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Chemical Characterization and Antioxidant Activity of Mountain Pine (*Pinus mugo* Turra, Pinaceae) from Republic of Macedonia

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Abstract: Chemical composition and antioxidant activity of essential oils and methanol extracts of needles of *Pinus mugo* native to R. Macedonia were investigated. Plant material was collected on Karadzica Mountain during two seasons. Essential oil was obtained by steam distillation and was analyzed by GC/FID/MS. 8 major constituents were identified: Δ^3-carene, α-pinene, limonene+β-phellandrene, germacrene D, *trans*-caryophyllene, δ-cadinene, bicyclogermacrene and α-cadinol. Identification of flavonoids and phenolic compounds in methanol extracts was made by LC-DAD/ESI-MS^n. 14 compounds were identified (2 phenolic acids, 2 procyanidins, 7 flavonol glycosides and 3 flavonol acylated glycosides). Total phenolic content (TPC) and total flavonoid content (TFC) were measured by Folin-Ciocalteau and aluminum chloride assay, respectively. TPC and TFC values ranged from 11.41-12.17 mgGAE/g and from 4.65-6.03 mgCE/g for dried plant material, respectively. Evaluation of antioxidant activity was made using DPPH and TBARS test. IC_{50} values for the methanol extract ranged from 11.58-16.01 mg/mL for DPPH and from 9.41-15.76 mg/mL for TBARS assay. IC_{50} values for the essential oil ranged from 2.51-4.26 mg/mL and 2.59-4.14 mg/mL for DPPH and TBARS assay, respectively. The needles from Macedonian *P. mugo* could be considered as a potential plant source for isolation of essential oil as well as flavonoids and other phenolics as they exhibit promising antioxidant activity. Additionally, further investigation and evaluation of chemical composition as well as biological activity should be done in order to provide more data for their possible medicinal, pharmaceutical and commercial utilization.

Keywords: Essential oil; flavonoids; GC/FID/MS**;** LC/DAD/ESI-MS^n, methanol extract, *Pinus mugo*.

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1. Introduction

Mountain or Dwarf pine, *Pinus mugo* Turra (Pinaceae) is a very variable pine species which comprises several subspecies [1]. According to Christensen, well defined subspecies are *Pinus mugo* subsp. *uncinata* (Ramond) Domin and *Pinus mugo* nothosubsp. x *rotundata* (Link) Janch. and H. Neumayer [2], but the most important of all is *Pinus mugo* subsp. *mugo,* which is spread in the southern and eastern Alps and Balkan Peninsula. This conifer is a low, shrubby, multi-stemmed plant, 3-6 m tall with symmetrical cone and hard and heavy wood. In general, it is usually used to protect soil against erosion and to retard avalanching. As an ornamental, this species is very popular with its rock landscapes, particularly in Scadinavia, Holland and Germany, where is widespread in municipal parks and gardens [3].

Essential oil of Mountain pine is a clear, colorless to slight yellowish liquid. Obtained from needles, it consists of a mixture of about 60 compounds, which mostly belong to the group of terpene hydrocarbons. The main components are α- and β-pinene, Δ^3-carene, β-phellandrene and limonene [4]. In terms of its biological activity, this oil shows beneficial effects in cases of catarrh as well as in a supportive therapy in rheumatic diseases. Additionally, Mountain pine oil shows strong effects on blood circulation, which especially enhanced after a topical administration of the oil [5]. According to Grasmann, a stimulation of blood circulation is mainly caused by Δ^3-carene. Furthermore, in combination with α-tocopherol and β-carotene, terpinolene which is integral part of the essential oil of *P. mugo*, effectively prevents oxidation of the LDL [6]. As it is revealed in the experimental studies with guinea pigs, the oil also possesses antimicrobial, spasmolytic and expectorant properties [7].
Based on the positive results of studies, the essential oil of *P. mugo* is seems to be an active ingredient of different medicinal preparations for topical use [8]. This pine oil is very suitable for inhalations, but it is also available in some cosmetic products. Due to the stimulating effects on skin and blood vessels, mountain pine oil is considered as a main ingredient in massage oils, soaps, shower gels as well as preparations for foot care. It is suppose that the special relaxing and stimulating effects of these preparations are mainly caused by monoterpenes Δ^3-carene and α- and β-pinene [8].

With the exception of chemical composition and biological properties of the *P. mugo* essential oils, there is a lack of data concerning the phenolic compounds of this species. Among the huge spectrum of secondary metabolites, it is well known that polyphenols may contribute to different health benefits of plant extracts. Regarding this, one of the most famous pine species, *Pinus pinaster*, contains 65-75 % of proanthocyanidins in its dietary extract (Pycnogenol) [9] and is used for treatment of inflammatory diseases [10]. In the last decade, polyphenolic compounds in various pine species have been intensively examined. In this order, Turkish authors reported the presence of catechin in the bark of *P. brutia* [11], while other reports focused on the phenolic components of French *P. halepensis* and Italian *P. laricio* needles indicated presence of phenolic acids such as *p*-coumaric, vanillic and ferullic acid [12]. In addition, total phenolic compounds have been also studied in Norwegian pine needles [13]. A very high range of total polyphenols, especially tyrosol, was detected in the bark of *Pinus eldarica*, native to Iranian region, what made this species an interesting source of natural compounds that stimulate resistance to oxidative stress and has anti-aging effect as well [14]. Recently results on ability of polyphenol-rich extracts of *P. sylvestris* bark to reduce viability and to induce apoptosis in HeLa cells suggested the presence of compounds that own antitumor properties [15].

According to Micevski, huge population of *Pinus mugo* can be found in R. Macedonia, but only in the central part of the country, on Karadzica Mountain. Withal, this location is the southernmost extensive point of distribution for this plant [16]. Until now, some preliminary data on essential oil composition as well as flavonoids and other phenolic compounds of Macedonia pine species including *Pinus mugo* were published [17, 18]. Evaluation of possible biological activity of the essential oil or plant extracts of Macedonian *Pinus mugo* have not been performed yet, thus the main objective of the present study was chemical characterization and evaluation of antioxidant activity of the needle essential oil as well as methanol extracts of Macedonian *P. mugo*.

2. Materials and Methods

2.1. Plant Material

The terminal twigs with needles of Mountain pine were collected on Karadzica Mountain during the two consecutive years (I and II). Two samples from plants growing on different altitudes, first (1) at an altitude of 2039 m and second (2) at 1640 m a.s.l. were collected each year and were marked as: Pm-1/I year, Pm-2/I year, Pm-1/II year and Pm-2/II year for those collected at first (1) and second (2) altitude, respectively. Plant identity was verified as *Pinus mugo* by Prof. Gjoshe Stefkov and herbarium voucher specimen (N°1/2008/Pm) was deposited at the Department of Pharmaceutical Botany, Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, R. Macedonia.

Plant material was dried at room temperature and on draft. After drying, the needles were separated from the branches and properly grounded to a fine powder in a steel blender. One part of it was used for essential oil isolation and other for extract preparation, as well.

2.2. Essential Oil Isolation

Essential oil isolation from plant needles was made by steam distillation in special all-glass Clevenger type apparatus. For that purpose, 20 g of minced and dried needles were distilled for 4 hours. After isolation, anhydrous sodium sulfate was added to remove residual water from the oil. For GC/FID/MS analysis, the essential oil was dissolved in xylene to obtain 1 μL/mL of oil solution.

2.3. Extraction Procedure

In order to analyze flavonoids and other phenolic compounds, samples containing 1.0 g of powdered dried plant material were processed. The extraction procedure for sample preparation was performed with 10 mL of 70 % methanol (HPLC grade), 30 min in ultrasonic bath (50/60 Hz, 720 W). The obtained methanol extracts were used for determination of total phenolic and total flavonoid content and for their identification by LC/DAD/ESI-MS^n analysis as well as for evaluation of antioxidant activity.

2.4. GC/FID/MS Analysis of the Essential Oil

Essential oil samples were analyzed on Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector (both controlled by GCMSD ChemStation software G1701EA E.01.00.237) as well as capillary flow technology which enables simultaneous analysis of the samples on both detectors. For that purpose, HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm) was used. Operating conditions were as follows: oven temperature at 60 °C (held 5 min), then increased to 80 °C at rate of 1 °C/min (held 2 min) and to 280 °C at 5 °C/min (held 5 min); helium as carrier gas at a flow rate of 1 mL/min; injector temperature 260 °C and that of the FID 270 °C. 1μl of each sample was injected at split ratio 1:1. The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 230 °C, transfer line temperature 280 °C and mass range from 50 - 500 Da. The MS was operated in scan mode.

Identification of the components present in the essential oils was made by comparing mass spectra of components in the investigated oils with those from Nist, Wiley and Adams mass spectra libraries, by AMDIS (Automated Mass Spectral Deconvolution and Identification System) and by comparing literature and estimated Kovat′s (retention) indices that were determined using mixture of homologous series of normal alkanes from C_9 to C_{25} in hexane, under the same above mentioned conditions. The percentage ratio of the identified components was computed by the normalization method of the GC/FID peak areas without any correction factors.

2.5. LC/DAD/ESI-MS^n Analysis of Flavonoids and Other Phenolic Compounds

Plant extracts were analyzed using an Agilent 1100 HPLC system equipped with G1315D DAD detector (controlled by a ChemStation software v.08.03) as well as G2445A Ion-trap Mass spectrometar (controlled by LCMSD software v.6.1.) fitted with an electrospray ionization (ESI) system. Chromatographic separations were carried out on XDB-C18 Eclipse column (150 mm x 4.6 mm, particle size 5 μm). The mobile phase consisted of two solvents: 0.1 % (*v/v*) water-formic acid (A) and 0.1 % (*v/v*) acetonitrile-formic acid (B). A gradient elution, starting with 20 % B, was installed to reach 40 % B at 7 to 20 min, then 75 % B at 30 min and 100 % B at 45 min. The flow rate was 0.5 mL/min, the injection volume 20 μL and the column temperature 30 °C. Spectral data for all peaks were accumulated in the range of 190-600 nm and chromatograms were recorded at 260, 280, 300, 330 and 350 nm.

MS data were acquired in the negative ionization mode. The full scan covered the range from *m/z* 100-1200. Nitrogen was used as nebulizing gas at pressure of 65 psi and the flow was adjusted to 12 L/min. The heated capillary and the voltage were maintained at 325 °C and 4 kV, respectively. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 5, respectively.
The identification and peak assignment of all phenolic compounds were based on comparison of retention times as well as UV-Vis and mass spectral data with those of standard substances and published data.

2.6. Determination of Total Phenolic and Total Flavonid Content

The total phenolic content (TPC) of the Mountain pine needles was determined with the Folin-Ciocalteu reagent according to a procedure described by Singleton et al. [19] with slight modifications. To 1.0 mL of the test sample (methanol extract), 0.5 mL Folin-Ciocalteau reagent (1:10 *v/v* diluted with distilled water) was added and stirred for 5 min at room temperature. Subsequent, 0.4 mL 7.5 % sodium carbonate was added and made up to 10 mL with distilled water. These mixtures were incubated at room temperature, in the dark, for 2 hours. After incubation, absorbance of blue color was measured at 765 nm using an Agilent 8453 UV-Vis spectrophotometer. The TPC was determined as mg of gallic acid equivalents per gram of dried plant material (mg GAE/g) using an equation obtained from standard gallic acid calibration curve.

The total flavonoid content (TFC) was determined using the aluminum chloride assay described by Talari et al. [20] with slight modification. To an aliquot of the test sample (1.0 mL methanol extracts), 4.0 mL distilled water and 0.3 mL 5.0 % sodium nitrite were added and allowed to stand for 5 min. Later, 0.3 mL 10.0 % aluminum chloride was added and the mixture was incubated for 6 min. Then, 2.0 mL 1 M sodium hydroxide was added and the volume was made up to 10.0 mL with distilled water. After incubation of 15 min, the mixture turned to pink and the absorbance was measured at 510 nm using an Agilent 8453 UV-Vis spectrophotometer. The TFC was expressed in mg of catechin equivalents per gram of dried plant material (mg CE/g) using an equation obtained from standard (+)-catechin calibration curve.

2.7. Antioxidant Activity

2.7.1. Free Radical Scavenging Activity - DPPH Assay

The scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals of *P. mugo* needle extracts was done according to method reported by Gyamfi et al.[21] with minor modifications. 200 μL of different concentrations of the tested samples (0.5, 5, 10, 25 and 50 mg/mL) prepared in methanol were placed in a cuvette and 4 mL 100 μM methanol solution of DPPH radical was added. Mixtures were shaken vigorously for 1 min and left to stand 10 min in the dark at ambient

temperature. Subsequently, the absorbance of the solutions was measured at 517 nm. Methanol was used as control. L-Ascorbic acid and butylated hydroxyl anisole (BHA) were used as standards. The percentage of inhibition of DPPH free radicals was calculated from the absorbance of the control (A_c) and of the sample (A_s) using the following equation: inhibition (%) = $[(A_c\text{-}A_s)/A_c]$ x 100 and the IC_{50} values (extract concentration providing 50 % of radical scavenging activity) were estimated by the interpolation from the graph obtained by plotting the percentage of inhibition against extract concentration.

The scavenging activity of DPPH free radical of *P. mugo* needle essential oil was measured using the above mentioned method in a series of oil concentrations of 0.005, 0.05, 0.5 and 5.0 mg/mL prepared in methanol.

2.7.2. Inhibition of Lipid Peroxidation - TBARS Assay

A modified thiobarbituric acid reactive substances (TBARS) assay was used to measure the potential antioxidant capacity of the methanol extracts and essential oil, using an egg yolk homogenate as lipid rich media obtained as described by Dorman et al. [22]. An aliquot of yolk material was made up to concentration of 10 % (*w/v*) in 1.15 % (*w/v*) KCl. The yolk was then homogenized for 30 s, followed by ultra sonification for further 5 min. 500 µL of 10 % (*w/v*) homogenate and 100 µL of different concentrations of tested extracts (0.5, 5, 10, 25 and 50 mg/mL) solubilized in methanol were added to a test tube and made up to 1 mL with distilled water. Subsequent, 50 µL 0.07 M $FeSO_4$ was added to a number of samples to induce lipid peroxidation and incubated for 30 min at 37 °C. Then, 1.5 mL 20 % acetic acid (pH adjusted to 3.5 with NaOH), 1.5 mL 0.8 % thiobarbituric acid (TBA) prepared in 1.1 % sodium dodecyl sulphate (SDS) and 20 % trichloroacetic acid (TCA) were added. This mixture was stirred in a vortex and heated at 95 °C for 60 min. After cooling to room temperature, 5 mL 1-butanol was added to each tube, stirred and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. Methanol was used as control. L-Ascorbic acid and butylated hydroxyl anisole (BHA) were used as standards. The percentage inhibition of lipid peroxidation was calculated from the absorbance of the control (A_c) and of the sample (A_s) using the following equation: inhibition (%) = $[(A_c\text{-}A_s)/A_c]$ x 100. The IC_{50} values (extract concentration needed to achieve 50 % of inhibition) were determined by the interpolation from the graph obtained by plotting the percentage of lipid peroxidation inhibition against extract concentration.

Antioxidant capacity of *P. mugo* needle essential oil was done using the above mentioned method in a series of oil concentrations of 0.005, 0.05, 0.5 and 5.0 mg/mL prepared in methanol.

2.8. Data Analysis

Data obtained from determination of TPC and TFC as well as antioxidant activity were expressed as mean values ± SD as all experiments were carried out in triplicate. Statistical analyses were performed by the statistical package STATGRAPH 21.0. The *p* value less than 0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Essential Oil Composition

The obtained essential oils were transparent, light yellowish liquids with specific and very strong turpentine odor. The yield of isolated essential oil ranged from 0.15% to 0.65%.

According to GC/FID/MS analysis, total of 74 components were identified in the investigated samples of *Pinus mugo* essential oils, representing 93.16-95.87 % of the oils. Data analysis of the chemical composition revealed 8 major constituents: Δ^3-carene (12.11-18.74 %), α-pinene (7.21-12.92

%), sum of limonene+β-phellandrene (3.05-5.72 %), germacrene D (2.38-11.81 %), *trans*-caryophyllene (5.65-6.44 %), δ-cadinene (4.03-6.58 %), bicyclogermacrene (3.03-6.84 %) and α-cadinol (3.42-4.98 %). These components represented the largest part of the essential oil composition since they accounted for 55.23 %, 56.47 %, 57.24 % and 63.58 % for Pm-2/II year, Pm-2/I year, Pm-1/II year and Pm-1/I year, respectively. The remaining part of the oil contains monoterpenes: β-pinene, α-terpinolene, bornyl acetate, α-terpenyl acetate and β-myrcene, and sesquiterpenes: α-hummulene, γ-muurolene, γ-cadinene, α-muurolol and *epi*-α-cadinol+*epi*-α-muurolol, in amounts larger than 1 % (Table 1).

Table 1. Chemical composition of the needle essential oil of Macedonian *Pinus mugo* (Pm)

RI[a]	RI[b]	Compound	Pm-1/I year (%)[c]	Pm-2/I year (%)[c]	Pm-1/II year (%)[c]	Pm-2/II year (%)[c]
926	959.9	Tricyclene	tr	tr	-	-
939	983.7	*α*-Pinene	7.89	12.92	12.33	7.21
953	988.5	Camphene	1.18	1.56	1.19	0.87
975	1000.3	Sabinene	0.52	0.69	0.54	0.34
980	1002.8	*β*-Pinene	2.39	3.30	1.66	1.26
983	1003.8	Hexanoic acid	tr	tr	-	tr
991	1010.9	*β*-Myrcene	1.85	1.94	1.57	1.19
1000	1016.7	*n*-Decane	0.91	0.86	0.60	0.72
1003	1019.8	*α*-Phellandrene	0.38	0.66	0.33	0.31
1007	1024.0	Δ^3-Carene	18.74	14.22	16.18	12.11
1018	1028.8	*α*-Terpinene	0.13	0.14	0.26	tr
1026	1035.2	*p*-Cymene	0.21	0.42	0.29	0.23
1031	1039.0	*D*-Limonene+*β*-Phellandrene	5.17	5.72	4.22	3.05
1033	1040.8	1,8-Cineole	0.13	0.12	-	-
1040	1064.3	*β-cis*-Ocymene	0.73	0.55	0.52	0.46
1062	1064.5	*γ*-Terpinene	0.44	0.40	0.50	0.36
1067	1084.0	*m*-Tolualdehide	0.71	0.64	0.68	0.78
1079	1084.4	*p*-Tolualdehide	tr	0.27	0.38	0.44
1085	1091.0	*p*-Mentha-2,4(8)-diene	tr	tr	tr	tr
1088	1092.2	*α*-Terpinolene	2.36	2.08	2.95	2.27
1099	1106.8	*n*-Undecane	1.33	1.27	0.85	0.99
1122	1136.4	*α*- Campholenal	tr	tr	tr	tr
1165	1172.5	Borneol	0.20	0.37	0.29	0.34
1177	1181.9	Terpinene-4-ol	0.22	0.26	0.50	0.23
1178	1184.1	Naphthalene	tr	tr	tr	tr
1183	1189.4	*p*-Cymene-8-ol	tr	0.43	0.28	0.15
1189	1191.6	*α*-Terpineol	0.12	0.18	0.15	0.32
1198	1199.2	Dodecane	0.35	0.35	0.20	0.24
1204	1207.1	Verbenone	tr	0.15	tr	tr
1235	1237.1	Thymol methyl ether	0.11	tr	tr	tr
1249	1254.6	Piperitone	-	tr	0.18	-
1256	1257.6	Linalyl acetate	tr	tr	-	-
1260	1263.1	2*E*-Decenal	-	tr	0.13	tr
1289	1285.6	Bornyl acetate	1.97	2.28	3.72	3.13
1293	1292.1	2-Undecanone	tr	0.15	0.15	0.12
1294	1296.1	Tridecane	tr	tr	tr	tr
1315	1312.5	2*E*,4*E*-Decadienal	tr	tr	0.12	tr

1339	1336.1	*δ*-Elemene	0.20	0.26	0.50	0.61
1350	1347.5	*α*-Terpenyl acetate	1.58	2.03	1.43	1.23
1373	1371.1	*α*-Ylangene	tr	tr	tr	tr
1376	1374.0	*α*-Copaene	0.14	0.10	0.11	0.17
1387	1383.3	*β*-Bourbonene	0.34	0.19	0.19	0.41
1391	1389.2	*β*-Elemene	1.01	1.26	1.95	2.63
1407	1403.4	Longifolene	-	-	tr	tr
1418	1418.0	*trans*-Caryophyllene	6.44	5.65	5.83	6.40
1430	1430.1	*β*-Copaene	0.35	0.21	0.14	0.39
1439	1444.3	Aromadendrene	tr	tr	0.20	0.37
1454	1453.6	*α*-Hummulene	1.34	1.23	1.28	1.78
1458	1456.3	*Allo*-aromadendrene	0.24	0.50	0.29	0.90
1477	1475.8	*γ*-Muurolene	1.11	0.61	0.86	0.91
1480	1481.7	Germacrene *D*	11.81	7.26	2.38	9.62
1485	1493.9	*β*-Selinene	0.28	0.31	0.46	0.37
1494	1496.3	Bicyclogermacrene	3.43	3.03	4.75	6.84
1495	1497.9	*α*-Muurolene	tr	tr	tr	tr
1513	1513.7	*γ*-Cadinene	1.22	1.18	1.81	1.32
1524	1525.3	*δ*-Cadinene	5.90	4.03	6.57	6.58
1532	1532.0	*trans*-Cadina-1,4-diene	0.13	0.15	0.28	0.32
1538	1540.6	*α*-Cadinene	0.23	0.23	0.45	0.41
1547	1546.4	*α*-Calacorene	tr	tr	0.23	tr
1561	1561.4	*E*-Nerolidol	0.50	0.45	0.58	0.71
1565	1564.8	Dodecanoic acid	tr	0.22	tr	tr
1574	1577.0	Germacrene-4-ol	0.96	0.56	1.10	0.45
1576	1583.0	Spatulenol	tr	1.15	0.54	1.50
1581	1586.4	Caryophyllene oxide	0.54	0.48	0.45	0.43
1606	1613.0	Humulene epoxide II	0.19	0.11	-	-
1627	1637.8	1-*epi*-Cubenol	0.08	0.04	0.11	0.14
1640	1647.4	*epi*-*α*-Cadinol+ *epi*-*α*-Muurolol	2.45	2.80	3.76	4.43
1645	1651.0	*α*-Muurolol	1.63	2.02	2.49	2.68
1653	1657.0	*α*-Cadinol	4.20	3.64	4.98	3.42
1700	1693.1	Amorpha-4,9-dien-2-ol	0.16	0.02	tr	0.13
1880	1884.5	3*Z*-Hexenyl cinnamate	tr	tr	tr	tr
1987	2009.4	Manool oxide	0.74	1.16	1.13	0.89
2080	2101.4	Abietadiene	tr	tr	tr	tr
2220	2128.4	Sclareol	-	0.39	0.25	tr
		Total percentage of components	**95.24**	**93.20**	**95.87**	**93.16**

RI[a] - Retention Index from literature [40]; RI[b] - Retention Index calculated from retention times relative to that of n-alkane series (C_9-C_{25}); [c] - Percentages obtained from FID data; tr < 0.01%.

It is well known that essential oil composition depends on many factors and usually varies a lot, thus geographical origin of the plant source could be important for recognizing oils with certain characteristics. According to literature data, *P. mugo* needle essential oil mainly consists of terpene hydrocarbons such as: Δ^3-carene (amount up to 35 %), α- and β-pinene (up to 20 %) and β-phellandrene (amount about 15 %) [4]. According to some older standards, the quality of this oil depends on the percentage amount of bornyl acetate which was expected to be present up to 10 % [23]. Furthermore, Greek authors pointed out on the presence of larger amounts of α-pinene (33.30 %) in essential oil of *P. mugo* as they distinguish this oil from essential oil of *P. pumilio* which was characterized by larger amounts of Δ^3-carene (14.9 %), β-phellandrene (10.7 %) and terpinolene (37.7

%) [24]. On the other hand, authors from Serbia found Δ^3-carene (23.9 %), α-pinene (17.9 %), β-pinene (7.8 %) and β-phellandrene (7.2 %) as predominant constituents of *P. mugo* needle essential oil [25]. In addition, Venditti et al. emphasize that needle essential oil of *P. mugo* growing wild on central Apennines in Italy is characterized by larger percentages of alcohols and esters such as α-terpineol (7.3 %) and bornyl acetate (11.5 %), followed by larger percentages of sesquiterpenes *trans*-caryophyllene (5.9 %) and α-cadinol (4.1 %). Besides mono and sesquiterpene, this essential oil was marked by larger percentage of diterpenes, notably abietane derivatives which accounted for 25.8 % of the oil [26]. Recently, the essential oil composition of needles of *P. mugo* from Canada was published where authors pointed out on Δ^3-carene (36.54 %), α-pinene (9.0 %) and α-terpinolene (18.0 %) as major constituents of the oil [27]. Comparing all these data to the essential oil composition of Macedonian *P. mugo*, similarities could be found as Macedonian species contained the same components mentioned above as major constituents of the oil (Δ^3-carene 12.11-18.74 %, α-pinene 7.21-12.92 %, limonene+β-phellandrene 3.05-5.72 %). Moreover, the sesquiterpenes *trans*-caryophyllene and α-cadinol represented an important part of the essential oil composition of Macedonian *P. mugo*. On the other hand, only four components (manool oxide, abietadiene, sclareol and dihidroabietal) were identified in the fraction of diterpenes. These components were present in a very low amount as the sum of total diterpenes ranged from 0.74 to 1.55 %.

3.2. Flavonoids and Other Phenolic Compounds

14 flavonoids and other phenolic compounds were identified in the methanol extracts of *P. mugo* using UV and mass spectral data (deprotonated molecular ions as well as corresponding fragments and losses) by LC-DAD/ESI-MS^n. Two identified phenolic acids were: quinic acid (component **1**) and *p*-coumaric acid (component **2**) while the identified procyanidins were: a catechin trimer (component **3**) and methylated catechin-catechin dimer (component **4**). The largest group of identified polyphenolic compounds were the flavonoids (total of 10 components) represented by glycosides of kaempferol, myricetin, quercetin, laricitrin and isorhamnetin (Table 2). Seven of these components (components **5, 6, 7, 8, 9, 11, 12**) contained a sugar moiety and three of them (components **10, 13, 14**) contained acylated sugars with coumaric or ferulic acid.

The identified phenolic acids showed characteristic absorption at 264 and 290 nm (component **1**) and at 312 nm (component **2**). These components had an intense $[M-H]^-$ ion at *m/z* 191 (for component **1**) and at *m/z* 163 (for component **2**) in negative mode that correspond to quinic acid as well as *p*-coumaric acid (component **1** and **2**, respectively). Components **3** and **4** showed UV-Vis spectra with absorption maxima at 232 and 280 nm and at 288 nm, respectively with $[M-H]^-$ ion at *m/z* 865 (for component **3**) which fragmented in MS^2 to *m/z* 739 and 577 characteristic for catechin trimer and with $[M-H]^-$ ion at *m/z* 591 (for component **4**) which fragmented in MS^2 to *m/z* 289 that correspond to methylated catechin-catechin dimer.

Components **5-14** had absorption maxima between 220-330 nm and fragmentation patterns characteristic for flavonoid glycosides. A deprotonated molecular ion $[M-H]^-$ at *m/z* 465 was observed in negative mode for component **5**. Further fragmentation in MS^2 experiments yielded a fragment ion at *m/z* 447 and the main fragment ion at *m/z* 285 implying that this compound was kaempferol hexoside. The MS^2 spectrum of the pseudomolecular ions at *m/z* 447 of compound **6**, isorhamnetine pentoside showed an intensive ion at *m/z* 315 due to the loss of a pentose moiety. Compound **7**, with $[M-H]^-$ at *m/z* 625, produced ions at *m/z* 463 and 317, the latter corresponding to a loss of rutinose unit, thus this compound was identified as myricetin rutinoside. The MS of compound **8** also had the characteristic fragmentation pattern of a flavonoid glycosides. It was identified as quercetin rutinoside as it showed a deprotonated molecular ion at *m/z* 609. Further MS^2 fragmentation leads to *m/z* 463 and 301 due to a loss of a rutinose unit. The MS^2 spectrum of compound **9** with $[M-H]^-$ at *m/z* 639 exhibited three ions at *m/z* 463, 445 and at *m/z* 331 corresponding to laricitrin and resulting from a loss of rutinose, so this compound was identified as laricitrin rutinoside. Compounds **10** and **13**, showed deprotonated molecular ions $[M-H]^-$ at *m/z* 593 and at *m/z* 739, respectively. In the MS^2, the same main peak at *m/z* 285 was detected, indicating the presence of coumaroyl unit linked to one hexose

([M-H]-146-162) for component **10** as well as rutinose ([M-H]-146-308) for component **13**. In this order, these compounds were identified as kaempferol coumaroyl hexoside and kaempferol coumaroyl rutinoside, respectively. Additionally, a deprotonated molecular ion $[M-H]^-$ at *m/z* 769 was observed in the negative mode for compound **14** which was attributed to kaempferol feruloyl rutinoside as further MS^2 experiments yielded a main fragment ion at *m/z* 285 obtained by loss of a feruloyl and rutinose units ([M-H]-176-308). Considering UV and mass spectra, component **11** was identified as isorhamnetin rutinoside with $[M-H]^-$ at *m/z* 623. The loss of *m/z* 308 (rutinose unit) resulted in a fragment ion at *m/z* 315 (isorhamnetin). Another flavonoid compound **12** with a deprotonated molecular ion at *m/z* 607 produced the characteristic fragment ion ([M-H]-308) at *m/z* 299 after a loss of a rutinose, thus this component was identified as methoxy kaempferol rutinoside.

Table 2. Flavonoids and other phenolic compounds in needles of Macedonian *Pinus mugo*.

No.	Component	t_R (min)	UV max (nm)	$[M-H]^-$	MS^2
1	Quinic acid	3.14	264, 290	191	-
2	*p*-Coumaric acid	6.59	312	163	-
3	Catechin trimer	7.05	232, 280	865	739, **577***
4	Methylated catechin dimer	9.34	288	591	**289**
5	Kaempferol hexoside	9.24	288	465	447, **285**
6	Isorhamnetin pentoside	12.38	230, 282	447	**315**
7	Myricetin rutinoside	13.22	240, 276	625	463, **317**
8	Quercetin rutinoside	15.00	220, 266, 314	609	463, **301**
9	Laricitrin rutinoside	15.57	250, 330	639	463, 445, **331**
10	Kaemferol coumaroyl hexoside	16.53	268, 314	593	447, **285**
11	Isorhamnetin rutinoside	17.72	256, 268, 314	623	477, 459, **315**
12	Metoxy kaempferol rutinoside	21.12	272, 320	607	461, **299**
13	Kaempferol coumaroyl rutinoside	28.03	230, 268, 314	739	593, 575, 453, **285**
14	Kaempferol feruloyl rutinoside	28.31	230, 266, 322	769	623, 315, **285**

*main MS^2 fragments in bold

According to literature data related to the polyphenolic profile of pine species, only few articles could be found. Up to now, there is a lack of data referred to the flavonoids and other phenolic compounds of *P. mugo* thus the obtained results were compared to data published for other pine species. In this way, Slimestad reported that the main flavonoid components in *Pinus cembra* were kaempferol 3-glucoside, isorhamnetin 3-glucoside, kaempferol 3-(6″-coumaroyl-glucoside) and kaempferol 3-(3″,6″-di-coumaroyl-glucoside) [28]. Additionally, taxifolin 7-glucoside was identified in *P. koraiensis* [28]. Further, Maimoona et al. identified quercetin and myricetin in needles of *P. wallichiana* and *P. roxburghii* [29] while Naeem *et al*., identified kaempferol, rhamnetin and isorhamnetin in the same *Pinus* species [30]. Moreover, Kaundun et al., found flavonol aglycones laricitrin and syringetin in *P. brutia, P. halepensis* and *P. eldarica* as well as two proanthocyanidins, prodelphinidin and procyanidin [31-32].

Compared to these results, similarities could be found as glycosides of kaempferol, isorhamnetin, quercetin, myricetin and laricitrin were identified in Macedonian *P. mugo*. The flavonol kaempferol was also identified in acylated glycoside form as kaempferol coumaroyl hexoside. In the class of procyanidins, the identified compounds (catechin trimer and methylated catechin-catechin dimer) were similar to the procyanidins identified in other *Pinus* species.

3.3. Total Phenolic and Flavonoid Content

The needle samples of *P. mugo* analyzed in the present study showed a total phenolic content (TPC) in range of 11.41-12.17 mg of gallic acid equivalents per gram of dried plant material

(mgGAE/g) (Table 3). Only minor variations in the TPC values were noticed in the samples from different year of collection as well as in the samples collected from the locations with different altitude as samples collected at higher altitude contained slightly higher TPC. The quantitative analysis of total flavonoids content (TFC) revealed that they contain TFC from 4.65-6.03 mg catechin equivalents per gram of dried plant material (mgCE/g) (Table 3). It was noticed that samples collected in the first year contained lower TFC compared to those collected in the second.

Regarding available data, only one literature report could be found related to the total phenolic content (TPC) in needles of different pine species [33]. In this order, Apetrei *et al*. recorded that *P. cembra* needle extract contained 78.22 mg GAE/g of dried plant material. Compared to this result, the TPC values for our tested samples were much lower. The total flavonoids content (TFC) in the same *P. cembra* needle extracts was 19.84 mg CE/g of dried plant material. Compared to this, the TFC values of our tested *P. mugo* samples were slightly lower.

Table 3. Total phenolic and flavonoids content of methanol extract of needles of Macedonian *Pinus mugo*.

Extract	Total phenolic content (mg GAE/g)	Total flavonoids content (mg CE/g)
Pm-1/I year	12.17 ± 0.4	5.25 ± 0.6
Pm-2/I year	12.03 ± 0.7	6.03 ± 0.7
Pm-1/II year	11.86 ± 0.5	4.65 ± 0.3
Pm-2/II year	11.41 ± 0.6	5.61 ± 0.4

3.4. Antioxidant Activity

The antioxidant action of methanol extracts and essential oils has been evaluated by two methods, the first known as 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, based on the measuring of antioxidant reducing ability towards the DPPH free radicals by UV/Vis spectrophotometry and the second, known as thiobarbituric acid reactive substances (TBARS) assay that concern the spectrophotometric detection of malonaldehyde, one of the secondary lipid peroxidation products. Methanol extracts of *P. mugo* needles showed radical scavenging activity in DPPH assay with IC_{50} values ranged from 11.58-15.54 mg/mL and 12.18-16.01 mg/mL for the samples collected in the first and in the second year, respectively. The reducing ability of methanol extracts against DPPH radicals was compared to the radical scavenging activity of two referent substances, ascorbic acid and BHA with IC_{50} of 11.26 μg/mL and 13.65 μg/mL, respectively (Table 4). Methanol extracts of *P. mugo* needles showed antioxidant activity in TBARS assay with IC_{50} ranged from 9.41-15.76 mg/mL which was inferior to antioxidant activity of ascorbic acid and BHA with IC_{50} of 9.05 μg/mL and 11.27 μg/mL, respectively (Table 4), but still important as the percentage of inhibition of the tested methanol extracts with concentration of 50 mg/mL were very high and ranged from 90.71-95.92 % for DPPH and from 78.54-87.45 % for TBARS assay.

Antioxidant activity of *P. mugo* needles essential oils showed that they have IC_{50} values ranging from 2.51-4.26 mg/mL and from 2.59-4.14 mg/mL as they were measured by DPPH and TBARS assay, respectively (Table 4). Compared to the IC_{50} values of methanol extracts, these values were lower, but still inferior to those of ascorbic acid and BHA. The highest value of inhibition percentage of the tested essential oils was found in samples with concentration of 5 mg/mL and was 70.87 % for DPPH and 69.85 % for TBARS assay.

Concerning literature data, very little is known about the antioxidant activity of the methanol extract and the essential oil of needles of *P. mugo*. Nevertheless, pine species possess capacity for this activity and essential oils isolated from needles and twigs of *P. cembra* showed radical scavenging activity against DPPH radical with half-maximal response 19.93±0.75 mg/mL and 18.66±0.70 mg/mL, respectively. In both cases, the antioxidant activity was declared as weak as the essential oils were less active than the positive control BHA (3.3±0.1 μg/mL) [34]. The essential oil of *P. radiate* [35] also performed poor radical scavenging activity with an average inhibition percentage lower than 25 %.

According to Ruberto and Baratta, the poor antioxidant performance has been already shown by essential oils with dominated fraction of monoterpene hydrocarbons [36]. The main constituents of *P. radiata* essential oil were found to be α- and β-pinene (21.9 % and 35.21 %, respectively) and β-phellandrene (12.6 %). Our investigated essential oil also consisted of large percentages of pinenes as well as monoterpene hydrocarbon Δ^3-carene thus this is probably the reason for weak to moderate antioxidant activity of the oil that results in high IC_{50} value. Nevertheless, this essential oil is still important as the concentration of 5 mg/mL of tested essential oil samples has showed almost 71% of inhibition of free DPPH radical. According to Grassmann et al., weak or moderate antioxidant activity of *P. mugo* essential oil is probably due to the aqueous environment when it is tested by methods like DPPH assay. When antioxidant activity is tested in more lipophilic environment (e.g. inhibiton of cooper induced oxidation of LDL), this essential oil exhibits good antioxidative capacity. In this way, the isolated monoterpene terpinolene exhibited remarkable protection against LDL-oxidation, even it is usually a minor component of the *P. mugo* essential oil [6]. On the other hand, essential oil of *P. pinaster* showed strong radical-scavenging activity against DPPH radical with IC_{50} value of 15 μg/mL. The microwave extracts of the same species, obtained by hydro diffusion and gravity, contained 78.5 and 74.6 mg GAE/g of TPC, respectively. These extracts showed much better antioxidant capacity and the values of TPC correlated with DPPH activity indicating that TPC highly contributed to the antioxidant activity [37]. Additionally, alcoholic extract as well as *n*-butanol insoluble fraction of *P. roxbughii* needles possess significant radical scavenging activity comparable to the activity of trolox [38]. Methanol extracts of needles of *Pinus* spp. (*P. nigra, P. brutia* var. *elderica* and *P. wallichiana*) showed strong antioxidant activity in TBARS assay in final concentration 0.02 % w/v, comparable to the activity of butylated hydroxyl toluene (BHT) [39]. According to our findings, low TPC values of the tested *Pinus mugo* needle extracts affect their antioxidant activity as the values of IC_{50} were high. Moreover, these methanol extracts are still under consideration as concentrations of 50 mg/mL have showed almost 96 % of reducing ability towards DPPH free radicals.

Table 4. Antioxidant activity of methanol extract and essential oil of needles of Macedonian *Pinus mugo*

Extract/referent substance/essential oil	**DPPH assay IC_{50} (mg/mL)**	**TBARS assay IC_{50} (mg/mL)**
Extracts		
Pm-1/I year	11.58 ± 0.5	9.41 ± 0.5
Pm-2/I year	15.54 ± 0.7	15.08 ± 0.6
Pm-1/II year	12.18 ± 0.7	11.64 ± 0.4
Pm-2/II year	16.01 ± 0.6	15.76 ± 0.3
Reference substance		
Ascorbic acid	11.26* ± 0.3	9.05* ± 0.4
Butylated hydroxyl anisole (BHA)	13.65* ± 0.6	11.27* ± 0.6
Essential oil		
Pm-1/I year	2.65 ± 0.6	2.59 ± 0.5
Pm-2/I year	3.22 ± 0.4	3.12 ± 0.6
Pm-1/II year	2.51 ± 0.3	2.42 ± 0.2
Pm-2/II year	4.26 ± 0.5	4.14 ± 0.3

*μg/mL

3. Conclussion

The needles from Macedonian *Pinus mugo* comprise essential oil with predominant presence of monoterpene hydrocarbons, mainly: Δ^3-carene, α-pinene and limonene+β-phellandrene, followed by sesquiterpene hydrocarbons: germacrene D, *trans*-caryophyllene, δ-cadinene and bicyclogermacrene. Methanol extracts from needles contained TPC from 11.41-12.17 mgGAE/g and

TFC from 4.65-6.03 mgCE/g of dried plant material. 14 flavonoids and phenolic compounds were identified (2 acids, 2 proacyanidins, 7 flavonol glycosides and 3 flavonol acylated glycosides). The most prevalent components were glycosides of kaempferol, myricetin, quercetin, laricitrin and isorhamnetin. Both essential oil and methanol extracts possessed ability for radical scavenging acitivity as well as inhibition of lipid peroxidation. In the future, subsequent assessment of chemical composition and evaluation of biological activity should be done in order to provide more data for better knowledge as well as possible medicinal, pharmaceutical and commercial utilization of *Pinus mugo* from Macedonian flora.

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