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Seasonal variation in the essential oil of *Satureja kitaibelii* determines chemotypes

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ABSTRACT

The aim of the present study was a chemical and chemometric analysis of *Satureja kitaibelii* Wierzb. ex Heuff. essential oil during different stages of development, with emphasis on chemotaxonomy. The most abundant compound class in the *S. kitaibelii* oil during examined phenological stages was oxygenated monoterpenes (21.3–61.2%), except vegetative stage, in which monoterpene hydrocarbons are dominant (51.1%). Geraniol, limonene, *p*-cymene and borneol are the most abundant compounds of essential oil during different stages of development. The highest variation in content, during plant development, was shown by the most dominant component, geraniol (coefficient of variation 112.3%). Chemometric analysis indicated two chemotypes: limonene/*p*-cymene/geraniol and limonene/*p*-cymene/borneol. We are of the opinion that the three most abundant EO components should determine the chemotype. Our results lead to the conclusion that essential oil isolated from the plant in 1 month should not be used to determine the chemotype.

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KEYWORDS
Satureja kitaibelii; essential oil; chemometric analysis; chemotaxonomy

1. Introduction

Chemotaxonomy helps distinguish plants that cannot be separated by morphological evidence, but that are easily distinguished by significant differences in the chemical composition of their essential oils. There was no agreed procedure for the classification of chemotypes. In most studies, the content of the dominant component of the essential oil is a criterion for chemotaxonomic classification. Obviously, it is difficult to distinguish "essential oil chemotypes" based on one dominant compound, since there are usually two or more main compounds that may be present at almost equal amounts (1).

The genus *Satureja* L. includes more than 30 species, with large number of polymorphic species and a number of infraspecific species. *Satureja kitaibelii* Wierzb. ex Heuff. is a perennial semi-bushy plant that inhabits dry, sunny and rocky areas, with the endemic distribution in southeast Serbia, southwest Romania, and northwest Bulgaria (2). Plants of this taxon are 30–50 cm high, characterized by white trichomes on opposite sides of a square stem. The leaves are entire, elongated lanceolate and rigid, densely covered on both sides with glandular trichomes containing essential oils (3). *S. kitaibelii* is commercially available under the name 'Rtanj tea' and has been used in traditional medicine for many years to treat diarrhea, nausea, cramps, indigestion, respiratory and infectious diseases, and in the Mediterranean kitchen as a culinary herb (4,5).

The chemical composition of *S. kitaibelii* essential oil (EO) is quite variable. The main component of *S. kitaibelii* EO (flowering stage of the plant life cycle) from several localities in southeastern Serbia is geraniol (6–10). In recent work, *S. kitaibelii* EO (June 2018) from Kravlje village, southeast Serbia, after multiple checks, contains no geraniol (11). According to some authors, *S. kitaibelii* essential oil from Serbia showed three potential chemotypes: geraniol, *p*-cymene and limonene (9).

Having in mind these facts, also given the importance of *S. kitaibelii* as a medicinal remedy and culinary herb, the aim of the present study was chemical and chemometric analysis of essential oil from natural populations *S. kitaibelii* at the Kravlje village, southeastern Serbia, during different stages of development, with emphasis on chemotaxonomy.

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2. Experimental

2.1. Plant material and chemicals

The aerial parts of *Satureja kitaibelii* Wierzb. ex Heuff. family Lamiaceae were collected during 2020 from a natural population at the Kravlje village (southeast Serbia), 43°27′16″ N, 21°54′20″ E, elevation 332 m a.s.l., quartz habitat with temperate continental climate. The plants were collected on the 15th in the months, at three different stages of development: vegetative stage (June); flowering stage (July and August); after flowering stage (September, October and November). At the studied locality, 48 shrubs were marked (12 on each cardinal point). The aerial parts of *Satureja kitaibelii* were collected by multi-point mixing method, hence 150 g sample, which was repeated three times.

Dr Marija Marković did identification of plant material, and the voucher specimen (accession number 13220) is deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš (Herbarium Moesiacum Niš – HMN).

All chemicals, reagents and standards were of analytical reagent grade and purchased from Sigma-Aldrich Chemical Company (Germany).

2.2. Oil isolation

Aerial parts of the plant (dried and pulverized) were subjected to hydro-distillation for 3 h, using the Clevenger-type apparatus, according to the European Pharmacopoeia (12). The resulting EOs were dried over anhydrous sodium sulfate and stored at 4°C.

2.3. Gas chromatographic and gas chromatographic-mass spectrometric analysis

The gas chromatographic analysis of the oils was carried out on a GC HP5890 II apparatus, equipped with the split – spitless injector, HP-5 MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) with helium as the carrier gas (1 mL/min) and flame ionization detector. Operating conditions were injector temperature of 250°C and interface temperature of 280°C and temperature program from 50°C (3 min) to 250°C at a rate of 3°C min^{-1}.

Gas chromatographic-mass spectrometric analysis analyses were performed on an Agilent Technologies apparatus, Model GS 6,890 N at 70 eV coupled with a mass selective detector MSD 5975C, under the same gas chromatographic conditions.

2.4. Identification of compounds

The identification of compounds was based on a comparison of Kovats retention indexes using calibrated automated mass spectral deconvolution and identification system software AMDIS ver. 2.64 in combination with selective ion analysis (SIA) resolution method (13) compared with those from available literature (14), and by comparing their mass spectra to those from Wiley 275 and NIST/NBS libraries, applied on Agilent Mass Hunter Workstation (B.06.00) and AMDIS (2.1, DTRA/NIST, 2011) software. Retention indexes were obtained by co-injection with alkanes (C_8 –C_{40} standard mixture).

2.5. Statistical analysis

The statistical analyses were carried out using Statistica software 8.0. To understand the correlation between the chemical composition of essential oils and the different stages of development, we would also highlight the existence of chemotype(s), hierarchical cluster analysis and principal component analysis were used. HCA (hierarchical cluster analysis) and PCA (principal component analysis) are the most widely used tools to explore similarities and hidden patterns among samples where the relationship between data and grouping is still unclear (15). A significant correlation between compounds and variability for dominant compounds was made at a 95% significance level ($P \leq 0.05$).

3. Results and discussion

3.1. Essential oil variability

The yield of EO ranged from 0.03% (October) to 0.33% w/w in June (Figure 1), highest at the vegetative stage and lowest after the flowering stage. In investigations (16,17), the same dynamics of changing the content of essential oil from wild *Satureja*. montana was established, during the stages of development. The variation of essential oil yield at different stages of vegetation cycle may be primarily due to genetic factors, developmental stages, plant origin, time collection, and other factors: drying and storage methods, extraction and analysis methods (18). It should be noted that in this research a higher EO yield was recorded in November, compared to October, which can be explained by drought. In the area of the village of Kravlje the average value of precipitation in November 2020 was 2.8 mm (19). In a study evaluating the effects of water stress levels on growth, photosynthesis and essential oil yield of *Matricaria recutita* and *Cichorium intybus,* the author concluded that under drought stress quantity of

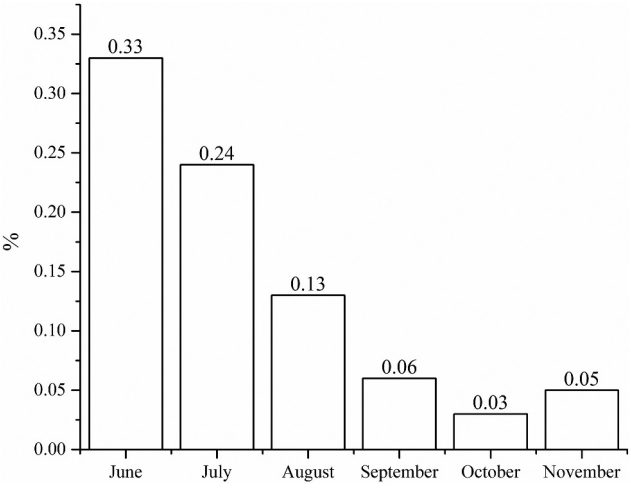

Figure 1. The yield of essential oil during different stages of development (%, W/w).

chamomile and chicory oil was improved (20). The fact is that the most intensive synthesis of secondary metabolites occurs during the flowering stage. However, from the economic aspect of the use of essential oil, *S. kitaibelii* should be collected in June, in the vegetative stage. Therefore, essential oil accumulation should be studied during plant development.

3.2. Essential oil composition during different stages of development

Fifty-one compounds were detected in the EO composition, accounting for 93.3% to 98.6% of the oil obtained at the vegetative, flowering, and after flowering stages, respectively. The components of *S. kitaibelii* EO were separated into six classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, phenolic compounds and others (Table 1). The oxygenated monoterpenes were the most abundant compound class in the oil (21.3–61.2%), and they were dominated by geraniol (2.9–42.1%) and borneol (4.1–16.4%). The second abundant compound class, monoterpene hydrocarbons (24.1–51.1%) was mainly dominated by limonene (8.7–15.3%) and *p*-cymene (4.9–11.9%). At the same time, these four compounds are the most abundant in *S. kitaibelii* EO. The percentage of these main components varies according to the growth stage (Figure 2). The highest variation in content, during the plant development, was shown by the most dominant component, geraniol (coefficient of variation 112.3%). The highest content of geraniol was noted in July, 42.1%. Limonene and *p*-cymene show relatively stable concentrations during the season (coefficients of variation 22.8% and 26%), while borneol concentration shows a permanent tendency to grow during the season (coefficient of variation 49.8%), reaching a maximum in November, 16,4% (Figure 3). As we have said, the oxygenated monoterpenes were the most abundant compound class in the *S. kitaibelii* oil during examined phenological stages, except vegetative stage, in which monoterpene hydrocarbons are dominant (51.1%). Our results agree with the number of studies regarding the chemical composition of *S. kitaibelii* EO, obtained in southeastern Serbia from samples collected in the flowering stage. Also, in most of these studies, geraniol is the dominant component (2–6). The exogenous factors are generally related to the environment such as light, precipitation, growing site, pH and soils constituents affecting the chemical composition of EO (21). In review of factors affecting chemical variability of essential oils Barra states that exogenous factors could generate ecotypes and chemotypes in the same plant species. Ecotypes are populations of the same species living under different environmental conditions. On the other hand, the chemotype represents a chemical variation of a plant's secondary metabolites induced by geographical location. The long-time exposure of an aromatic plant to different exogenous factors could cause variation in the chemical composition of essential oil determining chemotype. Therefore, ecotypes of the same species growing in different environments could be identified as different chemotypes (1). In most research, only the main compound of essential oils has been considered for chemotype determination (22). Ševarda et al. (23) based on composition of four populations of *S. kitaibelii* from Serbia, separated EOs in two chemotypes: chemotype of aromatic hydrocarbons like *p*-cymene and chemotype of aliphatic monoterpene products like geraniol. Other authors recognize three potential chemotypes of *S. kitaibelii* EO from Serbia: geraniol, *p*-cymene and limonene. They point out that, *p*-cymene has rarely been one of three dominant components in geraniol chemotype. More precisely, only two populations are stated: one from Suva planina mountain and the other from Stara planina mountain (9). It is known that there are almost always two or more major constituents of essential oil. Second, the pharmacological activity of essential oil can be a consequence of the synergistic action of minor constituents (24). Grayer et al. (25) proposed to assign *Ocimum basilicum* essential oil profile classes based on all the major constituents, even if there are three or four dominant compounds. If we applied the mentioned criteria in our research, we would get five chemotypes. If we were talking about monodominant chemotypes, as usual, we would get four chemotypes (Table 2). So, in the flowering stage (July and August), the geraniol/limonene/*p*-cymene

Table 1. The composition of the essential oil of the aerial parts of *S. kitaibelii*.

Compound	KI[a]	KI[b]	Class	June	July	August	September	October	November
α-Thujene	927.7	924	M	0.3	n.d.	0.1	0.1	0.1	n.d.
α-Pinene	934.7	932	M	3.4	1.9	4.1	2.4	2.5	2.2
Camphene	949.6	946	M	0.9	0.6	1.6	1.5	1.5	1.6
Sabinene	974.5	969	M	0.2	0.3	0.2	0.3	0.3	0.2
1-Octen-3-ol	978.5	974	O	0.6	0.5	0.7	0.6	0.7	0.6
Myrcene	991.5	988	M	1.1	0.4	0.5	0.2	0.2	0.2
3-Octanol	995.4	988	O	0.1	n.d.	0.1	0.1	0.2	0.2
α-Phellandrene	1005.7	1002	M	0.4	n.d.	0.3	0.1	0.1	n.d.
α-Terpinene	1017.9	1014	M	0.9	0.5	0.9	1.1	1.0	0.4
p-Cymene	1026.5	1020	M	11.9	4.9	9.1	10.7	10.8	9.3
Limonene	1031.2	1024	M	14.4	10.1	15.3	8.7	10.4	10.5
1,8-Cineole	1033.3	1026	M	1.5	0.9	1.4	1.7	1.6	2.0
(*Z*)-β-Ocimene	1038.8	1032	M	5.0	1.6	1.2	0.2	0.2	0.2
(*E*)-β-Ocimene	1049.2	1044	M	4.6	1.3	1.0	0.2	0.1	0.2
γ-Terpinene	1060.7	1054	M	6.3	1.3	1.6	2.0	1.8	0.9
(Z)-Sabinene hydrate	1068.6	1065	MO	1.4	3.6	1.3	2.9	4.3	2.5
Terpinolene	1089.9	1086	M	0.2	0.3	0.5	0.6	0.7	0.5
Linalool	1100.2	1095	MO	1.3	1.4	1.5	1.5	2.1	1.4
(*Z*)-*p*-menth-2-en-1-ol	1123.1	1122	MO	0.2	0.3	0.5	1.0	1.1	1.0
α-Campholenal	1128.3	1122	MO	n.d.	n.d.	0.3	0.5	0.6	0.7
allo-Ocimene	1129.9	1128	MO	0.6	n.d.	n.d.	n.d.	n.d.	n.d.
cis-*p*-Mentha-2,8-dien-1-ol	1137.0	1133	MO	n.d.	n.d.	n.d.	n.d.	0.4	0.6
trans-Sabinol	1141.1	1137	MO	0.1	n.d.	0.4	0.8	0.9	1.1
Camphor	1147.2	1140	MO	0.2	0.3	1.0	2.0	2.7	4.3
Borneol	1169.0	1165	MO	4.1	4.5	8.7	10.7	11.7	16.4
Terpinen-4-ol	1180.0	1174	MO	2.3	3.2	5.3	8.2	7.6	4.4
p-Cymen-8-ol	1187.0	1179	MO	0.2	n.d.	0.5	0.9	0.8	1.0
trans-*p*-Mentha-1(7),8-dien-2-ol	1189.8	1187	MO	n.d.	n.d.	0.3	0.6	0.6	1.0
α-Terpineol	1192.8	1186	MO	0.5	0.4	0.6	0.6	0.6	0.4
(*Z*)-Dihydrocarvone	1199.4	1191	MO	0.6	0.4	1.0	1.3	1.4	2.0
(*E*)-Dihydrocarvone	1207.5	1200	MO	1.2	0.8	1.4	1.1	1.2	1.7
Verbenone	1212.9	1204	MO	n.d.	n.d.	0.1	0.1	0.2	0.3
(*E*)-Carveol	1221.0	1215	MO	0.3	0.4	1.0	1.8	1.9	2.4
Nerol	1229.5	1227	MO	0.3	1.7	0.7	0.7	0.4	0.6
(*Z*)-Carveol	1232.8	1226	MO	0.1	n.d.	0.4	0.6	0.8	1.1
(*E*)-Verbenol	1243.0	1240	MO	0.2	0.9	0.7	0.8	0.6	0.6
Carvone	1247.3	1239	MO	n.d.	n.d.	1.5	2.2	2.2	2.8
Geraniol	1257.0	1249	MO	7.5	42.1	15.4	8.1	2.9	3.2
Geranial	1272.2	1264	MO	0.2	1.2	0.7	0.8	0.5	0.5
Thymol	1292.5	1289	PC	2.6	0.1	0.1	0.1	0.1	0.1
Carvacrol	1303.0	1298	PC	6.2	0.3	0.2	0.3	0.3	0.2
α-Copaene	1381.0	1374	S	0.2	n.d.	0.2	0.3	0.2	0.2
β-Bourbonene	1391.0	1387	S	0.6	1.3	1.8	3.1	2.5	2.3
β-Elemene	1396.0	1389	S	0.3	0.3	0.4	0.2	0.2	0.2
Caryophyllene	1426.8	1417	S	2.3	2.7	2.9	1.4	1.3	1.1
β-Copaene	1435.9	1432	S	0.2	n.d.	0.2	0.2	0.2	0.2
Germacrene D	1488.7	1484	S	4.3	4.3	4.2	1.3	1.0	0.7
Bicyclogermacrene	1503.9	1500	S	1.3	1.0	1.2	0.4	0.4	0.2
Spathulenol	1585.8	1577	SO	1.2	1.0	1.4	2.5	3.2	2.8
Caryophyllene oxide	1595.2	1582	SO	1.5	1.8	2.9	5.2	6.5	6.0
Torilenol	1614.0	1604	SO	0.2	n.d.	0.3	0.6	0.6	0.6
Total				**94.0**	**98.6**	**97.3**	**93.3**	**94.2**	**93.6**
Monoterpene hydrocarbon			M	51.1	24.1	37.8	29.8	31.3	28.2
Monoterpene oxygenated			MO	21.3	61.2	43.3	47.4	45.9	50.4
Sesquiterpene hydrocarbon			S	9.6	9.6	11.4	7.5	6.4	5.1
Oxygenated Sesquiterpene			SO	3.3	3.1	5	9	11	9.6
Phenolic compounds			PC	8.8	0.4	0.3	0.4	0.4	0.3
Others			O	0.7	0.5	0.8	0.8	1.2	1.2

n.d.-not detected; KI[a] (Kovats index) measured relative to *n*-alkanes (C9–C28) on the HP-5 column; KI[b], (Kovats index) from literature.

chemotype dominates. It is known that the flowering stage is the most important in plant development, but the question is whether a flowering stage is enough to determine the chemotype of the essential oil? In general, can the essential oil chemotype be determined based on only one stage of plant development? In the 'Introduction'section, we pointed out one of the reasons for this research. At location, the Kravlje village in June 2018, we did not find geraniol in *S. kitaibelii* EO (11). During multi-year research, γ-terpinene, one of the main constituents of *S. kitaibelii* EO from the Rtanj mountain (Serbia), was recorded with a significant content only 1 year (26). Manufacturers of tea tree oil (*Melaleuca alternifolia*) in Australia need to be assured that transplants will grow into terpinen-4-ol plants that are richer in terpinen-4-ol than

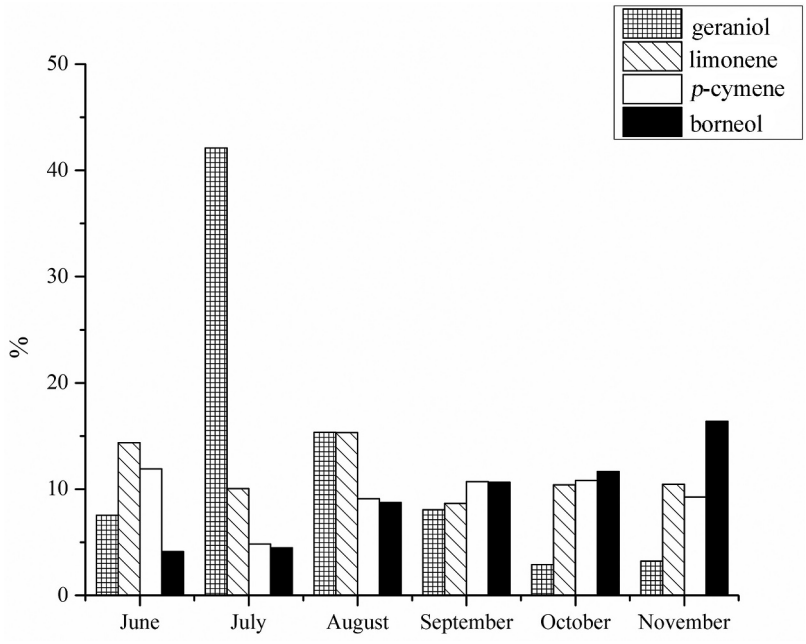

Figure 2. Changes in the content of the most abundant components of essential oil during different stages of development (%).

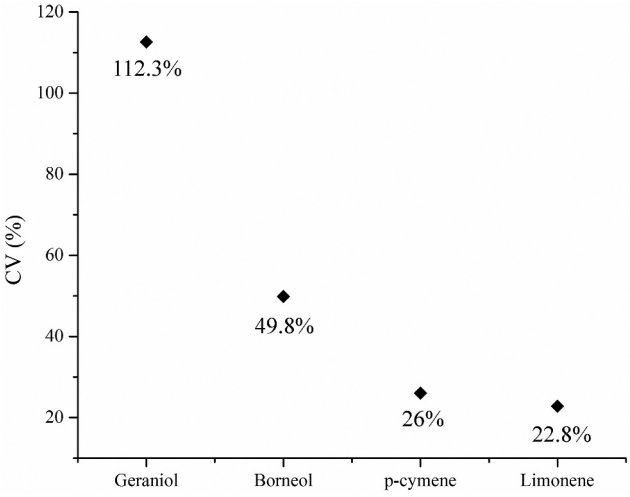

Figure 3. Coefficient of variation of the most abundant components of essential oil.

Table 2. Potential chemotypes of essential oil of *S. kitaibelii*, based on research in 1 month of plant development.

Months	Chemotype		
June	Limonene	*p*-Cymene	Geraniol
July	Geraniol	Limonene	*p*-Cymene
August	Geraniol	Limonene	*p*-Cymene
September	*p*-Cymene	Borneol	Limonene
October	Borneol	*p*-Cymene	Limonene
November	Borneol	Limonene	*p*-Cymene

terpinolene or 1,8-cineole. They certainly should know that terpenoid biogenetic pathways were found to be initiated at different stages of ontogeny (27). The results in Table 2 refer only EO chemotypes of individual months, not to the systematic and continuous research of *S. kitaibelii* EO during the three phases of development (6 months). We used chemometric methods – hierarchical cluster analysis (HCA) and principal component analysis (PCA) – to properly respond to these challenges.

3.3. Hierarchical cluster analysis

The relationship between the chemical composition of essential oils and stages of development of the *S. kitaibelii* was determined by hierarchical cluster analysis. In other words, cluster analysis contributes to a better understanding of the chemical profile of EO, i.e. the influence of the dominant component, and provides relationships among all the EOs studied. In the present research, all analyzed essential oil compounds were used for HCA. Ward's method and Pearson's correlation coefficients were used to classify variables to compute the similarity. The distance was reported as D_{link}/D_{max}, representing the quotient between the linkage distances for a particular case divided by the maximal linkage distance (28). In this way, it can be seen which pairs show the most similarity. The obtained results are given in the dendrogram presented in Figure 4. Months, representing the EOs obtained in the appropriate stage of vegetation cycle, are divided into two significant clusters, A and B $(D_{link}/D_{max}) \times 100 = 50)$. Cluster A is divided into two sub-clusters (A1 and A2), while cluster B is divided into B1 and B2. The strongest linkage is observed for EOs from November and October, belonging to cluster A. This indicates a significant

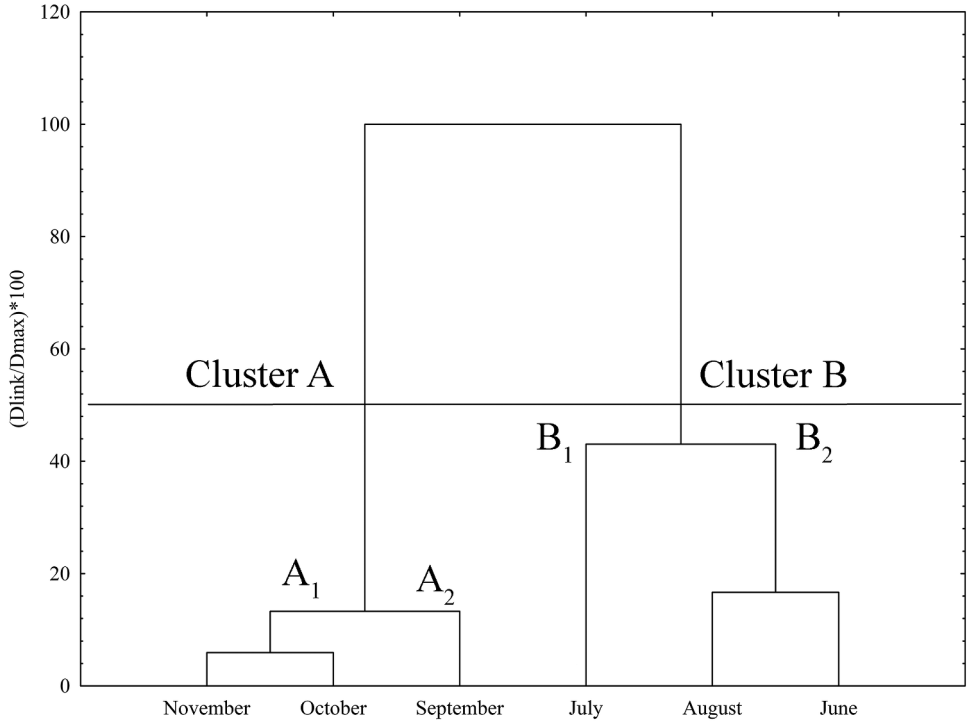

Figure 4. Dendrogram of EOs obtained in different stages of development.

similarity among the chemical composition of EOs in these 2 months, with a dominant content of borneol. Contents of *p*-cymene, limonene and borneol are similar in cluster A. EO obtained in September differs from the EOs in subcluster A1 in domination of geraniol, which was found in lower content in mentioned oils. Cluster B linked oils obtained in vegetative and flowering stages (June, July, and August), which indicates the similarity of the chemical composition of EOs during the mentioned stages of development. These EOs are characterized by higher geraniol and limonene content than cluster A. Another reason for the formation of cluster B is a lower content of borneol in EOs obtained during these 3 months. Subcluster B_1 is EO obtained from a plant harvested in July, which is highlighted by the content of geraniol (42.1%). The lower content of *p*-cymene in July could be another reason to differentiate it from subcluster B2. EOs in subcluster B_2 were separated due to lower content of geraniol and very similar content of *p*-cymene and limonene. Division to these two clusters is in accordance with the chemical profiles of essential oils. Cluster analysis suggests two different chemical profiles of essential oil depending on the harvest time of the same plant. Cluster A (EOs obtained after flowering stage (September, October and November) is characterized by limonene, *p*-cymene and borneol as the main constituents. Cluster B (EOs obtained in flowering stage) is characterized by geraniol, limonene, and *p*-cymene as the main constituents. Individual variability between the chemical composition of EOs within the same species, during different stages of development, could be significant in determining the chemotype of EOs correctly.

3.4. Principal component analysis

Twelve compounds with average relative abundance ≥1% were used (Figure 5) for principal component analysis. Essential oils obtained during different stages of development were separated, accounting for 89.12% of the total variance by combining PC1 and PC2. They have eigenvalues greater than 1. The remaining principal components, with eigenvalues less than 1, have little contribution to the total variance of the original data and these values are considered to explain the errors in the analysis (29). The PC1 is better correlated with variables and explained 65.10% of the total variance, while PC2 explained 24.02%. This is expected because PCs are extracted successively, each one accounting for as much of the remaining variance as possible (30). Correlation coefficients of *S. kitaibelii* EOs obtained using PCA show the relationship between the chemical composition of essential oils (Table 3). Pearson coefficient higher than 0.7 can be considered a significant correlation and a coefficient having a value above 0.9 indicates a strong correlation between variables (31). PCA helps to find a correlation between EOs and dominant compounds and to examine changes in variable correlations. PCA supported the result obtained by cluster analysis. PCA pointed out two groups of

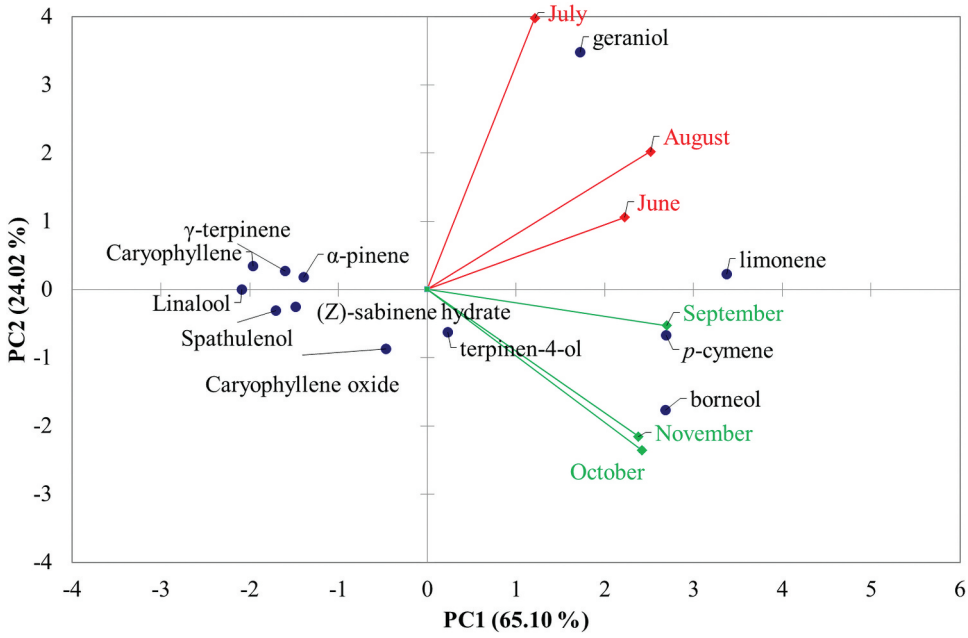

Figure 5. Principal component analysis carried out on data of chemical composition of essential oils in the loading plot.

Table 3. Correlation coefficients obtained between essential oils of *S. kitaibelii* using PCA at different vegetation stages.

Variables	June	July	August	September	October	November
June	**1**					
July	0.361	**1**				
August	**0.792**	0.729	**1**			
September	0.611	0.364	0.765	**1**		
October	0.533	−0.054	0.530	0.884	**1**	
November	0.460	0.017	0.552	0.830	**0.926**	**1**

Note: Bold correlations are signifcant at $p < 0.05$.

Table 4. Contribution of variables and observation to the formation of PC1 and PC2 (%).

Contribution of the variables (%)			Contribution of the observations (%)		
Variables	PC1	PC2	Observations	PCl	PC2
June	15.7	3.6	Geraniol	6.3	70.1
July	4.7	50.2	Limonene	24.2	0.3
August	20.0	13.0	*p*-Cymene	15.4	2.6
September	23.1	0.9	Borneol	15.4	18.1
October	18.6	17.7	Terpinen-4-ol	0.1	2.3
November	18.0	14.7	γ-Terpinene	5.4	0.4
			Spathulenol	6.2	0.5
			Caryophyllene oxide	0.5	4.4
			Caryophyllene	8.2	0.7
			α-Pinene	4.1	0.2
			(*Z*)-Sabinene hydrate	4.7	0.4
			Linalool	9.3	0.0

variables characterizing two chemical patterns, based on the data. EOs close to each other in the PCA diagram have the same chemotype. All EOs positioned on the positive side of PC1 are separated. All variables are separated on the first axis based on the abundances of geraniol, limonene, *p*-cymene and borneol. EOs obtained in June, July and August can be distinguished on the positive score value of PC2. Contents of geraniol and limonene are the most important contributors, influencing the separation of EOs obtained in the vegetative and flowering stages (June, July and August), compared to EOs obtained in the after flowering stage (September, October and November) (Table 4). The highest contribution on PC2 had geraniol (70.1%), and the variable July (50.2%). Such a distribution is expected because the EO obtained this month was proved to have the highest content of geraniol (higher than 40%), which is co-located in the same area. This EO is located a considerable distance away from all the other samples of EOs, indicating that its composition differs from the other samples. Limonene had the most significant contribution to PC1 (24.2%). The vectors of variables June and August (representing the EOs obtained in these months) occupy an acute angle, which indicates a significant correlation between them ($R = 0.792$). These EOs are co-located in the higher right-hand quadrant of Figure 5, together with limonene suggesting that they have a high content of this monoterpene. EOs obtained in the after flowering stage are positioned on the negative side of PC2. Two compounds significantly influenced such a distribution of EOs: *p*-cymene and borneol (Table 4). However, two groups of EOs also separated on the second axis, different in chemical composition, though only slightly compared to each other. As illustrated in Figure 5, the vectors of the variables October and November are parallel with a slight angle between them, indicating a strong correlation ($R = 0.926$, Table 3) (32). This is mainly due to the content of borneol, which is a dominant compound in both essential oils.

The results obtained by HCA and PCA indicate that *S. kitaibelii* has different chemical compositions of essential oil during different stages of development. This fact concludes that essential oil isolated from the plant in 1 month should not be used to determine the chemotype. Lakušić et al. (33) came to the same conclusion in research on seasonal variations in the composition of the essential oils of *Rosmarinus officinalis*. They concluded that the same genotype, during different stages of development, can synthesize essential oils that are so different that can be classified as different chemotypes. The following important fact is the number of the most abundant compounds of essential oil that determine the chemotype. We are of the opinion that the three most abundant EO components should determine the chemotype. This research proposes the criteria that compounds whose content varies the least, during the studied stages of development, have an advantage over those most abundant compounds but their content varies significantly. Based on the above, it follows that two chemotypes, limonene/*p*-cymene/geraniol and limonene/*p*-cymene/borneol, were found in the essential oil of *S. kitaibelii* from Kravlje village, southeast Serbia. The first chemotype is characteristic of the vegetative and flowering stages, while the second was established after the flowering stage.

4. Conclusions

In the present study, combined chemical and chemometric analysis of *S. kitaibelii* essential oil during different stages of development, over the course of 6 months, was done with an emphasis on chemotaxonomy. The yield of EO ranged from 0.03% to 0.33% w/w, highest at the vegetative stage and lowest after flowering stage. The oxygenated monoterpenes were the most abundant compound class in the oil. The four most abundant essential oil components are geraniol, limonene, *p*-cymene and borneol. The percentage of these main components varies according to the growth stage. The highest variation in content, during plant development, was shown by the most dominant component, geraniol. The results of the chemometric analysis indicate two chemotypes: limonene/*p*-cymene/geraniol and limonene/*p*-cymene/borneol. We are of the opinion that the three most abundant EO components should determine the chemotype. This research proposes the criteria that compounds whose content varies the least, during the studied stages of development, have an advantage over those most abundant compounds but their content varies significantly. The obtained results lead to the conclusion that essential oil isolated from the plant in 1 month should not be used to determine the chemotype. We suggest that chemotaxonomic studies of essential oils are based on monitoring the composition of essential oils during plant development, at least in three stages of vegetation cycle. Finally, we point out the need to use efficacious statistical and chemometric methods, for a complete and objective interpretation of the results of chemical analysis.

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Disclosure statement

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