VARIATION OF MONOTERPENE CONTENT AND SPECIFIC INTERGENIC REGION OF cp-DNA GROUP NATURAL POPULATIONS OF *SALVIA OFFICINALIS* L. OF NORTHERN ALBANIA IN A SIMILAR ORDER

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ABSTRACT

Essential oils were analyzed from sage populations of northern Albania, which were previously verified for their genetic relatedness based on the RFLP of a specific intergenic region of cpDNA. Samples were collected in May 2016 and dried in the shade. The essence was isolated using hydro-distillation for 16 hours in Clevenger apparatus as recommended by the European Pharmacopoeia. Essential oils were extracted using toluene, and injected into Varian 450 GC equipped with PTV injector, capillary columns and FID detector. VF-1ms capillary column (30 m x 0.33 mm x 0.25 um) was used for the separation of chemical components of the essential oils. Compounds of major content were α-thujone (18.67%-36.1%), camphor (10.98% - 22.95%), 1,8-cineole (8.4% -22.23%), camphene (2.14% - 11.43%), β-thujone (2.29% - 7.67%), α-pinene (2.2% -9.42%). The content of minor monoterpenes is up to 5%; Sesquiterpenes are presented by β-caryophyllene and humulene, which count for less than 10%; The contribution of terpenoid groups varied among populations (monoterpene components 0.02- 36.1%, and sesquiterpenes from 0.25-8.02%).Based on chemical profiles populations can be considered as very close (Postriba-Valbona and Torovica-Malësi e Madhe); can be grouped (Shkodra-Rubik; F.Milot-Shëngjin); or are completely distinct (Balldren, Kruja, Lohe, Ulza, Lezha). Grouping of populations according to their genetic (RFLPs of intergenic cp-DNA) or chemical composition of essential oils are in accordance. According to ISO 9909 and the German Drug Codex for medicinal use, most of the natural populations of northern Albania have the proper amount of α-thujone, champhor, 1.8-cineole, β-thujone, α-humulene, linalool, borneol and bornyl acetate, and higher amounts of α-pinene, camphene, and limonene.

Keywords: *Salvia officinalis*, essential oils, monoterpene, GC/FID, α-/β-thujone, camphor.

INTRODUCTION

Dalmatian sage or common sage (*Salvia officinalis* L.) from *Lamiaceae* family is one of 1,000 Salvia species (Walker *et al.*, 2007) native to the east side of Adriatic (Ristic *et al.*, 1999) and Ionian seas (Karousou *et al.*, 2000). It is also cultivated in temperate regions all around the world (Echeverrigaray *et al.*, 2006; Cunha *et al.*, 2003), and widely used in food, pharmaceutical, and cosmetic industries and has a wide range of medical effects (Bouaziz *et al.*, 2009).

A number of papers report on gastroprotective (Mayer *et al.*, 2009), antidiabetic (Eidi*et al.*, 2009), anti-obesity (Ninomiya *et al.*, 2004), anti-inflammatory (Baricevic*et al.*, 2001), antispasmatic (Todorov*et al.*, 1984), virucidal (Tada *et al.*, 1994), fungicidal, and bactericidal (Bouaziz *et al.*, 2009); (Delamare *et al.*, 2007); (Pinto *et al.*, 2007) properties.It is believed that sage has a great impact to improve Alzheimer's disease and decreases irritability (Akhondzadeh *et al*., 2003), improves mood and cognitive performance, reduces anxiety of young people (Kennedy *et al*., 2006), improves memory and attention of older people (Scholey *et al.*, 2008), and inhibits HIV-1 reverse transcriptase (Watanabe *et al.*, 2000).The pharmaceutical properties of aromatic plants included sage are mainly attributed to their essential oils. The term 'essential oil' was first used by Paracelsus von Hohenheim in 16th century to name the effective component of a drug, 'Quinta essential' (Guenther, 1950). These complex mixtures, constituted by terpenoid hydrocarbons, oxygenated terpenes and sesquiterpenes, which originate from the plant secondary metabolism,areobtained with distillation and extraction (Chamoro *et al*., 2011). The major constituents identified in sage essential oils are 40, the most commonly found of which are α- and β-thujone, camphor, 1,8-cineole, humulene, borneol acetate, limonene, viridiflorol, caryophyllene, manool (Asllani, 2000; Velickovic *et al*., 2003; Grausgruber-Gröger *et al*., 2012; Said-Al Ahl*et al*., 2015; Couladis *et al*., 2012,etc).

However, reports from different parts of the world show a great variability in essential oil composition. Thus, sage from Lithuania has the essential oil richest in manool (20.9%) (Bernotiene *et al*., 2007) with dominant constituents 1,8- cineole and cis-thujone. The sage from Brazil has major oil constituents: α-thujone, camphor, α-pinene and β-thujone (Porte *et al*., 2013).

Samples from Egypt revealed the presence of camphor, α-thujone, sclareol, β-thujone, 1, 8-cineole, γ-selinene, α-humulene, caryophyllene, borneol, limonene and humulene epoxide (Said-Al Ahl *et al*., 2015). According to Couladis (2002) the main compounds in samples from Serbia were oxygenated monoterpenes as α-thujone, β-thujone, 1.8-cineol, camphor, borneol and bornyl acetate. Among the dominant sesquiterpenes were α-humulene, viridiflorol and manool.

Among the 40 identified constituents in Montenegro's samples (Stesevic *et al.*, 2014) the most abundant were *cis*-thujone, camphor, 1,8-cineol, *trans*-thujone, camphene, borneol, viridiflorol, limonene, *α*-pinene, and *α*-humulene. In all samples analyzed in Central Italy (Menghini *et al*., 2012) 1,8 cineol is present in constant range, larger differences are found in α-pinene content, and α-thujone is always the main component. Jug-Dujakovic´ *et al*, (2012), reported that among the 62 compounds detected in Bosnia-Herzegovina, eight of them (cis-thujone, camphor, trans-thujone, 1, 8-cineole, b-pinene, camphene, borneol, and bornyl acetate) made 78.13–87.33% of essential oils of individual populations.The chemical composition of essential oils from Albanian sage was also examined,and over 40 compounds were detected in the oils, from which about 30 could be identified. The main components identified were α-thujone, β-thujone, camphor and 1,8-cineole (Asllani, 2000; Kongjika *et al*., 2005; Bacu *et al*., 2005; Babani *et al*., 2005). In a report of 2015,Cvektovikj showed that in Southeast Europe among 80 detected compounds the major were *β*-pinene, 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor, borneol, *trans*-caryophyllene, *α*-humulene, viridiflorol, and manool, which represented 42.60 to 85.70% of the components in the analyzed essential oils. Also, strong correlations were observed between the contents of *trans*-caryophyllene and *α*-humulene, *α*-humulene and viridiflorol, and viridiflorol and manool.

Nowadays, ISO 9909 and the German Drug Codex regulate the amounts of constituents in the sage essential oils for trade purposes. The Codex regulates the amounts of five, while ISO 9909 of eleven compounds. The lower limit of camphor in the German Drug Codex is 3 times and the upper limit by 1.5 times higher than that recommended by ISO 9909. The medicinal

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properties of the essential oils of sage have been properly investigated (Kintzios, 2000; Bowles, 2003; Lawrence, 2005; Tegmeier *et al*., 1995). Considering the pharmaceutical importance of each component of the essential oils, a number of studies considered the evaluation of their variation during phenological periods. The best moment for harvesting is the initial flowering period, if the objective is a high content in alpha-thujone, and during full flowering if the objective is a high yield in essential oil, concludes Arraiza (2012). Other studies consider the seasonal influence on the formation of main monoterpenes, and conclude that all monoterpene synthases and monoterpenes were significantly influenced by cultivar and season (Grausgruber-Gröger *et al*., 2012). Major seasonal changes were found in the composition of oil distilled from a flowering type of Dalmatian sage, but oil yields from healthy, established plants did not vary greatly. Total thujone levels were lowest around flowering in spring and summer, so autumn or winter was the best harvest time to obtain oils with high thujone levels (Perry,1999). A number of studies consider the genomic organization and regulation of terpene synthase coding genes, and on the possible use of these gene families to distinguish among close populations of sage. Monoterpenoid essential oils are biosynthesized in plants by a pathway which is different from the established mevalonic acid route (Eisenreich, 1997), and many reports prove that monoterpene synthases are compartmentalized in plastids and monoterpene synthesis can occur in photosynthetic paranchyma cells (Bouvier, 2000). The nuclear ribosomal internal transcribed spacer region and three chloroplast regions (rbcL, matK and trnH-psbA) were used (Li *et al*., 2013; Ibrahim *et al*., 2012; Papa &Bacu, 2016; etc) to describe the phylogenetic relationships among sage populations.

Aiming to understand factors, which regulate the expression of terpene synthases coding genes, Grausgruber-Gröger (2012) reported that terpene synthase mRNA expression and respective end product levels were concordant in the case of 1,8-cineole and camphor indicating basically transcriptional control, but discordant for α-/β-thujone.

Considering the variability of the qualitative and quantitative data on the essential oils constituents from sage of different origin, and knowing that behind that there is the complexity of gene families coding for terpene synthases, their regulated expression, and possible post-translational modification, here we compare the genetic variability among sage populations of northern Albania based on cpDNA-RFLP of a specific *trn* region (Papa, 2016), with chemical variability of their essential oils.

METHODOLOGY
Plant Material

Dried plant material from Dalmatian sage (*Salvia officinalis*, L.) were collected in 13 areas of northern Albania (13 populations) as described in Table 1. Samples were collected during spring-summer 2016 and were dried in shadow for a better preservation of their chemical compounds.

Isolation of Essential Oils for *Salvia officinalis* L.

50g of areal parts from sage plants dried in shadow were a subject of hydro-distillation for 16 hours with Clevenger apparatus (recommended from Pharmacophea Europea, 2014) for essential oil isolation. Essential oil was collected in 1 ml toluene as an extraction solvent. Water was eliminated by adding 1gr of sodium sulfate (Asllani, 2004). It was preserved in dark vials in 4^0C. Essential oil dissolved in toluene was used in GC/FID analysis.

Gas-chromatography Analysis

Varian 450 GC apparatus, equipped with PTV injector FID was used to evaluate essential oil composition. Essential oil composition was determined in capillary column VF-1m (30 meter length x 0,3 mm interne diameter x 0,25µm film), adapted for their separation. Injector and detector temperature were programmed 280^{0}C and 300^{0}C respectively. The method of injection was selected *split* (1:100). Nitrogen was used as a carrier gas and helper gas with a total flow respectively 1ml/min and 24 ml/min. Initial temperature of the oven was maintained 50^{0}C for 2 minutes, then it was increased in 150^{0}C with 60^{0}C/min. After that the temperature increased in 280^{0}C with 8^{0}C/min and at last in 300^{0}C with 10^{0}C/min. When the temperature reached 300^{0}C it was stopped in that level for 2 minutes. It was injected for each sample a volume of 2 µl. In order to determine a quality and quantity evaluation it was used a mixture of n-octane (C8) up to (C20) to calculate Kovats index. Kovats index with literature dates were used to identify principal components (Adams, 1995; David *et al*, 2010;). Quantitative results of the analyzed components are presented in percentage against the total value.

Table 1.GPS coordinates of sampled populations.

Populations	Latitude	Longitude	GPS coordinations
Krujë	41.493402	19.771796	41° 29' 36.2472" N19° 46' 18.4656" E
Torovica	41.892877	19.530494	41° 53' 34.3572" N19° 31' 49.7784" E
Shkodër (Shirokë)	42.066447	19.428860	42° 3' 59.2092" N19° 25' 43.896" E
Rubik	41.766822	19.777343	41° 46' 0.5592" N19° 46' 38.4348" E
M.Madhe (Grishaj)	42.281956	19.453365	42° 16' 55.0416" N19° 27' 12.114" E
Balldren	41.799867	19.630841	41° 47' 59.5212" N19° 37' 51.0276" E
Lohe	42.273489	19.536202	42° 16' 24.5604" N19° 32' 10.3272" E
F.Milot	41.700425	19.725456	41° 42' 1.53" N19° 43' 31.6416" E
Postribë	42.145131	19.592587	42° 8' 42.4716" N19° 35' 33.3132" E
Shëngjin	41.824211	19.577079	41° 49' 27.1596" N19° 34' 37.4844" E
Valbona	42.441253	19.883999	42° 26' 28.5108" N19° 53' 2.3964" E
Ulza	41.677475	19.864193	41° 40' 38.91" N19° 51' 51.0948" E
Lezhë	41.777724	19.658028	41° 46' 39.8064" N19° 39' 28.9008" E

RESULTS AND DISCUSSION

The essential oils content for thirteen natural populations of sage of northern Albania showed that from 20 components of essential oils analyzed with GC/FID (figure 1, table 1), the major compounds are α-thujone, β-thujone, camphor, 1,8-cineole, α-pinene, and camphene.
Alfa-thujone is the most predominant compound with a range from 18.67% (Shëngjin population) to 36.1%(Postriba population), followed by camphor with a range 10.98% (Balldren) to 22.95% (Rubik);1.8-cineole with 8.4% (Valbona) to 22.23% (Shkodër);camphene with 2.14% (Balldren) to 11.43% (Rubik); β-thujone with2.29% (Ulza) to 7.67% (Valbona), and α-pinene with 2.2% (Postriba) to 9.42% (Kruja). Other monoterpenes found were borneol (1.21% in Balldren-3.89% in Shëngjin), β-pinene (0.88% in Torovica-2.44% in Lezha), myrcene (0.4% in Ulza-1.96 in Lezha), bornyl acetate (0.33%

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in Shkodër-2.77% in Lohe). Two out oftwenty analyzed compounds were sesquiterpenes, β-caryophyllene and humulene, found in a quantity which corresponds to 0.25% (Malësi e Madhe, Grishaj) to 7.62% (Balldren), and 0.32% (Shëngjin) to 8.02% (Ulza), respectively. The rest of components were in amounts less than 1%. The contribution (in percentage) of terpenoid groups varied among populations. Monoterpene componentsranged from 0.02-36.1%,and sesquiterpenes from 0.25-8.02%. Minordifferences were observed for the oxygenated monoterpenes. Myrcene was the monoterpene found in lower amount (0.4%-2.84%). (Table 1) The results on the essential oils content for populations of northern Albania (Table 1, Fig 1,2,3) are in accordance to other reports regarding the main terpenoid compounds (Said-Al Ahl*et al*., 2015; Porte et *al*., 2013; Couladis *et al*., 2002; Stesevic *et al*., 2014; Menghini *et al*., 2012; Jug-Dujakovic´ *et al*., 2012). Meanwhile, the quantities of the compounds, as expected, differ from those reported in Mediterranean countries and other areas of the world, as well as among local populations under study.Important difference in amount was displayed for α-thujone, which in northern Albania's samples resulted to be in higher level compared to Brasil populations (Porte *et al*., 2013); Egyptian populations (Said-Al Ahl *et al*., 2015); Serbian populations (Couladis *et al*., 2002), Montenegro populations (Stesevic *et al*., 2014), and Central Italy (Menghini *et al*., 2012). While populations of Bosnia-Herzegovina show higher percentage for α-thujone (Jug-Dujakovic´*et al*., 2012) compared to our samples.

Name	Korca	Kruja	Torovica	Shkoder	Rubik	Malesi Madhe (Grishaj)	Balldren	Shengjin	Lohe	Ulza	Postriba	F.Milot	Valbona	Lezha
Alpha-pinene	5,52	9,42	3,72	8,28	5,86	4,01	4,46	4,17	3,22	2,76	2,2	3,4	2,93	4,19
Camphene	7,4	7,93	6,21	11,02	11,43	7,31	2,14	3,32	4,46	6,3	4,1	6,3	3,93	5,14
Beta-pinene	2,1	1,34	0,88	1,19	1,05	0,93	2,37	0,98	2,01	1,52	2,1	1,4	1,42	2,44
Myrcene	1,2	0,86	1	1,36	1,21	1,41	0,97	0,76	2,84	0,4	0,94	1,24	0,69	1,96
Limonene	0,13	0,02	0,02	0,12	0,3	0,08	0,11	1,48	1,22	0,59	2	2,3	1,3	1,81
alfa-Terpinene	0,81	0,15	1,28	1,17	1,36	0,11	0,95	3,53	2,39	0,57	2,41	0,92	0,43	0,32
1,8- Cineole	12,9	13,92	13,86	22,23	12,46	10,47	12,99	13,4	10,9	19,04	9,4	10,8	8,4	16,17
p-Cymene	0,1	0,25	0,13	0,32	0,07	2,35	0,27	1,34	0,6	2,29	0,43	1,31	0,92	0,58
gamma-Terpinene	0,32	0,13	0,08	0,24	0,9	0,006	0,04	0,43	0,55	0,04	0,28	0,72	0,51	0,66
Cis-Sabinene hydrate	0,1	0,1	0,02	0,12	0,2	0,001	0,4	0,51	0,32	0,05	0,3	0,52	0,42	0,48
Linaleol	0,25	0,1	0,01	0,1	0,08	0,23	0,09	0,59	0,12	0,17	0,2	0,4	0,43	0,52
Alfa-Thujone	32,9	26,32	31,17	18,73	27,52	32,15	32,9	18,67	29,15	27,64	36,1	21,7	32,43	22,95
Beta-Thujone	5,6	5,86	5,73	4,27	3,42	6,66	8,45	2,53	3,64	2,29	4,7	3,7	7,67	4,19
Camphor	16,5	16,94	21,55	22,61	22,95	22,68	10,98	21,97	21,48	22,84	17,6	26,1	16,02	17,92
Borneol	2,1	1,56	2,44	1,24	1,86	2,68	1,21	3,89	1,32	2,4	1,6	3,2	3,14	2,39
Terpinen-4-ol	0,52	0,46	0,56	0,22	2	0,19	0,14	0,28	1,05	1,21	0,32	0,37	0,45	0,28
alfa-Terpineol	0,1	0,11	0,22	0,82	0,02	0,13	0,18	2,51	0,75	0,19	1,34	0,83	0,71	0,89
Bornyl acetate	1,2	1,67	1,02	0,33	0,49	1,63	2,08	1,75	2,77	2,64	1,5	2,3	1,16	1,64
Caryophyllene	2,4	3,6	0,8	2,07	0,95	0,25	7,62	6,36	2,68	4,5	3,2	3,5	3,74	5,23
Humulene	2,7	3,64	1,44	2,71	0,93	0,98	6,43	0,32	0,46	8,02	6,7	5,6	5,39	4,27

Table 2. Essential oils content (20 compounds) for populations of *Salvia officinalis* L. of Northern Albania.

The total amount of monoterpenes found in northern Albania populations is lower than that reported from samples from Central Herzegovina and Southern Europe (Cvektovikj *et al*., 2015; Jug-Dujakovic´ *et al*., 2012). Considering the sesquitepene content, the essential oils from northern Albania contain two compounds, humuleneand β-caryophyllene, while samples from Eastern Lithuania (Bernotien *et al*., 2007), Serbia (Couladis *et al*., 2007), Southern Europe (Cvektovikj *et al*., 2015), and Montenegro (Stesevic *et al*., 2014) have a small percentage of three extra constituents.

Considering differences in major constituents among local Albanian populations, it is obvious that samples from Torovicë, Malësi e Madhe, Lohe, Postribë, Balldren, Valbonë have the highest amount of α-thujone (Table 1, Fig 2). Populations of Balldren and Valbonë have the highest values of β-thujone; Eight out of 13 the populations have camphor content more than 20%; There is a high variability in 1, 8-cineole and α-pinene content.

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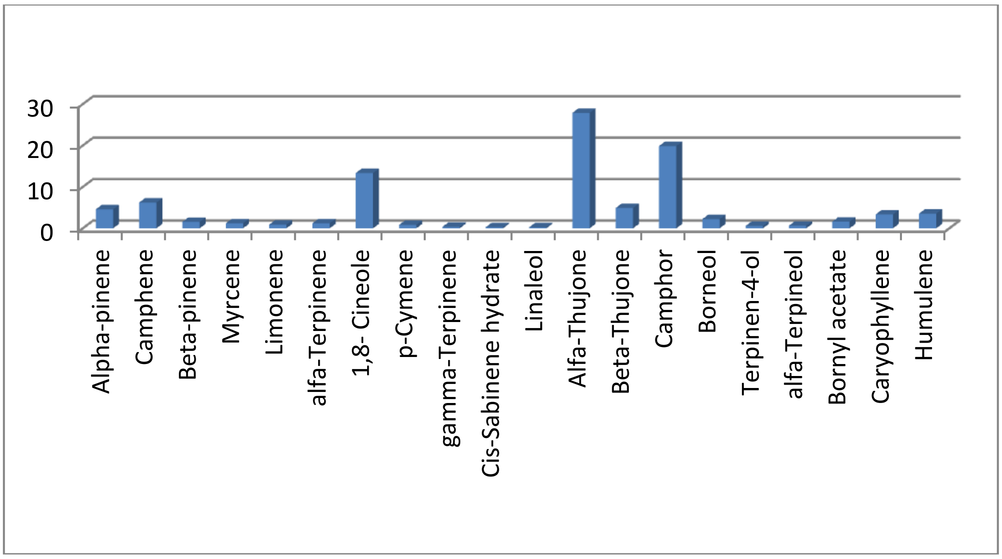

Fig. 1. Mean average values of essential oil components for populations of sage of northern Albania.

The camphene content shows a peek for two populations (Shkodra, Rubik), while the rest of populations have about half of the amount.The comparison of the essential oils content at the 13 populations under study can serve to group them in six main groups, which are not connected by geographical proximity or altitude above the sea level (Fig2).

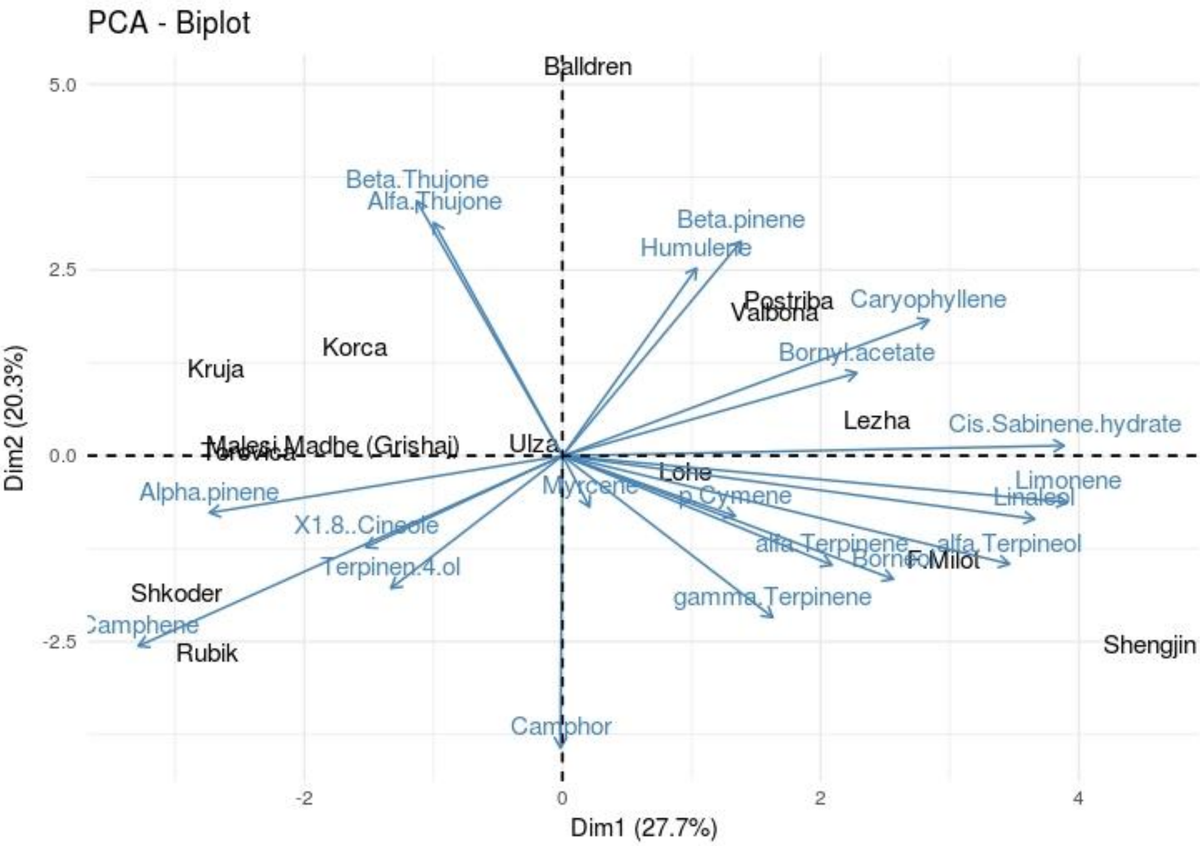

Fig 2. Grouping of populations of natural sage of northern Albania according to the essential oils content.

According to ISO 9909 for medicinal use, not all the components of the sage essential oils under study match the requirements of ISO 9909 andthe German Drug Codex (Introduction). Most of the natural populations of northern Albania have the proper amount of α-thujone,

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champhor,1, 8-cineole, β-thujone, α-humulene, linalool, borneol and bornyl acetate, but other components (Figure 1) such as α-pinene, camphene, and limonene resulted in higher amounts.

The comparison of the grouping of sage populations based on the RFLPs of trn of cp-DNA (Papa *et al*., 2016) to the one described in this work, shows that they are grouped in a very similar way. A number of reports consider the use of intergenic regions of cpDNA as a very important tool for systematic studies at population level because of the high variability of these regions compared to the conservancy of coding regions of cpDNA. Thus, the variability of monoterpenes, as the end products of monoterpene synthase coding genes, which corresponds to the variability of *trn* regions of cp-DNA, could be explained with possible roles of these non-coding areas on the regulation of the expression of monoterpene coding genes.

CONCLUSIONS

1. Results show that major components of the essential oilsof populations of northern Albania were α-thujone, β-thujone,camphor, cineol, α-pinene, and camphene.
2. Important difference in amount was displayed for α-thujone, which in northern Albania's samples resulted to be in higher level compared to Brasil, Egyptian, Serbian, and Central Italy populations, but lower than those from Montenegro.
3. The content of minor monoterpenes is up to 5%; The presence of sesquiterpenes is verified for β-caryophyllene and humulene, which count for less than 10%;
4. Based on chemical profiles populations can be considered as very close (Postriba-Valbona and Torovica-Malësi e Madhe); can be grouped (Shkodra-Rubik; F.Milot-Shëngjin); or are completely distinct (Balldren, Kruja, Lohe, Ulza, Lezha).
5. Grouping of populations according to RFLPs of intergenic cp-DNA or chemical composition of essential oils are in accordance.

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