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Altitude Variation in the Composition of Essential Oils, Fatty Acid Methyl Esters, and Antimicrobial Activities of Two Subspecies of *Primula vulgaris* Grown in Turkey

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In this study, the changes caused by variation of altitude to the essential oils (EOs), fatty acid methyl esters (FAMEs), and antimicrobial activities of *Primula vulgaris* Huds. subsp. *vulgaris* (*Pvv*) and *P. vulgaris* Huds. subsp. *sibthorpii* (Hoffmanns) W.W.Sm. & Forrest (*Pvs*)) grown in Turkey were investigated. Major fluctuations in the composition of *Pvv* and *Pvs* oils included methyl-4-methoxy salicylate (4.5-35.3%; *Pvv* and 3.2-37.2%; *Pvs*), (*Z*,*Z*,*Z*)-7,10,13-hexadecatrienal (5.1-21.8%; *Pvv* and 4.4-15.2%; *Pvs*) and flavone (5.5-14.9%; *Pvv* and 1.6-18.0%; *Pvs*). Fatty acid profile ($C_{6:0}$-$C_{26:0}$) changes were noted in *Pvv* and *Pvs*. Methyl hexadecanoate (2.4-9.3%) and methyl octadecanoate (1.0-4.7%) were present in all the FAME samples of the plants. The antimicrobial activity of the EOs of *Pvv* and *Pvs* were tested against nine bacterial species, which showed activity against *Mycobacterium smegmatis* with minimum inhibitory concentrations (MIC) varying from 8.5 to 59.2 µg/mL in all samples, respectively, depending on the altitude at which the oils were obtained.

Keywords: *P. vulgaris* subsp. *vulgaris*, *P. vulgaris* subsp. *sibthorpii*, Altitude variation, Essential oils, Fatty acid methyl esters, Antimicrobial activities.

Primula L. belongs to the Primulaceae family, which consists of 430 genera [1], nine of which are found in Turkey [2]. *P. vulgaris* Huds. is one of the most widely grown species in Turkey. Its leaves are in the form of rosettes at the base, growing in short bursts, and it is a cold-resistant, perennial herbaceous plant. Flowers of *P. vulgaris* can be various colors such as white, yellow, pink, lilac, and purple. This species grows in the north or in north-facing meadows located in open areas within the forest [1]. *P. vulgaris* is of Euro-Siberian origin, and grows in the north and south of Western Turkey, Southern and Central Europe, North Africa, Transcaucasia and Lebanon, showing a wide geographical spread. This species grows up to 2400 m above sea level in Turkey. However, in Europe, the species is not usually found above 1500 m. The flowering stage of this species, in general, is between March and May, but may depend on environmental and climatic conditions.

P. vulgaris is represented by five subspecies, two of which, *P. vulgaris* Huds. subsp. *vulgaris* (*Pvv*) and *P. vulgaris* subsp. *sibthorpii* (Hoffmanns) W.W.Sm. & Forrest (*Pvs*), are present in Turkey [2]. *Pvv* and *Pvs* are spread out in the northern and southern regions of Turkey at altitudes of ~300-2100 m and ~100-1300 m, respectively. *Pvv* and *Pvs* are named at the sub-species level, although they grow in the same habitat and show quite similar morphological properties in the northern part of Turkey. These two subspecies are morphologically very similar. However, *Pvs* differs from *Pvv* by its basal leaves with dense reticular venation and dense tomentose-pilose surface [3]. *P. vulgaris* has been used as an ornamental and medicinal plant all over the world. Intraspecies crosses obtain stable hybrids which are widely used as ornamental plants [4]. Extracts obtained from *P. vulgaris* species have been commonly used in traditional medicine [5].

The EOs and FAMEs of some *Primula* species have been mentioned in the literature [6-8]. Many studies have shown that the EOs and the components of the plants can be influenced by environmental factors such as soil, temperature, light and altitude. There are many studies on the seasonal variation of the plants which show differences in their chemical components [9-12], as in our case. The overall fatty acid components of these *Primula* taxa are consistent with those generally observed in other plant species [13-20]. Unsaturated fatty acids have many beneficial effects on human health [21]. Thus, investigations of the fatty acid contents of plants have become a topic of great interest. The various biological activities of the EOs have been mentioned [22-24].

To our knowledge, there is no previous published report for the altitude variation in the chemical composition of the EOs (Table 1 and Table 2) and FAMEs (Table 3) of *Pvv* and *Pvs*. This article presents the first report on altitude variation on the chemical composition, FAMEs and antimicrobial activities of the EOs obtained from *Pvv* and *Pvs* grown in southern Turkey.

The obtained crude EOs of *Pvv* and *Pvs* were analyzed by GC-MS with an HP-5 column. Identification of the compounds was made by a typical library search (NIST and Wiley 7NL) and literature comparison [6-10, 20]. The general chemical profile of the EOs, the percentage content, and retention indices of the constituents are summarized in Tables 1 and 2. In total, 24 and 50 constituents were identified and represented an average of 85.4-99.7% of the EOs in *Pvv* and *Pvs,* respectively.

The antimicrobial activities of EOs from *Pvv* and *Pvs* were assayed against nine bacterial species. Activity was shown against *Mycobacterium smegmatis* with minimum inhibitory concentration

Table 1: Identified components in the essential oils of *P. vulgaris* subsp. *vulgaris*[a,b].

Compounds	300 m % Area	600 m % Area	700 m % Area	1100 m % Area	1600 % Area	1700 m % Area	1900 m % Area	2100 m %Area	Ex.RI	Lit. RI
α-Pinene	0.4	0.9	0.4	0.1	0.5	0.5	0.3	0.2	932	939
β-Pinene[c]	0.2	0.2	-	-	-	0.8	-	0.1	973	979
2-Amyl furan	-	-	-	-	-	0.2	-	-	991	991
α-Phellandrene	-	0.4	-	-	-	0.4	-	0.2	1001	1003
Para-mentha,1(7),8 diene	0.4	-	-	-	-	-	-	-	1003	1004
Limonene[c]	0.7	1.0	0.5	0.2	-	0.6	0.7	0.3	1025	1029
Nonanal	-	-	-	-	-	0.4	-	0.6	1101	1101
Methyl salicylate	6.8	0.6	-	-	0.8	-	-	0.5	1190	1192
Methyl-4-methoxy salicylate	35.3	15.5	21.6	24.1	5.3	5.5	4.5	35.2	1440	MS[1]
Tetradecanol	0.5	0.7	0.6	-	1.8	0.4	0.9	0.5	1670	1673
Chalcone	-	-	-	0.8	-	0.8	-	1.2	1802	MS[2]
Hexahydrofarnesyl acetone	0.9	-	1.0	1.0	1.5	1.1	1.3	1.6	1840	1847
Ethyl linoleolate	1.4	2.5	4.5	1.9	13.5	1.4	4.3	2.6	1885	1893
Methyl hexadecanoate	0.9	2.3	1.1	1.1	2.2	0.6	2.4	0.6	1920	1922
Eicosane[c]	4.9	9.2	1.1	2.9	6.8	5.1	5.5	2.9	2000	2000
7-Tetradecyne	2.3	2.8	2.2	1.4	2.6	1.2	1.7	1.1	2090	MS[3]
Heneicosane[c]	0.8	0.9	3.2	-	1.8	1.8	0.7	2.0	2100	2100
(Z,Z,Z)-7,10,13-Hexadecatrienal	10.0	18.6	10.7	5.1	13.6	5.6	21.8	5.2	2091	MS[4]
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3.0	3.7	5.0	1.6	3.1	-	7.3	2.9	2105	MS[5]
Flavone	10.7	8.2	14.9	13.0	5.5	13.0	12.9	7.0	2170	MS[6]
Docosane[c]	5.4	5.0	11.4	16.5	10.0	18.9	8.0	0.7	2200	2200
Tricosane[c]	4.2	6.3	2.6	9.3	9.2	10.3	7.2	10.7	2300	2300
Tetracosane[c]	9.1	9.4	14.0	16.5	15.0	19.7	10.9	16.7	2400	2400
Pentacosane[c]	1.8	4.2	4.8	3.3	6.5	7.0	4.9	5.0	2500	2500
Total	99.7	88.2	99.6	98.8	99.7	95.3	95.3	97.8		
	% and N.C.	% and N.C.	% and N.C.	% and N.C.	% and N.C.	% and N.C.	% and N.C.	% and N.C.		
Monoterpene hydrocarbons	1.7: 4	2.5: 4	0.9: 2	0.3: 2	0.5: 1	2.3: 4	1.0: 2	0.8: 4		
Terpene related compounds	11.6: 2	8.2: 1	15.9: 2	14.8: 3	7.0: 2	14.9: 3	14.2: 2	9.8: 3		
Hydrocarbons	26.2: 6	30.8: 6	37.1: 6	48.5: 5	49.3: 6	62.8: 6	37.2: 6	37.0: 6		
Others	60.2: 8	46.7: 8	45.7: 7	35.2: 6	42.9: 8	15.3: 8	42.9: 7	50.2: 9		
Total number of compounds	20	19	17	16	17	21	17	22		

MS[1]: 182(45), 150(100),122(50), 107(35), 95(5),79(15); MS[2]: 208(90), 179(20) ,31(45),103(55), 77(100),51(50); MS[3]: 165(15), 141(35), 109(50), 95(70), 54(75), 81(90), 67(100; MS[4]: 234(5), 121(15), 108(35), 93(50), 79(100), 55(55), 41(75), 67(90); MS[5]: 121(20), 108(50), 95(55),79(100),67(65),55(60); MS[6]: 222(100), 194(50), 165(10),120(55), 92(45),63(20). [a]RI calculated from retention times relative to that of *n*-alkanes (C_6-C_{32}) on the non-polar HP-5 column. [b]Percentages obtained by FID peak-area normalization. [c]Identified by authentic samples.

(MIC) values (Table 4) varying from 8.5 to 59.2 µg/mL in all samples, depending on the altitude at which the oils were obtained.

The data from the present study demonstrated that the components of the EOs in *Pvv* and *Pvs* varied significantly with elevation, as seen in Tables 1 and 2. Monoterpene hydrocarbons were predominant among the terpenes in all the analyzed EOs of *Pvv* and *Pvs*. The main component of the EOs of *Pvv* varied depending on the height (300-2100 m). In general, methyl-4-methoxy salicylate (4.5-35.3%), (*Z*,*Z*,*Z*)-7,10,13-hexadecatrienal (5.1-21.8%), flavone (5.5-14.9%), docosane (0.7-18.9%), tricosane (2.6-10.7%), and tetracosane (9.1-19.7%) were found to be the major compounds in varying amounts. The results revealed that no regular increase or decrease in the amounts of components depends on high elevation.

The major compounds in the EOs of *Pvs* differed depending upon altitude (100-1300 m). In the EOs of *Pvs*, methyl-4-methoxy salicylate (3.2-37.2%), flavone (1.6-18.0%), docosane (2.4-11.4%), tricosane (3.9-12.1%), tetracosane (4.0-12.5%), and pentacosane (2.3-10.0%) were the main compounds. There was no regular increase or decrease in the amounts of parent compounds in the essential oil of *Pvs* due to different elevations. However, the percentage of methyl-4-methoxy salicylate decreased from 37.2% to 3.2% (300-1300 m height), whereas the percentage of tetracosane increased from 4.0% to 12.5% at 100 m to 1300 m altitude, respectively.

Hydrocarbons were components of the EOs of *Pvv* and *Pvs*. The amount of hydrocarbons in the EOs of *Pvv* regularly increased from 26.2% to 62.8% at heights from 300 m to 1700 m, respectively. However, the ratio of hydrocarbons in *Pvv* remained almost the same (37.2 and 37.0%, respectively) at altitudes of 1900 m and 2100 m. The amount of hydrocarbons in the EOs of *Pvs* increased from 25.2% to 48.3% with increases in altitude (300 m to 1300 m).

α-Pinene (0.1-0.9%), methyl-4-methoxy salicylate (4.5-35.3%), ethyl linoleolate (1.4-13.5%), methyl hexadecanoate (0.6-2.4%), eicosane (1.1-9.2%), 7-tetradecyne (1.1-2.8%), (*Z*,*Z*,*Z*)-7,10,13-hexadecatrienal (5.1-21.8%), flavone (5.5-14.9%), docosane (0.7-18.9%), tricosane (2.6-10.7%), tetracosane (9.1-19.7%), and pentacosane (1.8-7.0%) were present in all eight samples (*Pvv*) that were collected from different altitudes (300 m to 2100 m) of Trabzon. Also, α-pinene (0.3-1.0%), limonene (0.3-1.3%), methyl-4-methoxy salicylate (3.2-37.2%), methyl hexadecanoate (0.6-2.7%), eicosane (2.5-8.2%), heneicosane (0.6-3.2%), flavone (1.6-18.0%), docosane (2.4-11.4%), tricosane (3.9-12.1%), tetracosane (4.0-12.5%), and pentacosane (2.3-10.0%) were found in all five samples of *Pvs* from 100 m to 1300 m altitude. All identified components (24 compounds) in the EOs of *Pvv* were present in the EOs of *Pvs* (50 components), but with different ratios.

In comparison with published data, the EO analyses of *Pvv* and *Pvs* are in good agreement [9-12] with reported variations in the composition of the EOs obtained from *Thymus vulgaris* L., *Schinus terebinthifolius* Raddi., *Tetradenia riparia* (Hochst.) Codd, and *Myrcia salzmannii* Berg. (Myrtaceae). Monoterpene hydrocarbons were the most represented chemical class in the flowers of *Primula spectabilis* [6]. α-Pinene and methyl salicylate were major components with different ratios in the EOs of *P. spectabilis* [6]. Methyl 2,4-dihydroxy-5-methylbenzoate (30.41%) and methyl 2,6-dihydroxy-5-methylbenzoate (29.27%) were the main compounds in the essential oil of *Primula obconica* [7]. It is known that many factors, such as altitude, season, growing conditions, temperature and reproductive stage, can lead to qualitative and quantitative differences in the EOs produced [6-12].

The observed chemovariation might be environmentally determined by seasonal and altitudinal influence.

Table 2: Identified components in the essential oils of *P.vulgaris* subsp. *sibthorpii* [a,b].

Compounds	100 m	300 m	700 m	1200 m	1300 m	Ex.RI	Lit. RI
	% Area	% Area	% Area	% Area	% Area		
Nonane	1.0	-	-	-	-	900	900
α-Pinene[c]	1.0	0.4	0.3	0.5	0.6	939	939
Camphene	-	-	-	-	0.8	954	954
Benzaldehyde	-	0.3	0.3	0.4	0.4	960	960
β-Pinene[c]	1.3	0.7	0.4	-	-	975	979
2-Amyl furan	-	-	-	0.2	0.4	991	991
Mesitylene	0.6	0.1	-	-	-	990	996
α-Phellandrene	0.8	-	-	-	0.2	1003	1003
para-mentha-1(7),8- diene	-	0.2	0.2	0.3	-	1003	1004
ortho-Cymene	0.2	-	-	-	-	1020	1026
Limonene[c]	1.3	0.6	0.3	0.6	1.3	1029	1029
Acetophenone	0.3	-	-	0.4	-	1064	1065
3-Carene	0.4	0.4	-	-	-	1099	MS[1]
Nonanal	0.4	-	0.2	1.2	1.4	1101	1101
Camphor[c]	-	-	-	-	0.2	1146	1146
Methyl salicylate	-	1.9	2.8	-	-	1190	1192
Bornyl acetate	-	-	-	-	1.6	1289	1289
Tridecane	0.9	-	-	-	0.6	1300	1300
α-Cubebene	-	-	-	-	0.3	1351	1351
Tetradecane	0.5	-	-	-	-	1400	1400
E-Caryophyllene	-	-	-	-	1.5	1419	1419
Methyl-4-methoxy salicylate	21.0	37.2	30.8	8.2	3.2	1440	MS[2]
E-β-ionene	0.6	-	-	-	0.3	1480	1489
Eugenol asetat	-	-	-	-	0.4	1523	1523
α-Muurolol	2.0	-	-	-	-	1640	1646
Tetradecanol	1.7	-	-	-	-	1670	1673
Anthracene	2.2	-	0.2	-	-	1744	MS[3]
Octadecene	-	-	0.3	-	-	1780	1790
Chalcone	-	-	0.4	1.1	-	1802	MS[4]
Hexahydrofarnesyl acetone	-	0.7	0.7	1.0	1.7	1840	1847
Hexadecanal	2.5	-	-	-	-	1870	1876
11,14,17-Eicosanoic acid methyl ester	6.1	-	-	2.6	-	1875	MS[5]
Ethyl linoleolate	-	-	2.3	-	2.1	1890	1893
Nonadecane	-	-	0.2	-	-	1900	1900
Methyl hexadecanoate	2.7	1.2	0.6	1.5	2.5	1920	1922
Eicosane[c]	8.2	4.4	2.5	5.4	5.3	2000	2000
Methyl eicosa-5,8,11,14,17-pentaenoate	-	3.0	-	-	-	2062	MS[6]
Dodeca-1,8-dien-4-yne	-	-	2.0	-	-	2063	MS[7]
(*E,E,E*)-1,4,8-dodecatriene	-	-	-	6.2	-	2063	MS[8]
(Z,Z,Z)-9,12,15-octadeca-trien-1-ol	-	-	-	-	6.4	2064	MS[9]
7-Tetradecyne	-	1.2	1.1	2.1	2.5	2090	MS[10]
9,12,15-Octadecatrienoic acid methyl ester	-	-	-	-	9.4	2097	MS[11]
Heneicosane[c]	3.2	0.9	1.8	0.6	1.3	2100	2100
(Z,Z,Z)-7,10,13-hexadecatrienal	15.2	5.9	4.4	9.7	-	2101	MS[12]
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-	1.2	2.0	-	-	2105	MS[13]
Flavone	1.6	7.4	13.7	18.0	13.1	2170	MS[14]
Docosane[c]	5.2	2.4	4.5	6.7	11.4	2200	2200
Tricosane[c]	8.2	3.9	5.2	6.5	12.1	2300	2300
Tetracosane[c]	4.0	5.8	5.8	9.3	12.5	2400	2400
Pentacosane[c]	10.0	7.7	7.7	2.3	5.1	2500	2500
Total	94.9	87.9	90.7	85.4	98.6		
	% and N.C.	% and N.C.	% and N.C.	% and N.C.	% and N.C.		
Monoterpene hydrocarbones	5.0: 6	2.3: 5	2.2: 5	2.8: 4	3.7: 5		
Monoterpenoid	-	-	-	-	0.2: 1		
Sesquiterpene hydrocarbones	0.5: 1	-	-	0.3: 1	0.8: 2		
Sesquiterpenoid	2.0: 1	-	-	-	-		
Terpene related compounds	4.4: 3	8.1: 2	15.0: 4	20.1: 3	17.1: 5		
Hydrocarbones	33.0: 9	25.1: 6	28.0: 8	30.8: 6	48.3: 7		
Others	50.5: 9	52.4: 9	45.5: 9	32.0: 9	26.5: 8		
Total number of compounds	29	22	26	24	28		

MS[1]: 136(20), 121(25), 105(15), 93(100), 79(35), 67(10); MS[2]: 182(45), 150(100), 122(50), 107(35), 95(5), 79(15); MS[3]: 178(100), 152(5), 89(15),76(10); MS[4]: 208(90), 179(20), 131(45), 103(55), 77(100), 51(50); MS[5]: 121(20), 108(50), 95(55), 79(100), 67(65), 55(60); MS[6]: 119(45), 108(50), 91(75), 79(100), 67(60), 55(35); MS[7]: 162(5), 119(20), 105(10), 93(60), 79(100), 67(65); MS[8]: 133(20), 119(20), 105(10), 93(55), 79(100), 67(60); MS[9]: 121(10), 108(35), 93(40), 79(100), 67(70), 55(60); MS[10]: 234(5), 121(15), 108(35), 93(50), 79(100), 55(55), 41(75), 67(90); MS[11]: 108(35), 93(50), 79(100), 67(80), 55(55), 41(60); MS[12]: 121(20), 108(50), 95(55),79(100),67(65),55(60);MS[13]: 252(20), 98(35), 83(30), 71(85), 55(100), 25(15); MS[14]: 222(100), 194(50), 120(55), 92(45), 63(20). [a]RI calculated from retention times relative to that of *n*-alkanes (C_6-C_{32}) on the non-polar HP-5 column. [b]Percentages obtained by FID peak-area normalization. [c]Identified by authentic samples. N.C. Number of compounds.

The fatty acid methyl ester (FAMEs) compositions of the two *Primula* subspecies (*Pvv* and *Pvs)* are given in Table 3. In total, 13 FAMEs were detected by using GC and GC-MS in all species tested herein. The major FAMEs were palmitic acid methyl ester ($C_{16:0}$) (2.4-9.1% and 3.6-9.3%), and stearic acid methyl ester ($C_{18:0}$) (1.0-3.8% and 2.0-4.7%) in *Pvv* and *Pvs*, respectively. The total FAMEs percentages of *Pvv* and *Pvs* were between 5.8-24.9% in *Pvv* and 12.2-28.8% in *Pvs*. Oleic acid methyl ester ($C_{18:1}$) was detected only in *Pvs* (2.1-6.7%) at heights of 100 m and 1200 m, as well as caprylic acid methyl ester ($C_{8:0}$), which was determined at an elevation of 300 m in *Pvv* (0.8%) and 100 m in *Pvs* (0.8%). Caproic acid methyl ester ($C_{6:0}$) was found only in *Pvv* at a height of 700 m. Other fatty acids such as $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{18:3}$, $C_{18:2}$, $C_{20:0}$, $C_{22:0}$ and $C_{26:0}$ were also found in all of the tested species, but all of them were in small quantities (less than 3.5%). In the literature, the fatty acid contents of *P. sibthorpii* [25] were mentioned and oleic, linoleic and palmitic acids were reported to be major FAMEs. Stearidonic acid was reported in *P. florindae* [26], but, in our case, palmitic and stearic acids were the main FAMEs with different ratios in *Pvv* and *Pvs.*

The results of the antimicrobial assays of the EOs obtained from *Pvv* and *Pvs* collected at various altitudes, and assayed by the agar diffusion method [27-28] are shown in Table 4. The EO was

Table 3: FAMEs of *P. vulgaris* subsp. *vulgaris* and *P. vulgaris* subsp. *sibthorpii*[a,b].

FAMEs		*P. vulgaris* subsp. *vulgaris*								*P. vulgaris* subsp. *sibthorpii*					Ex. RI	Lit RI
		300 m	600 m	700 m	1100 m	1600 m	1700 m	1900 m	2100 m	100 m	300 m	700 m	1200 m	1300 m		
		%	%	%	%	%	%	%	%	%	%	%	%	%		
Methyl hexanoate	$C_{6:0}$	-	-	0.1	-	-	-	-	-	-	-	-	-	-	926	927
Methyl octanoate	$C_{8:0}$	0.8	-	-	-	-	-	-	-	0.8	-	-	-	-	1125	1127
Methyl decanoate	$C_{10:0}$	0.7	-	-	-	0.2	0.1	-	-	0.7	0.3	-	0.2	-	1323	1326
Methyl dodecanoate	$C_{12:0}$	0.8	0.3	0.4	0.3	0.9	0.4	-	0.3	0.5	0.4	0.6	0.6	-	1524	1526
Methyl tetradecanoate	$C_{14:0}$	1.9	1.5	0.6	0.7	1.1	0.5	-	0.3	1.4	1.3	1.9	6.1	1.5	1722	1724
Methyl hexadecanoate	$C_{16:0}$	8.2	5.2	2.7	4.0	9.1	5.9	3.8	2.4	9.3	3.6	5.2	6.1	6.2	1920	1922
Methyl linolenoate	$C_{18:3}$	0.9	-	2.0	-	5.7	-	-	-	-	-	-	1.1	-	2076	MS[1]
Methyl oleate	$C_{18:1}$	-	-	-	-	-	-	-	-	6.7	-	-	2.1	-	2080	MS[2]
Methyl linoleate	$C_{18:2}$	0.6	-	1.1	-	3.4	-	-	1.5	3.5	-	-	-		2094	2096
Methyl octadecanoate	$C_{18:0}$	3.8	3.1	1.1	3.2	3.5	2.4	2.0	1.0	3.7	2.0	3.3	3.2	4.7	2125	2125
Methyl eicosanoate	$C_{20:0}$	0.7	1.6	0.7	0.5	1.0	-	-	0.9	1.1	1.0	0.9	0.6	1.4	2273	2272
Methyl docosanoate	$C_{22:0}$	0.8	0.6	0.3	0.5	-	0.6	-	0.6	1.1	0.5	0.6	0.5	-	2440	2441
Methyl hexacosanoate	$C_{26:0}$	-	-	0.6	0.7	-	-	-	-	-	3.1	0.9	3.1	1.6	2534	MS[3]
	Total	19.2	12.3	9.6	9.9	24.9	9.9	5.8	7.0	28.8	12.2	13.4	23.6	15.4		

[a]RI calculated from retention times relative to that of FAMEs (C_4-C_{24}) on the non-polar HP-5 column. [b]Percentages obtained by FID peak-area normalization. MS[1]:292 (5),109 (20), 5 (50), 81 (75), 41 (90), 67 (100); MS[2]: 296(5), 264 (10), 111(20), 29 (40), 83 (50), 41 (90), 55 (100); MS[3]: 367 (10), 97 (20), 143 (30), 57 (40), 410 (50), 74 (100).

generally effective against *M. smegmatis* (MIC values varied from 8.5 to 51.8 µg/mL for *Pvv* and 15.8 to 59.2 µg/mL for *Pvs*). The activity against *M. smegmatis* can be explained by the high concentration of hydrocarbons and aromatic ester present in the EOs. Kubo has mentioned that the carbon chain length influences disorder in the lipidic bilayer [29]. Experimental results suggested that maximum activity against *M. smegmatis* might be dependent on the number of carbon atoms in the hydrophobic chain.

In summary, altitude variation indicates different chemical profiles for *Pvv* and *Pvs* with respect to the levels of major components of the EOs. The major components identified in EOs produced by *Pvs* and *Pvv* were methyl-4-methoxy salicylate and (*Z*,*Z*,*Z*)-7,10,13-hexadecatrienal. The high concentrations of methyl-4-methoxy salicylate in the oils of *Pvv and Pvs* grown in Trabzon suggest that these EOs could be used as a natural source of methyl-4-methoxysalicylate, because of the natural abundance of *Pvv and Pvs* in the region. Thirteen FAME constituents were identified from *Pvs* and *Pvv* which showed palmitic and stearic acid methyl ester to be major FAMEs. The results obtained, following the measurement of MICs, indicated that *M. smegmatis* was the most sensitive microorganism, showing the largest inhibition, and the lowest MIC values varied from 8.5 to 51.8 µg/mL for *Pvv* and 15.8 to 59.2 µg/mL for *Pvs*.

Experimental

Sample collection and isolation of EOs: *Pvv* and *Pvs* were collected at the flowering stage (March-April, 2012) from 11 different localities in Zigana valley, Trabzon-Turkey (at heights of ~100-2100 m). The plants were authenticated by Prof. Kamil Coşkunçelebi [1-3]. Voucher specimens were deposited in the Herbarium of the Department of Biology, Karadeniz Technical University (KATUB-Coşkunçelebi). Altitude, collection dates, yields, and herbarium numbers of the studied *Primula* taxa are listed in Table 5.

EOs of *Pvv* and *Pvs* were obtained from fresh plants (~55 g and 80 g each, respectively) by hydrodistillation in a modified Clevenger-type apparatus with a cooling bath (-12°C) system (4 h) [yields: 0.08-0.42% and 0.24-0.63%, w/w, respectively]. The obtained oils were dissolved in HPLC grade *n*-hexane (1 mL), dried over anhydrous sodium sulfate and stored at 4-6°C in a sealed brown vial. Two µL of the EOs were directly injected into the GC-FID-MS instrument.

GC and GC-MS: GC-FID and GC-MS analyses were made as described previously [23,24].

Identification of constituents: The identity of each compound was supported by comparing their retention indices (RI) with published values. Retention indices of all the components in the EOs (Table 1 and Table 2) and FAMEs (Table 3) were determined by Kovats method using *n*-alkanes and FAMEs (C_6-C_{32} and C_4-C_{24}, respectively) as standards. The constituents of the oils and FAMEs were identified by comparison of their mass spectra with those of mass spectral libraries [30] (NIST and Wiley 7NL), authentic compounds (α-pinene, β-pinene, camphene, limonene, eicosane, heneicosane, docosane, tricosane, tetracosane, pentacosane, and FAMEs; C_4-C_{24}) and with published data [6-10, 20, 30]. The samples were analyzed twice, and the percentage compositions of the samples were computed from the GC peak areas without using correction factors.

Preparation of fatty acid methyl esters (FAMEs): The fresh *Pvv* and *Pvs* materials (each 30 g) were ground into small pieces and refluxed in diethyl ether using a Soxhlet apparatus. Then, the total ether extracts were concentrated in a rotary evaporator to obtain crude mixtures (yields: 0.19 g, 0.65%: 300 m; 0.16 g, 0.52%: 600 m; 0.12 g, 0.41%: 700 m; 0.14 g, 0.48%: 1100 m; 0.16 g, 0.52%: 1600 m; 0.13 g, 0.42%: 1700 m; 0.16 g, 0.53%: 1900 m; 0.10 g, 0.34%: 2100 m in *Pvv* and 0.09 g, 0.29%: 100 m; 0.31 g, 1.02%: 300 m; 0.26 g, 0.87%: 700 m; 0.17 g, 0.58%: 1200 m; 0.26 g, 0.87%: 1300 m in *Pvs*.) The obtained lipid samples were heated with 5% sodium hydroxide solution in methanol (5 mL) at 60-70°C for 3-4 h. The solutions were cooled and the aqueous mixture was neutralized with 2 N HCl and extracted with *n*-hexane-diethyl ether (1:1, v/v). The organic layer was separated and washed with water (10 mL), dried over anhydrous Na_2SO_4 and filtered. Approximately 30 mg of each sample was dissolved in 2 mL methanol in a test tube and the solutions were cooled in an ice bath; an excess of BBr_3 was then added, dropwise. The tube was heated in a boiling water bath at 100°C for 6 h and cooled. Then water (3 mL) was added and extracted with *n*-hexane (2 mL x 2 times), shaking briefly. The hexane layers were washed with potassium bicarbonate solution (4 mL, 2%), dried over anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure in a rotary evaporator to yield FAMEs [12-18] and other lipids (yields: 0.0054 g, 2.76%: 300 m; 0.0054 g, 3.4%: 600 m; 0.0130 g, 10.7%: 700 m; 0.0141 g, 9.8%: 1100 m; 0.0178 g, 11.4%: 1600 m; 0.0165 g, 12.9%: 1700 m; 0.0077 g, 4.8%: 1900 m; 0.0229 g, 22.4%: 2100 m in *Pvv* and 0.0081 g, 9.0%: 100 m; 0.0203 g, 6.6%: 300 m; 0.0122 g, 4.63%: 700 m; 0.0093 g, 5.3%: 1200 m; 0.0037 g, 1.4%: 1300 m in *Pvs*).

Antimicrobial activity: The antimicrobial activity of the EOs from *Pvv* and *Pvs* was evaluated using *Escherichia coli* ATCC35218, *Yersinia pseudotuberculosis* ATCC911, *Pseudomonas aeruginosa* ATCC43288, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* 709 Roma, *Mycobacterium smegmatis* ATCC607, *Saccharomyces cerevisiae* RSKK 251 and *Candida albicans* ATCC60193. All test microorganisms were obtained from the Hifzisihha Institute of Refik Saydam (Ankara, Turkey). EOs were weighed and dissolved in *n*-hexane to prepare extract stock solution of 33.100-186.100 µg/mL.

The antimicrobial effects of the EOs were tested quantitatively in the respective broth media by using double dilution and the minimal inhibition concentration (MIC) values (µg/mL) were determined [27]. The antibacterial and antifungal assays were performed in Mueller-Hinton broth (MH) (Difco, Detroit, MI) / Tween 20 (0.25%) at pH 7.3 and buffered Yeast Nitrogen Base (Difco, Detroit, MI)/ Tween 20 (% 0.25) at pH 7.0, respectively. The microdilution test plates were incubated for 18-24 h at 35°C**.** Brain Heart Infusion broth (Difco, Detriot, MI) with Tween 20 (0.25%) was used for *M. smegmatis,* and incubated for 48-72 h at 35°C [28]. The MIC was defined as the lowest concentration that showed no growth. Ampicillin (10.000 µg/mL), Streptomycin 10.000 µg/mL and fluconazole (2.000 µg/mL) were used as standard antibacterial and antifungal drugs, respectively. *n*-Hexane at a dilution of 1:10 was used as solvent control.

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Table 4: Screening for antimicrobial activity of the essential oils of *Pvv* and *Pvs* (µg /100 mL).

Altitude		Stock Sol. (µg/mL)	Microorganisms and Minimal Inhibition Concentrations								
			Ec	*Yp*	*Pa*	*Ef*	*Sa*	*Bc*	*Ms*	*Ca*	*Sc*
Pvv	300 m	33.100	-	-	-	-	-	207	51.8	-	-
	600 m	155.000	-	-	-	-	-	-	15.1	-	-
	700 m	175.000	-	-	-	-	-	-	8.5	-	-
	1100 m	124.000	-	-	-	-	-	-	24.2	-	-
	1600 m	142.900	-	-	-	-	-	-	13.9	-	-
	1700 m	186.100	-	-	-	-	-	-	18.2	-	-
	1900 m	139.500	-	-	-	-	-	-	13.6	-	-
	2100 m	181.900	-	-	-	-	-	-	17.8	-	-
Pvs	100 m	152.300	-	-	-	-	-	119	59.2	-	-
	300 m	135.000	-	-	-	-	-	-	13.2	-	-
	700 m	139.490	-	-	-	-	-	-	13.6	-	-
	1200 m	98.200	-	-	-	-	-	-	38.4	-	-
	1300 m	162.200	-	-	-	-	-	-	15.8	-	-
Ampicillin		10	8	32	>128	2	2	<1			
Streptomycin		10							4		
Fluconazole		5								<8	<8

Ec: *Escherichia coli*, *Yp*: *Yersinia pseudotuberculosis*, *Pa*: *Pseudomonas aeruginosa*, *Ef*: *Enterococcus faecalis*, *Sa*: *Staphylococcus aureus*, *Bc*: *Bacillus cereus* 702 Roma, *Ms*: *Mycobacterium smegmatis*, *Ca*: *Candida albicans*, *Sc*: *Saccharomyces cerevisiae*.

Table 5: Altitude, collection dates, yields, and herbarium numbers of studied *Primula* taxa (*Pvv* and *Pvs*).

P. vulgaris subsp. *vulgaris*				*P. vulgaris* subsp. *sibthorpii*			
Altitude, ~ m	Collection dates	Yields (w/w) (%)	KATUB numbers	Altitude, ~ m	Collection dates	Yield (w/w) (%)	KATUB numbers
300	31.03.2012	0.40	1160	100	23.03.2012	0.48	1173
600	04.04.2012	0.12	1166	300	31.03.2012	0.63	1159
700	04.04.2012	0.17	1161	700	04.04.2012	0.34	1162
1100	23.04.2012	0.29	1167	1200	18.04.2012	0.24	1165
1600	23.04.2012	0.21	1168	1300	18.04.2012	0.61	1164
1700	23.04.2012	0.08	1171				
1900	23.04.2012	0.42	1170				
2100	23.04.2012	0.16	1169				

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