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HEAVY METAL ACCUMULATION AND CHEMICAL COMPOSITION OF ESSENTIAL OIL OF *JUNIPERUS OXYCEDRUS* L. (CUPRESSACEAE) GROWN ON SERPENTINE SOILS IN BULGARIA

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Abstract

This study investigated the heavy metal concentrations and chemical compositions of the essential oils of Juniperus oxycedrus L. (Cupressaceae), growing on serpentine soils in the Eastern Rhodopes Mountains, Bulgaria. Elevated Ni content in soils does not affect the development of Juniperus oxycedrus L. and the quality and quantity of oil obtained from it. Sixty components representing 98.10-98.92% of the total oil were identified. The major compounds were determined limonene (12.10-13-84%), γ-himachalene (7.47-12.58%), manoyl oxide (6.60-12.80%), α-pinene (6.41-8.78%), dibutyl phthalate (1.48-8.14%), δ-cadinene (2.93-6.33%), γ-cadinene (3.64-5.00%), β-bisabolene (2.98-4.29%) in needles oil. The Juniperus oxycedrus L. can be considered as "excluder plant," containing relatively low metal concentrations in the needles even in cases of high elemental concentrations in the soils. Metal concentrations for toxic elements in plants and oils were below the permissible limits for pharmaceutical purposes. Therefore, Juniperus oxycedrus L. found on serpentine soils is recommended to be collected for pharmaceutical purposes.

Key words: *serpentine soils, essential oil composition, heavy metals, Juniperus oxycedrus L.*

INTRODUCTION

Juniperus oxycedrus (red juniper, prickly juniper), a member of the cypress family (Cupressaceae), is an evergreen, shrubby plant or small tree forming red galbules (Franco, 2002). The species belongs to the genus Juniperus and is widely distributed in the Mediterranean region (Adams, 2008; Farjon, 2013, Semerdjieva et al., 2019). The distribution of the species on rocky, stony slopes in poor skeletal soils is of practical importance as it prevents erosion and regulates soil water content (Franco, 2002; Gussev, 2015).

All plant parts, including the galbules, contain aromatic essential oil (Semerdjieva et al., 2019). *Juniperus oxycedrus* essential oil is produced by steam distillation of young twigs and stem bark. The oil is resinous with a very dark brown colour, and has a characteristic tarry odour. *J. oxycedrus* oil is used in the food industry and has antimicrobial and antifungal biological properties (Cavaleiro et al., 2006).

The content and composition of juniper oil are influenced by (1) origin (location), (2) sex, (3) stage of phenological development, (4) plant part, and (5) method and duration of oil extraction (Cantrell et al., 2013; Zhelyazkov et al., 2013).

Figueiredo et al. (2006) reported that environmental conditions, geographical variations and genetic factors could affect the oil content of plants. Furthermore, some heavy metals are reported to affect the yield of essential oils from aromatic medicinal plants (Zhelyazkov et al., 2008). Yeritsyan & Economakis (2002) found that high Fe concentration in juniper reduced essential oil yield. The main components in the oil are α-pinene, α-felandrene, sabinene, myrcene and others (Adams et al., 2005; Medini et al., 2009; Derwich & Chabir, 2011). The composition of oil varies widely, which affects biological activity.

The present work aims to conduct a comparative study that will allow us to determine the amounts of heavy metals, macro and trace elements in serpentine soils, the needles of *Juniperus oxycedrus* and the quality of oil from juniper growing on serpentine soils.

MATERIALS AND METHODS

The plant material used in this study was randomly selected plants and needles from *J. oxycedrus* growing on serpentine soils in the

Eastern Rhodopes. The serpentine soils are collected near the village of Kazak, the village of Golyamo Kamenyane, and the village of Avren,

All samples were collected in late June and early July and air-dried. Oils were extracted from each needle sample with a Clevenger apparatus by steam distillation for 2 hours. At the end of each distillation, the oil was separated from the water, collected in glass vials, and stored until gas chromatographic analyses were performed. The oils were analyzed for heavy metals, and their chemical composition determined. Determination of the chemical constituents of the oil was performed on a 7890A gas chromatograph (Agilent Technologies) and a 5975C mass spectral detector (Agilent Technologies). Compounds were identified by comparing retention times and Kovacs relative indices (RI) with those of standard substances and mass spectral data from the NIST'08 library (National Institute of Standards and Technology, USA).

Soil samples were prepared for analysis by treatment with aqua regia (ISO 11466). The plant and oil samples were treated by the method of microwave mineralization. To determine the content of heavy metal, macro and microelement of in the plant and soil samples, an inductively coupled emission spectrometer (Jobin Yvon Horiba "ULTIMA 2", France) was used.

RESULTS AND DISCUSSIONS

The contents of heavy metals, macro and microelements in the studied serpentine soils are presented in Table 1. The soils were neutral to slightly alkaline and had medium to high organic matter content (data are no shown) = Ultramafic (serpentine) soils are considered unfavourable habitats for plants due to low nutrient content, calcium deficiency, magnesium toxicity and high concentrations of potentially toxic elements such as chromium, nickel, and cobalt.

High concentrations of Mg relative to Ca and elevated concentrations of Ni are considered important factors affecting plant growth and survival on serpentine soils.

Eastern Rhodope soils are characterized by elevated Fe, Ni, Cr, Co and Mn levels, which is typical of serpentine soils (Table 1). The Ni content ranges from 1365.1 to 2397.2 mg/kg and is considered typical (500-5000 mg/kg) for serpentine and Mg- and Fe-rich soils. Nickel is known to be toxic in soils at levels above 500 mg/kg (Allen et al., 1974), although toxicity is dependent on Ni bioavailability.

The total Cr content of soils ranges from 493.7 to 2233 mg/kg) and exceeds the upper limit given for soils by Allen et al. (1974) and Brooks (1987) (500 mg/kg).

While the Cu content at all sites was within normal limits (Kabata-Pendias and Pendias, 1984), the Pb content was significantly higher and reached a maximum level of 31.7 mg/kg, which is in agreement with the data reported by Babalonas et al. (1984) from serpentine soils in northern Greece.

The Zn content falls within the range of normal soils, and ranges from 43.5-90.3 mg/kg.

The Cd content of the soils is below the limits given by Allen et al. (1974). Mn concentrations in soils ranged between 1294.7 and 2733 mg/kg.

Total Ca concentrations in serpentine soil samples ranged from 2653.8 mg/kg to 5599.5 mg/kg. The Mg content is higher than Ca and ranges from 29182.6 to 63512.8 mg/kg, and the Ca/Mg ratio is less than 1.

The Fe content of serpentine soils is high and ranges from 69.9 to 457.0 mg/kg, typical for the Balkan Peninsula's serpentine soils (Salihaj & Bani, 2008; Pavlova, 2001).

The element composition of serpentine soils from the Eastern Rhodopes is similar to serpentine soils from the Balkan Peninsula (Brooks 1987), with high concentrations of metals such as Ni and Cr and relatively low Ca/Mg ratios. Ca/Mg ratios range from 0.088 to 0.091. The Ca and Mg levels found in these Bulgarian serpentine soils are typical of such soils from other areas (Brooks, 1987). The total Ni content of the soil is similar to that of Italian (Vergnan Gambi et al., 1982), Greek (Babalonas et al., 1984) and Albanian (Shallari et al., 1998) serpentine zones.

Table 2 presents the results obtained for the heavy metal, macro and microelement contents in the needles of *Juniperus oxycedrus* growing on serpentine soils in the Eastern Rhodopes.

Table 1. Content of heavy metals, micro and macro elements (mg/kg) in serpentine soils from Eastern Rhodopes, Bulgaria

Element	Min	Max	Average
Pb	0.56	1.87	1.13
Cd	0.26	0.38	0.32
Zn	6.1	29.2	12.8
Cu	2.9	4.45	3.8
Fe	69.9	457.0	178.2
Mn	26.2	85.7	43.0
P	270.9	676.4	490.0
Cr	0.26	2.38	0.96
Ni	2.44	46.7	16.4
Ca	5374.2	14295.1	8878.1
Mg	814.1	1984.3	1516.0
K	1278.5	2900.1	2209.5
Co	0.59	2.68	1.097

Table 2. Contents of heavy metals, macro and microelements (mg/kg) in *Juniperus oxycedrus* needles

Element	Min	Max	Average
Pb	0.56	1.87	1.13
Cd	0.26	0.38	0.32
Zn	6.1	29.2	12.8
Cu	2.9	4.45	3.8
Fe	69.9	457.0	178.2
Mn	26.2	85.7	43.0
P	270.9	676.4	490.0
Cr	0.26	2.38	0.96
Ni	2.44	46.7	16.4
Ca	5374.2	14295.1	8878.1
Mg	814.1	1984.3	1516.0
K	1278.5	2900.1	2209.5
Co	0.59	2.68	1.097

The Ni content of juniper needles ranged from 2.44 mg/kg to 46.7 mg/kg, while the Cr content ranged from 0.26 to 2.38 mg/kg.

Despite the high Ni and Cr contents in the soil, the amounts of Ni and Cr assimilated by the plants were small and did not exceed the limits suggested by Kabata-Pendias and Pendias (1984).

The elevated levels of Pb in soils found in the Kazak area are likely due to traffic on the roadway but not due to the soil-forming rocks. The Pb and Cd content of juniper needles is within normal ranges. Mn concentrations in soils ranged from 1294.7 to 2733 mg/kg and from 26.2 to 85.7 mg/kg in juniper needles. The high Mn levels in the soils of the Kazak area are atypical and are likely due to the soil-forming rocks, topography, vegetation, and high limiting soil matter content. Plant Mn values are within the ranges given by Kabata-Pendias and Pendias (1984).

The Fe content in juniper needles was lower than the limit of 500 ppm suggested by Allen et al. (1974).

It is noteworthy that the amount of Ca taken up by the plants is significant (ranging from 5374.2 to 14295.1 mg/kg), although the amount of Ca in serpentine soils is low. According to Proctor (1971), Ca is one of the elements influencing the reduction of heavy metal toxicity. Probably plants growing on serpentine soils absorb more Ca to compensate for the toxic action of various toxic metals. Karataglis et al. (1982) suggested that plants have a mechanism that allows them to take up significant amounts of Ca

The high Mg content of serpentine soils determines the high Mg content of plant tissues (mean range 1516.0 mg/kg). The Ca/Mg ratio in all plant samples was > 1. Plants from serpentine soils can maintain a ratio greater than 1 despite the minimum Ca levels found in the soils. They either have very efficient uptake systems or the ability to exclude Mg despite high soil concentrations.

The heavy metal content of juniper essential oil has also been determined (data not shown). The results indicate that most of the heavy metals in red juniper needles do not pass into the oil during needle processing. Therefore their content in the oil is significantly lower. The results show that the essential oils' heavy metal content is lower than the aboveground part of juniper, and the amounts of Pb, Zn and Cd in juniper oil are lower than the accepted maximum permissible values the oil meets the criteria for an environmentally friendly product.

Juniperus oxycedrus can be classified as an exclusion plant, containing relatively low concentrations of metals in the aboveground parts, despite high concentrations of the elements in the soil.

The results of the chromatographic analysis of essential oils obtained by processing *Juniperus oxycedrus* needles growing on serpentine soils in the Eastern Rhodopes are presented in Table 3.

Table 3. Chemical composition (%) of *Juniperus oxycedrus* oil

№	Component		Min	Max	Average
1	α-Pinene	933	6.41	8.78	7.67
2	Sabinene	969	0.45	1.21	0.79
3	β-Pinene	974	0.51	0.70	0.61
4	β-Myrcene	991	1.06	1.45	1.27
5	δ-2-Carene	1001	0.13	0.18	0.15
6	α-Phellandrene	1006	0.27	0.37	0.32
7	δ-3-Carene	1018	0.06	0.09	0.07
8	p-Cymene	1025	1.17	1.60	1.40
9	**Limonene**	**1029**	**12.10**	**13.84**	**13.18**
10	β-Phellandrene	1030	0.11	0.16	0.14
11	α-Terpinolene	1087	0.20	0.27	0.24
12	p-Cymenene	1089	0.17	0.23	0.20
13	β-Linalool	1096	0.56	0.77	0.67
14	n-Nonanal	1110	0.28	0.38	0.33
15	trans-Pinene hydrate	1119	0.10	0.14	0.12
16	α-Campholenal	1122	0.27	0.36	0.32
17	cis-Limonene oxide	1132	0.38	0.52	0.45
18	trans-Pinocarveol	1135	0.47	0.64	0.56
19	cis-Verbenol	1138	0.35	0.48	0.42
20	Pinocarvone	1160	0.13	0.18	0.16
21	Terpinen-4-ol	1174	0.18	0.25	0.22
22	p-Cymen-8-ol	1177	0.22	0.30	0.27
23	Cryptone	1183	0.89	1.22	1.07
24	α-Terpineol	1187	1.35	1.84	1.61
25	Myrtenal	1195	0.26	0.36	0.31
26	Verbenone	1204	1.01	1.39	1.21
27	trans-Carveol	1215	0.98	1.34	1.17
28	Phellandral	1275	0.21	0.29	0.26
29	Bornyl acetate	1285	0.31	0.42	0.37
30	α-Copaene	1374	0.70	0.96	0.84
31	β-Bourbonene	1386	0.39	0.53	0.47
32	7-epi-Sesquithujene	1390	0.29	0.39	0.34
33	α-Cedrene	1410	0.20	0.27	0.24
34	β-Caryophyllene	1417	0.73	0.99	0.87
35	β-Cedrene	1419	0.48	0.66	0.58
36	trans-Geranylacetone	1452	0.24	0.33	0.28
37	(Z)-β-Farnesene	1454	0.50	0.69	0.60
38	α-Caryophyllene	1456	0.62	0.84	0.74
39	γ-Muurolene	1476	0.30	0.42	0.36
40	α-Curcumene	1480	0.22	0.30	0.26
41	**γ-Himachalene**	**1482**	**7.47**	**1.58**	**10.63**
42	Germacrene D	1484	0.36	0.49	0.43
43	α-Muurolene	1499	0.72	0.98	0.86
44	**β-Bisabolene**	**1505**	**3.13**	**4.29**	**3.74**
45	**γ-Cadinene**	**1513**	**3.64**	**5.00**	**4.36**
46	δ-Cadinene	1524	2.93	6,33	4.93
47	α-Calacorene	1545	1.45	1,98	1.73
48	±-trans-Nerolidol	1556	0.34	0.68	0.44
49	β-Calacorene	1564	0.38	0.54	0.46
50	(-)-Spathulenol	1577	2.66	3.55	3.12
51	Caryophyllene oxide	1582	1.26	3.43	1.76
52	α-Cedrol	1596	1.87	2.56	2.24
53	tau.-Cadinol	1619	1.21	1.95	1.50
54	tau.-Muurolol	1638	1.40	2.24	1.89
55	Cadalene	1675	1.09	2.23	1.43
56	10-nor-Calamenen-10one	1702	0.35	0.84	0.46
57	(Z.Z)-Farnesyl acetone	1860	0.27	0.37	0.33
58	n-Nonadecane	1901	2.03	2.78	2.43
59	Dibutyl phthalate	1919	**1.48**	**8.14**	4.94
60	Manoyl oxide	1992	**6.60**	**12.80**	9.77

Sixty components were identified in Juniperus oxycedrus needles oils. The oil composition was dominated by limonene (12.10-13-84%), γ-himachalene (7.47-12.58%), manoyl oxide (6.60-12.80%), α-pinene (6.41-8.78%), dibutyl phthalate (1.48-8.14%), δ-cadinene (2.93-6.33%), γ-cadinene (3.64-5.00%), β-bisabolene (2.98-4.29%).

The content of monoterpenes in Juniperus oxycedrus oil ranged from 29.81 to 38.69%. Semerdjieva et al. (2019) found that the predominant component in the oil of monoterpenes is α-pinene (9.4-24.5%), followed by limonene (1.8-15.2%) and caryophyllene oxide (0.73-13.1%). However, the results obtained showed that the oil was dominated by limonene (12.10-13-84%,)) followed by α-Pyrene (6.41-8.78%), p-Cymene (1.17-1.60%) and β-Myrcene (1.06 -1.45%).

Studies have shown that α-pinene in the oil of needles of J. oxycedrus varies over an extensive range, from 40% to 57% in Spain, 20.7-85.6% in Italy, 6.3-70.7% in Portugal, Croatia and Greece, 27.4-58.0% in Tunisia, 22.5%-27.1% in the Republic of North Macedonia and 17.8-29.0% in Bulgaria (Adams, 1998, 1999; Angioni et al., 2003; Medini et al., 2010; Salido et al., 2002; Sela et al., 2013; Semerdjieva et al., 2019). Differences in plant genetics explain differences in the amount of α-pinene (at subspecies and cultivar levels) and seasonal variations in oil composition (Medini et al., 2010). According to Adams et al. (2005) limonene is present in higher concentrations in oil, including in Bulgarian samples. Samples with higher limonene content were characterized by large amounts of Manoyl oxide or β-Caryophyllene. The limonene content of needles varies considerably; in the Republic of North Macedonia it is between 2.8% and 18.1%, in Algeria it is 5.8%, in Corsica it is 1.2-1.3%, in Italy it is about 30%, in Greece it ranges from 17.1% to 27.7%, and in Bulgaria it is between 5.8% and 13.6% (Adams et al., 1999; Valentini et al., 2003; Boti et al., 2006; Sela et al., 2013; Semerdjieva et al., 2019).

The oil's sesquiterpenes ranged from 34.5% to 56.12%. The amount of sesquiterpene hydrocarbons varied from 22.31 to 37.71%. The major components of this group are γ-Himachalene (7.47-12.59%), δ-Cadinene (2.93-6.33%), γ-Cadinene (3.64-5.00%), β-Bisabolene (3.13-4.29%), α-Calacorene (1.45-1.98%). The oxygen-containing sesquiterpenes vary from 10.82 to 18.41% of the total oil composition. The oil contains (-)-Spathulenol (2.66-3.53%), α-Cedrol (1.87-2.56%), tau.-Muurolol (1.40-2.24%), Caryophyllene oxide (1.26-3.43%), tau.-Cadinol (1.21-1.95%), Cadalene (1.09-2.23%).

Four chemotypes (a-pinene type, limonene type, sabinene type and trans-pinocarveol type) of J. oxycedrus were identified by Dob et al. (2006). The oils we studied belong to the limonene type chemotype. Limonene is considered to be specific to the oil of J. oxycedrus from the Balkan Peninsula, including Bulgaria (Adams et al., 2005; Adams and Tashev, 2012).

Figure 2 presents the classification of the identified compounds based on functional groups. The highest content of sesquiterpene hydrocarbons was found in juniper leaf essential oil (24.13-37.71%), followed by monoterpene hydrocarbons (22.64-28.87%), oxygen-containing sesquiterpenes (10.82-18.41%), diterpenes (6.60-12.80%), oxygen-containing monoterpenes (7.17-9.82%) and other classes of organic compounds (4.54-12.33%) (Figure1).

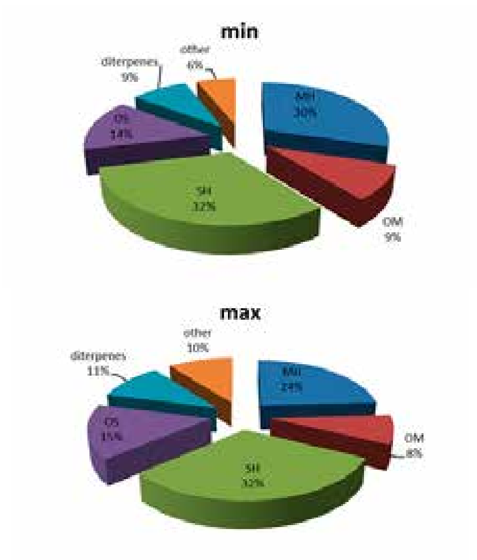

Figure 1. Classification of identified compounds based on functional groups in juniper needles oils

CONCLUSIONS

Based on the results obtained, the following conclusions can be drawn:

1. The chemical composition of serpentine soils from the Eastern Rhodopes is similar to serpentine soils from the Balkan Peninsula and is characterized by a high content of Ni (1365.1-2397.2 mg/kg), Cr (493.7-2233 mg/kg), and Mg (29182.6-63512.8 mg/kg).
2. The unfavourable soil characteristics do not affect the development of red juniper, nor the quality and quantity of the oil obtained.
3. *Juniperus oxycedrus* is an exclusionary plant containing relatively low concentrations of metals in aboveground parts despite high concentrations of elements in the soil.
4. The highest content of sesquiterpene hydrocarbons in juniper needles essential oil (24.13-37.71%), followed by monoterpene hydrocarbons (22.64-28.87%), oxygen-containing sesquiterpenes (10.82-18.41%), diterpenes (6.60-12.80%), oxygen-containing monoterpenes (7.17-9.82%), and other classes of organic compounds (4.54-12.33%).

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