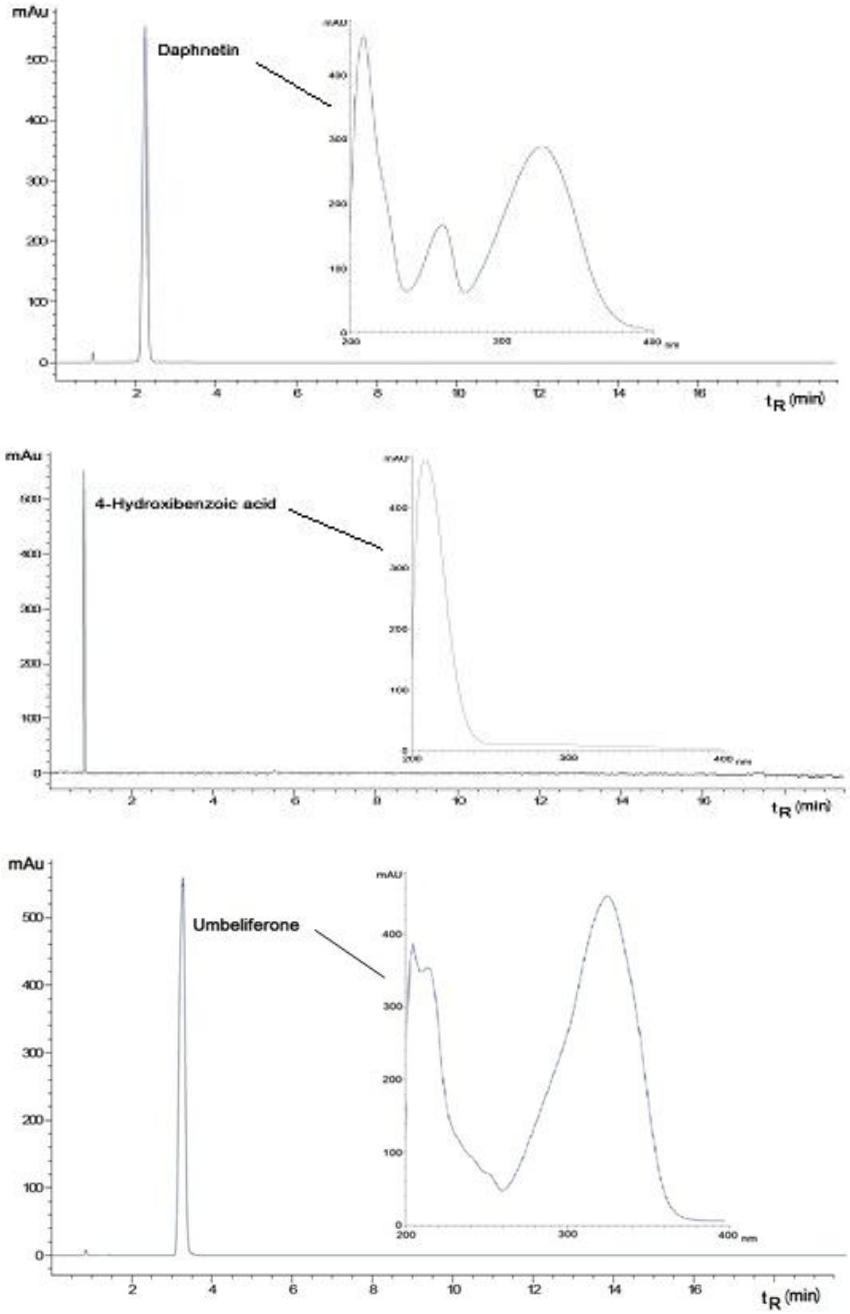

Figure 3: HPLC chromatograms and UV spectra of standards

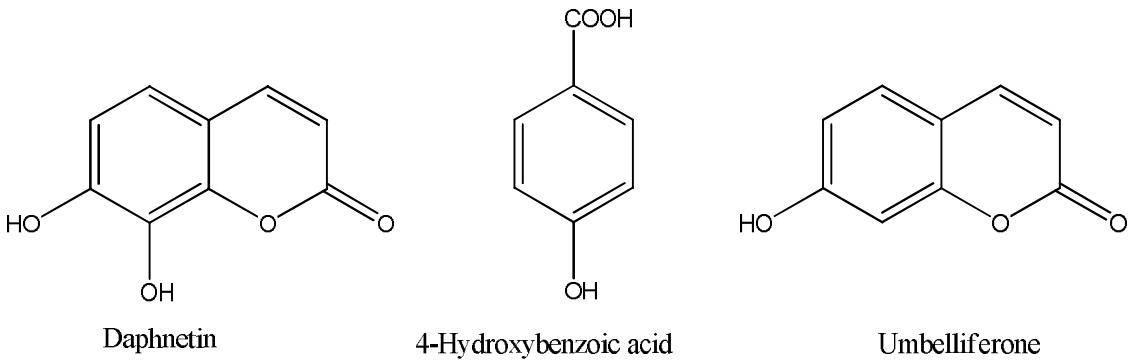

Figure 4: The structures of the identified compounds from HPLC chromatograms of *D. alpina* extracts

As it can be seen in the both the chromatograms and UV spectra, there were present of 4-hydroxybenzoic acid (t_R = 0.96 ± 0.10 min), and two hydroxylated derivative of coumarin, 7, 8 - dihydroxycoumarine (daphnetin, t_R = 2.25 ± 0.10 min) and 7-hydroxycoumarine (umbelliferone, t_R = 2.63 ± 0.10 min), as the most represented compounds in the tested extracts. Identification of the above-mentioned substances was achieved on the basic of their retention times (t_R) which was compared with the corresponding values for the standard substances. The UV absorbance maxima (from 200-400 nm) also corresponded with the values of standards. Their structures were shown in Fig 4.

Daphnetin has the most intense peaks in the HPLC chromatogram of the chloroform extracts of *D. alpina* twigs and leaf. This compound belongs to the coumarines and has three characteristic absorption maxima at 204, 261 and 325 nm.

Total antioxidant capacity

The total antioxidant capacity (TAC) was determined using phosphomolybdenum method. It was ranged from 69.71 ± 0.54 µg AA/g for the methanol extract of leaf to 73.55 ± 1.02 µg AA/g for the chloroform extract of twigs (Table 1). ANOVA showed significance in the total antioxidant capacity of the extract. Tukey's HSD post hoc comparison test indicates a statistically significant difference in the total antioxidant capacity of the CHLT/CHLL and CHLT/METT. The methanol extract of twigs had the highest activity (IC_{50} = 21.57±1.03 µg/mL), followed the methanol extract of leaf (IC_{50} = 23.15 ± 1.05 µg/mL), the chloroform extracts of twigs (IC_{50} = 25.45 ± 1.05 µg/mL) and the chloroform extract of leaf (IC_{50} = 25.45 ± 0.89 µg/mL).

DPPH scavenging

DPPH scavenging activity (IC_{50} values) in various extract of *D. alpina* leaf and twigs are shown in Table 2. These values for the all extracts were higher than 20µg/mL. The methanol extract of twigs had the highest activity (IC_{50} = 21.57 ± 1.03 µg/mL), followed the methanol extract of leaf (IC_{50} = 23.15 ± 1.05 µg/mL), the chloroform extracts of twigs and leaf (IC_{50} = 25.45 ± 1.05 µg/mL and IC_{50} = 25.45 ± 0.89 µg/mL). IC_{50} value determined for chloroform extracts of twigs and leaf were significantly different from methanol extract of leaf. However, the scavenging activities of all extracts were less than those of standard compounds-BHT. Table 2 shows the IC_{50} values (mean ± SD) of chloroform and methanol extracts of *D. alpina* compared with BHT. Data were analyzed by analyses of variance (ANOVA) procedure followed by Tukey`s HSD post hoc comparison test.

Anti-lipid peroxidation

The results of inhibitory activity against lipid peroxidation for the tested extracts of *D. alpina* are shown in Table 2. The methanol extract of leaf had highest lipid peroxidation inhibition (IC_{50} = 26.79 ± 0.34 µg/mL), followed the chloroform extract of leaf (IC_{50} = 27.87 ± 1.03 µg/mL), the chloroform extract of twigs (IC_{50} = 34.23 ± 0.89 µg/mL) and the methanol extract of twigs (IC_{50} = 35.24 ± 0.55 µg/mL). The methanol and chloroform extracts of leaf showed greater inhibition of lipid peroxidation then twigs extracts. The IC_{50} values obtained for the tested extracts were significantly higher than the IC_{50} value obtained for BHT.

Metal-chelating ability

Based on the obtained results in Table 2, it can be concluded that the constituents of *D. alpina* extracts has the ability to create complex with ferrous ion. As compared for IC_{50} values, metal chelating ability of methanol extracts (21.57 ± 1.03 µg/mL for twigs and 23.15 ± 1.05 µg/mL for leaf), were more effective than that of the chloroform extract of twigs (45.45 ± 1.15 µg/mL) and leaf (44.65 ± 0.99 µg/mL). Statistical analysis of the potential of the extracts for the ability to metal chelating indicate statistical significance between the IC_{50} values of the methanol and chloroform extracts of *D. alpina* (Table 2).

Hydroxyl radical scavenging activity

The results of hydroxyl radical scavenging activity are shown in Table 2. For the tested extracts, the chloroform extract of twigs had the highest IC_{50} value (98.86 ± 0.94 µg/mL), followed by the methanol extract of twigs (91.55 ± 1.05 µg/mL), the methanol extract of leaf (87.98 ± 1.07 µg/mL) and the chloroform extract of leaf (80.56 ± 1.05 µg/mL). The potential of all extracts to scavenge hydroxyl radical were significantly different from the IC_{50} values obtained for standard compounds.

Correlations

The results of correlation of total phenolic and

Table 2: Free radical scavenging activity of the chloroform and methanol extracts of *D. alpina*

Extracts of *D.alpina*	DPPH	Inhibitory activity against lipid peroxidation	Ferrous ion chelating ability	Hydroxyl radical scavenging activity
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
CHLT	25.45 ±1.05	34.23±0.89	45.45±1.15	98.86±0.94
CHLL	25.45±0.89	27.87±1.03	44.65±0.99	80.56±1.05
METT	21.57±1.03	35.24±0.55	21.57±1.03	91.55±1.05
METL	23.15±1.05	26.79±0.34	23.15±1.05	87.98±1.07
BHT	15.61±1.26	1.00±0.23	-	33.92±0.79
	***P*-value**			
ANOVA	*	*	*	*
Tukey`s HSD test				
CHLT/CHLL	n.s.	*	n.s.	*
CHLT/METS	*	n.s.	*	*
CHLT/METL	n.s.	*	*	*
CHLT/BHT	*	*	-	*
CHLL/METT	*	*	*	*
CHLL/METL	n.s.	n.s.	*	*
CHLL/BHT	*	*	-	*
METT/METL	n.s.	*	n.s	*
METT/BHT	*	*	-	*
METL/BHT	*	*	-	*

**p < 0.05; n.s. - not significant*

flavonoids contents with antioxidant assays of *D. alpina* are shown in Table 3. A significant correlation of IC_{50} values of metal chelating ability and DPPH was established with total phenolic contents, while a significant correlation exists between the hydroxyl radical scavenging activity and flavonoids content. A no significant correlation was found between IC_{50} values of anti-lipid peroxidation assay, and hydroxyl radical and total phenolic respectively anti-lipid peroxidation assay, DPPH and metal chelating ability and flavonoid contents.

Antimicrobial activity

The results reveal antimicrobial activity of the chloroform and methanol extracts of *D. alpina* within the concentration range from 15.62 to 125 µg/mL. The highest susceptibility to the chloroform extract of *D. alpina* twigs among the bacteria tested was exhibited by *B. subtilis* ATCC6633 (15.62 µg/mL) followed by strains of *K. pneumoniae* ATCC13883 and *E. coli* ATCC25922 (31.25 µg/mL) and *P. vulgaris* ATCC13315 and *P. mirabilis* ATCC14153 (62.5 µg/mL).

The chloroform and methanol extracts showed comparatively efficient MIC value 31.25 µg/mL against *S. aureus, K. pneumoniae, E. coli, P. vulgaris, C. albicans* and *A. niger* (Table 4). The highest susceptibility to the methanol extract of *D. alpina* leaf among the bacteria tested was exhibited by *P. vulgaris* ATCC13315 (MIC = 15.62 µg/ml). On the other hand, antifungal activity of the chloroform and methanol extracts of *D. alpina* was within the concentration range from 31.25 to 125 µg/mL.

Comparison of antimicrobial activity shows that chloroform extracts exhibited higher activity against selected strains of microorganisms then the methanol extracts, exception is the effect on *P. mirabilis* where MIC value is the same for all types of extracts.

DISCUSSION

A preliminary analytical investigation of the chloroform and methanol extracts obtained from the leaf and the stems of *Daphne alpina* was performed to determine total phenolic and flavonoid contents. The total phenolic contents of leaf and twigs extracts of *D. alpina* as gallic acid equivalents were found to be highest in the methanol extract of twigs (METT) followed by the chloroform extract of leaf (METL), the chloroform extract of twigs (CHLT) and the chloroform extract of leaf (CHLL).

The results also showed that the highest total flavonoid content were found in the chloroform extract of twigs and lowest in the chloroform extract of leaf. Our previous study on *Daphne*

blagayana extracts showed higher content of flavonoids and phenols in twigs than leaf extracts

Table 3: Correlation of the total phenolic and flavonoids content with antioxidant assays of the *D. alpina* extracts

	Correlation R^2	
Assays	**Total phenolic**	**Flavonoids**
IC_{50} of DPPH radical scavenging potential	0.774	0.160
IC_{50} of inhibition of lipid peroxidation	0.296	0.427
IC_{50} of ferrous ion chelating ability	0.941	0.103
IC_{50} of hydroxyl radical scavenging potential	0.192	0.890

Table 4: Minimum Inhibitory Concentrations (µg/ml) of chloroform and methanol extracts of *D. alpina* leaf and twigs

	MIC (µg/mL)					
Microorganism	***CHLT***	***CHLL***	***METT***	***METL***	***TET***	***KET***
S. aureus ATCC25923	62.5	31.25	62.5	31.25	0.98	nt
K. pneumoniae ATCC13883	31.25	31.25	62.5	125	0.49	nt
E. coli ATCC25922	31.25	31.25	62.5	31.25	0.98	nt
P. vulgaris ATCC13315	62.5	31.25	62.5	15.62	1.95	nt
P. mirabilis ATCC14153	62.5	62.5	62.5	62.5	1.95	nt
B. subtilis ATCC6633	15.62	15.62	62.5	125	0.24	nt
C. albicans ATCC10231	62.5	31.25	62.5	125	0.98	nt
A. niger ATCC 16404	62.5	31.25	62.5	125	Nt	0.98

[29]. The results of the HPLC analysis suggested that all *D. alpina* extracts containing 4 - hydroxybenzoic acid, 7 - hydroxy coumarine and 7, 8 - dihydroxy coumarine, as the major compounds Daphnetin has the most intense peaks in the HPLC chromatogram of the chloroform extracts of twigs and leaf. This compound belongs to the coumarines and has three characteristic absorption maxima at 204, 261 and 325 nm.

Results of the antioxidant and antimicrobial potential of extracts of *D. alpina* are similar to the results of other studied Daphne species from Serbia (*D. cneorum, D. blagayana*) [11,29,31] except endemic *D. malyana* [10]. These results can be explained by a similar chemical composition of the examined species Daphne dominated flavonoids and coumarins (daphnetin) which are the main carriers of biological activity in this genus.

Daphnetin has been found in several species of the genus Daphne and has been reported to possess different biological activity including antimicrobial, antioxidant, antimalarial, anticoagulation and immunomodulating activities [8,9,15,26,28].

The study for antimicrobial activity of *Daphne gnidium* extracts showed that the daphnetin is one of most active compounds [30]. His presence in the *D. alpina* extracts indicates that he is largely responsible for the manifested antimicrobial activity. However, the presence of other metabolites can contribute to the manifest activities.

CONCLUSION

D. alpina leaf and twig have high phenolic and flavonoid contents which contribute to the antioxidant and antimicrobial activities of this plant. HPLC–UV analysis of the leaves and twigs showed that their chloroform and methanol extracts contain 4-hydroxybenzoic acid, 7-hydroxycoumarine (umbeliferone) and 7,8-dihydroxycoumarine (daphnetin) as the major compounds. The extracts possess moderate antioxidant and antimicrobial activities and the phenolic compounds are believed to be responsible for these activities.

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