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The significance of minor components on the antibacterial activity of essential oil via chemometrics

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ABSTRACT

The significance of minor constituents of essential oils (EOs) for antibacterial activity by chemometric methods principal component analysis (PCA) and hierarchical clustering analysis (HCA) was researched. In this study, the antibacterial activity of six EOs from *Achillea* and *Artemisia* species was evaluated on nine laboratory control bacterial strains. Gas Chromatographic-Mass Spectrometric (GC/MS) data showed that these samples have similar chemical compositions, with highest content of oxygenated monoterpenes and lowest content of oxygenated sesquiterpenes and sesquiterpenes hydrocarbons. The strongest clustering is observed for *Achillea millefolium* and *Achillea crithmifolia* EOs. In PCA analysis, these two EOs are located at the considerable distance away from all of the other samples of EOs, indicating that their composition and activity differs significantly from the other samples. All bacteria for assessment of antimicrobial activity are grouped to the left side of the plot, located diametrically opposite to group I. This unique location can be pointed out as a reason for the lowest activity against bacteria, suggesting that the dominant EO components may not determine antibacterial activity. These findings were suggesting that the minor compounds (oxygenated sesquiterpenes and sesquiterpenes hydrocarbons) or a combination thereof were possibly responsible for the complete antibacterial activity of EOs.

1. Introduction

Asteraceae, also called Compositae, is one of the largest plant families, with 1620 genera and 23,600 species (Funk, Susanna, Stuessy, & Robinson, 2009). According to the pharmacological uses, *Achillea millefolium*, *Achillea clypeolata*, *Achillea crithmifolia*, *Artemisia absinthium*, *Artemisia annua* and *Artemisia alba* Turra have a long history of application in folk medicine (Nigam et al., 2019; Saeidnia, Gohari, Mokhber-Dezfuli, & Kiuchi, 2011).

Chemometrics is the chemical discipline that uses primarily mathematical and statistical methods employing formal logic to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analyzing chemical data (Héberger, 2008). Different from traditional statistical methods, chemometrics allows a multi-pronged approach, which includes the detection of hidden connections between variables and separates useful from useless information. Pattern recognition procedures can be either unsupervised (depending only on the structure of the entire data set) or supervised (using the presumed sample class identifications to establish classification rules) (Siebert, 2011). Unlike supervised classification methods that include class information in their models, unsupervised techniques determine the structure of a dataset based on measurements. That was the reason for choosing this type of classification.

Chemometrics can display variation within the complex essential oil GC/MS data graphically, thus allowing for a more precise interpretation of active biomarkers (Orchard, Sandasi, Kamatou, Viljoen, & van Vuuren, 2017). Two standard methods, such as principal component analysis and hierarchical clustering analysis, were applied to identify similarities in the chemical composition of samples, and in doing so, they highlight differences that can assist with the identification of chemical markers within the dataset (Granato, Santos, Escher, Ferreira, & Maggio, 2018).

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Hierarchical clustering analysis is a convenient method for studying the similarities (or dissimilarities) between objects in the variables space, or similarities (dissimilarities) between variables in the objects space (Smoliński, 2014). The methods of sub-clusters linking may be distinguished and depend on the data examined and the particular purpose of its application. Single linkage and Ward's method are most used to calculate the distance between two clusters. Ward's method minimizes the sum of squares of any two (hypothetical) clusters that can be formed at each step (Dimitrijevic et al., 2015). Different types of distances, such as Euclidian or Manhattan distances, can be used as a measure of the distance between the examined variables.

Principal component analysis is a mathematical method enabling data dimensionality reduction while retaining the discriminating power in the data and can be used to find differences between samples, to determine group associations, and to weigh relative contributions of compounds to the separation of the group (Wang et al., 2014).

The antimicrobial activity of essential oils (EOs) and plant extracts against the most varied microorganisms has been investigated over the last years (Kuhn et al., 2019). The presence of different classes of compounds means that the essential oils are effective against a diverse range of pathogens and the reactivity of essential oil depends upon the nature, composition, and orientation of its functional groups (Swamy, Akhtar, & Sinniah, 2016). It is known that major components of essential oil have key role in antibacterial activity. However, the significance of minor components on the antimicrobial activity of essential oils is mentioned as a possibility in a lot of studies, but it has never been proven by any analytical method. Using chemometric methods, we wanted to check this assumption, in the case of selected genera and species. This study is part of the research on the chemometric methods applied to the results of chemical and biological analyzes of plant secondary metabolites from Serbia (Dimitrijević, Mitić, Ranković, & Miladinović, 2019; Kocić, Stanković-Đorđević, Dimitrijević, Marković, & Miladinović, 2020). The objective of this paper is the use of PCA and HCA methods to establish the significance of minor constituents of essential oils for antibacterial activity. According to the authors' knowledge, this is the first report on a chemometric study of the antimicrobial activity of six essential oils from *Achillea* and *Artemisia* species on nine laboratory control bacterial strains, to establish the antibacterial importance of the minor components of essential oil.

2. Materials and methods

2.1. Plant material and chemicals

The aerial parts of *Achillea millefolium*, *Achillea clypeolata*, *Achillea crithmifolia*, *Artemisia absinthium*, *Artemisia annua* and *Artemisia alba* Turra were collected at the blooming stage in 2019. From natural populations at locations in Serbia. Voucher specimens are deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš (Herbarium Moesiacum Niš – HMN). Information on selected plant species and yields of isolated essential oils are presented in Table 1.

Table 1
Information on collected plants and essential oils yield.

Species	Locations	Voucher accession number	Essential oil yields (%, w/w)
A. millefolium	Kravlje village	13222	0.88
A. clypeolata	Rtanj mountain	8165	0.10
A. crithmifolia	Vidlič mountain	7793	0.42
A. absinthium	Belava mountain	14054	0.38
A. annua	Kravlje village	7154	0.80
A. alba Turra	Belava mountain	7960	0.31

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 4 h, using the Clevenger-type apparatus to produce oil. The resulting EOs essential oils were dried over anhydrous sodium sulfate and stored at 4 °C.

2.3. Gas chromatographic and gas chromatographic-mass spectrometric analysis

The GC analysis of the oils was carried out on a GC HP5890 II apparatus, equipped with the split–splitless injector, HP-5MS 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. The operating conditions were set as following: 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}.

GC-MS analyses were performed on an Agilent Technologies apparatus, Model GS 6890 N at 70 eV coupled with a mass selective detector MSD 5975C, under the same gas chromatograph conditions.

2.4. Identification of compounds

The identification of compounds was based on a comparison of Kovats retention indexes by the use of calibrated automated mass spectral deconvolution and identification system software AMDIS ver. 2.64 in combination with selective ion analysis (SIA) resolution method (Tan et al., 2010) compared with those from available literature (Adams, 2007), and by comparing their mass spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. Retention indexes were obtained by co-injection with an aliphatic hydrocarbons C9–C28 standard mixture.

2.5. Antibacterial testing

The activity of the essential oil samples was tested towards 9 different bacteria. Laboratory control strain was obtained from the American Type Culture Collection (ATCC). Gram-negative bacteria were represented by *Salmonella enteritidis* ATCC 13076, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumoniae* ATCC 10031 and *Enterobacter aerogenes* ATCC 13048, while the researched Gram-positive strains were *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *and Enterococcus faecalis* ATCC 19433. The inocula of the bacterial strains were prepared from the overnight broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7–10^8 CFU/mL, depending on a genera-consensus standard by the Clinical and Laboratory Standards Institute) (CLSI, 2009).

2.6. Micro-well dilution assay

A micro-well dilution assay was used to determine the minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC).

All tested oils were prepared in 70.0% ethanol in a microtiter plate over the range of 0.025–50.0 μL/mL in inoculated nutrient broth with the final volume of 100 μL. The final concentration of bacterial was 10^6 CFU/mL in each well. The microtiter plate was incubated at 37 °C for 24 h. All experiments were performed in triplicate.

Streptomycin and chloramphenicol, commercial antibiotics, were used as a positive control and medium with ethanol as a negative

control. Bacterial growth was determined by adding 20 μL of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews, 2001). To determine MBC, the broth was taken from each well without visible growth and inoculated in Mueller Hinton agar for 24 h at 37.

2.7. Statistical analysis of data

In order to understand the significance of minor constituents of essential oils for antibacterial activity, EOs components, MIC and MBC values were subjected to HCA and PCA using Statistica 8.0, StatSoft, Tulsa, Oklahoma, USA. A probability level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chemical composition of EOs

The yield of EOs essential oils ranged from 0.10% for *A. clypeolata* to 0.88% for *A. millefolium* (Table 1). The distribution of the EOs components and their relative amounts is represented in Table 2. Exactly 33 compounds were identified in six samples. The percentage of identified components of EOs ranged from 83.4% for *A. absinthium* to 92.9% for *A. millefolium*. All researched EOs contained predominantly oxygenated monoterpenes, as follows: *A. millefolium* (81.4%), *A. clypeolata* (66.6%), *A. crithmifolia*, (81.9%), *A. absinthium* (53.6%), *A. annua* (51.3%) and *A. alba* (63.9%). Two of the main dominant components in each of EOs are given in bold. The most dominant components in EOs were: 1,8-cineole (41.6%) in *A. millefolium* and (21.7%) in *A. clypeolata, trans*-chrysanthenyl acetate (35.2%) in *A. crithmifolia*, *cis*-β-epoxyocimene (21.5%) in *A. absinthium*, α-pinene (17.9%) in *A. annua* and camphor (18%) in *A. alba*.

3.2. Antibacterial activity of EOs

Results from the antibacterial assay show that examined EOs possessed antimicrobial activities against all tested microorganisms (Table 3). An essential oil from *A. millefolium* has been found to have antibacterial activity with a range of MIC values from 1372.8 to 22000 μg mL^{-1} and MBC values from 1372.8 to 22000 μg mL^{-1}. *A. clypeolata* EO demonstrated MIC values from 1326 to 5312.5 μg mL^{-1} and MBC values from 1326 to 10625 μg mL^{-1}. EO from *A. crithmifolia* has antimicrobial activity with a range of MIC values from 2629 to 21000 μg mL^{-1} and MBC values from 5250 to 42000 μg mL^{-1}. *A. absinthium* EO demonstrated MIC values from 647.4 to 5187.8 μg mL^{-1} and MBC values from 2597.9 to 20750 μg mL^{-1}. EO from *A. annua* has been found to have antimicrobial activity with a range of MIC values from 655.2 to 10500 μg mL^{-1} and MBC values from 655.2 to 21000 μg mL^{-1}. *A. alba* EO demonstrated MIC values from 670.8 to 10750 μg mL^{-1} and MBC values from 1341.6 to 21500 μg mL^{-1}. Reference antibiotic streptomycin was active in concentrations between 0.5 and 16 μg mL^{-1}, while chloramphenicol was active in concentrations between 1 and 16 μg mL^{-1}.

Table 2
Volatile compounds detected in essential oils.

No.	Component	*A. millefolium*	*A. clypeolata*	*A. crithmifolia*	*A. absinthium*	*A. annua*	*A. alba*
1.	α-pinene	2.6	1.8	2.2	–	17.9	2.1
2.	Camphene	1.1	–	–	–	1.2	4.9
3.	Sabinene	1.8	–	–	8.3	–	–
4.	β-pinene	3.1	2.5	2	–	1.9	–
5.	*p*-Cymene	–	2.9	4.2	2.5	0	1.6
6.	1,8-Cineole	41.6	21.7	27.4	3.1	5.6	13.2
7.	Artemisia ketone	–	–	–	–	17.3	–
8.	Linalool	–	2.8	–	2.1	0	–
9.	Artemisia alcohol	–	–	–	–	3.1	1.6
10.	β-Thujone	–	–	–	18.8	–	5.3
11.	*cis*-β-epoxyocimene	–	–	–	21.5	–	–
12.	*trans*-Pinocarveol	–	2	–	–	6.4	4
13.	Verbenol	–	12.9	–	–	–	–
14.	Camphor	30	5.1	10.4	–	6	18
15.	Pinocarvone	–	–	–	–	5.1	2.1
16.	*cis*-Chrysanthenol	–	–	–	–	5.6	2
17.	Borneol	4.8	9.8	2.8	–	1.2	4.3
18.	*cis*-pinocamphone	–	–	–	–	–	1.8
19.	Terpinen-4-ol	2.2	7.1	4.4	–	1	1.8
20.	α-Terpineol	2.8	3.8	1.7	–	–	–
21.	*trans*-Chrysanthenyl acetate	–	1.4	35.2	–	–	1.5
22.	*trans*-sabinyl acetate	–	–	–	8.1	–	–
23.	*cis*-Chrysanthenyl acetate	–	–	–	–	–	8.3
24.	β-caryophyllene	–	–	–	3.2	1.5	–
25.	neryl isobutanoate	–	–	–	2.9	–	–
26.	β-selinene	–	–	–	1	2.7	0
27.	Elemol	–	–	–	–	–	2.4
28.	neryl 3-methylbutanoate	–	–	–	6.5	–	–
29.	neryl 2-methylbutanoate	–	–	–	4.3	–	–
30.	Spathulenol	–	–	–	0	–	1
31.	Caryophyllene oxide	2.9	10.5	1.7	1.1	2.8	1
32.	*cis*-Cadin-4-en-7-ol	–	–	–	–	5.7	–
33.	β-Eudesmol	–	6.3	–	–	–	7.8
Total		92.9	90.6	92	83.4	85	84.7
Monoterpene hydrocarbons		8.6	7.2	8.4	10.8	21	8.6
Oxygenated monoterpenes		81.4	66.6	81.9	53.6	51.3	63.9
Sesquiterpene hydrocarbons		–	–	–	7.1	4.2	–
Oxygenated sesquiterpenes		2.9	16.8	1.7	11.9	8.5	12.2

Table 3
Antibacterial activity of essential oils (MIC/MBC in micrograms per milliliter of essential oils).

	Bacterial strains	Achillea			Artemisia			Reference antibiotics	
		A. millefolium	*A. clypeolata*	*A. crithmifolia*	*A. absinthum*	*A. annua*	*A. alba*	Streptomycin	Chloramphenicol
Gram (−)	*S. enteritidis*	22000/22000	1326/1326	10500/21000	1294.8/2597.9	10500/10500	670.8/1341.6	4.0/4.0	4.0/8.0
	E. coli	1372.8/1372.8	1326/2660.5	5250/10500	2597.9/20750	5250/21000	5375/10750	16.0/16.0	16.0/16.0
	P. aeruginosa	5500/11000	1326/2660.5	5250/10500	1294.8/2597.9	5250/21000	5375/10750	8.0/8.0	4.0/16.0
	P. mirabilis	11000/11000	5312.5/10625	21000/42000	5187.8/10375	10500/10500	5375/21500	16.0/16.0	16.0/16.0
	K. pneumoniae	11000/11000	5312.5/10625	21000/42000	5187.5/10375	10500/10500	10750/21500	16.0/16.0	2.0/2.0
	E. aerogenes	5500/5500	1326/2660.5	2629.2/5250	2597.9/2597.9	5250/21000	2691.8/5375	0.5/0.5	1.0/1.0
Gram (+)	*B. cereus*	2750/5500	2660.5/5312.5	2629.2/5250	647.4/2597.9	1310.4/1310.4	670.8/2691.8	0.5/0.5	1.0/4.0
	S. aureus	1372.8/1372.8	1326/1326	21000/42000	5187.5/10375	655.2/655.2	1341.6/2691.8	0.5/0.5	1.0/8.0
	E. faecalis	11000/11000	5312.5/10625	10500/21000	5187.5/10375	10500/10500	10750/21500	4.0/4.0	2.0/4.0

3.3. Chemometrics analysis

Chemical constituents and antibacterial activity of six essential oils of *Achillea* and *Artemisia* genera were subjected to chemometrics analysis to determine any relationship between them. To classify *Achillea* and *Artemisia* species in the space of studied parameters describing the chemical composition, as well as the antibacterial activity of essential oils, the HCA was applied. In this research, Ward's algorithm was used to produce cluster groups based on Euclidean distance. Based on this algorithm, we can see which pairs show the most similarity. The HCA results based on the chemical composition of essential oils are given in the dendrogram presented in Fig. 1. To verify the possible association between the essential oils we used class compounds as variables and plant species as a cases. Two main clusters were noticeable at $(D_{link}/D_{max}) \times 100 < 50$, which suggests distinct chemical differences within each of the species. This separation is of importance because it shows a clear division of samples into two recognizable clusters for every single species.

Also, the HCA analysis was applied to compare studied parameters, describing the antibacterial activity of essential oils. Also, plant species were used as case and bacterial as variables.

The results obtained following HCA are shown as a dendrogram

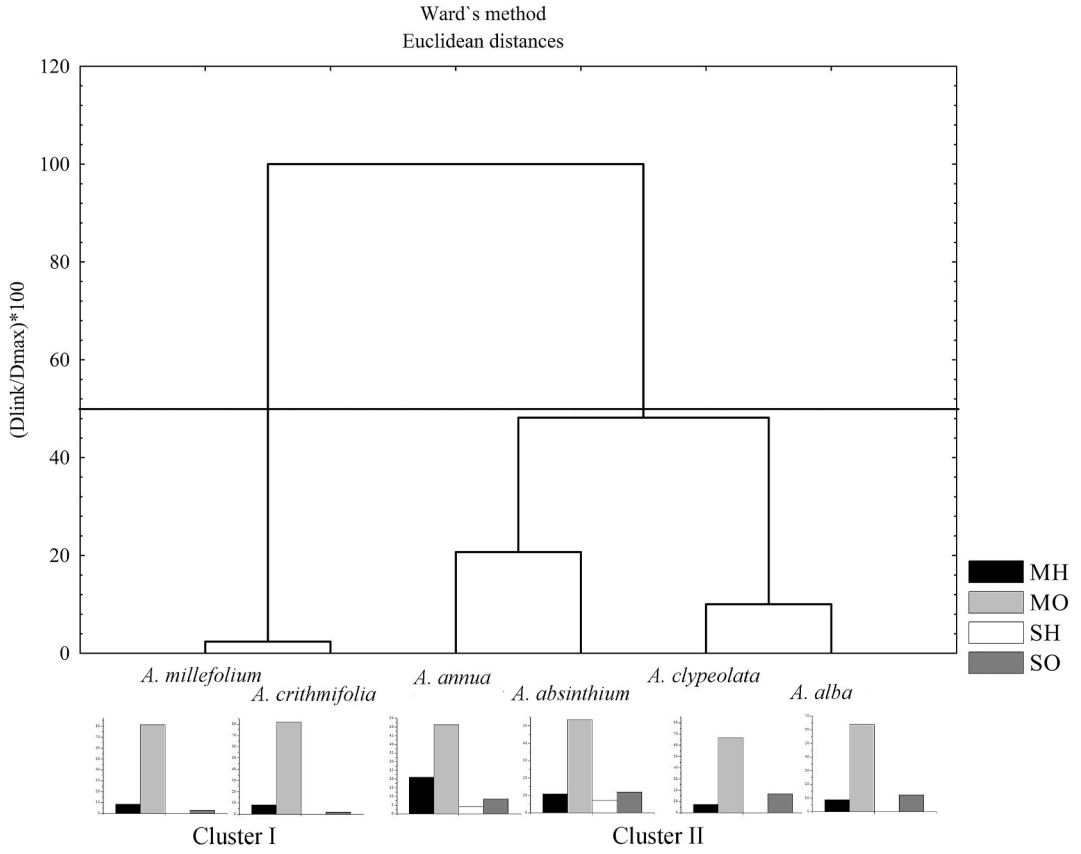

Fig. 1. Cluster analysis diagram of essential oils based on chemical composition.

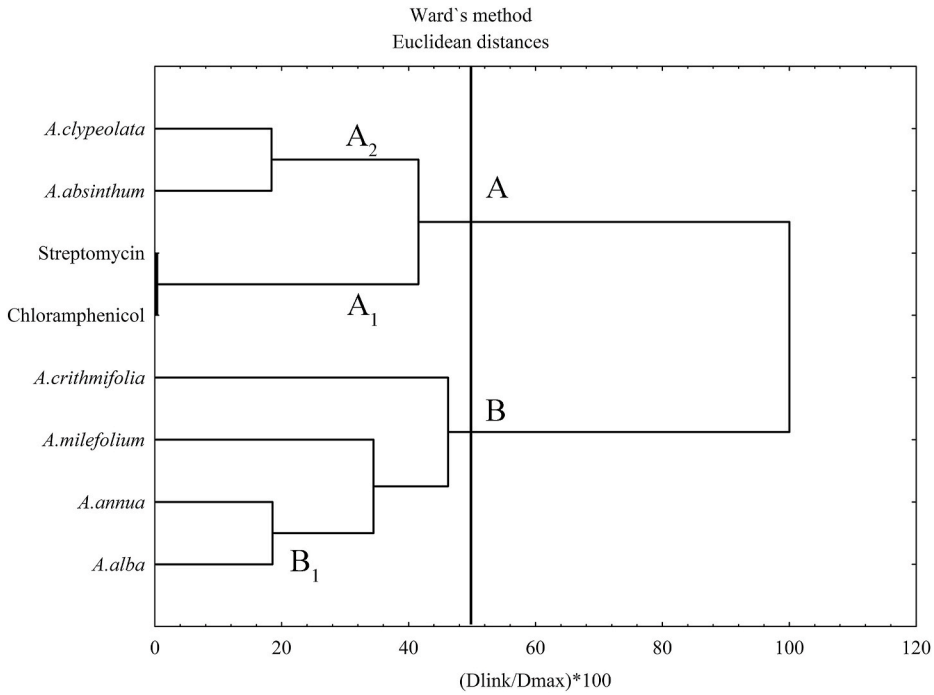

Fig. 2. Cluster analysis diagram of essential oils and standard antibiotics based on their antibacterial activity.

(Fig. 2). Two main clusters at $(D_{link}/D_{max}) \times 100 < 50$ were suggesting distinct antimicrobial activity within each of the species.

PCA method was applied to understand the real effect of EOs compound classes on antibacterial activity. As shown in Fig. 3, the PCA plot represents a graphical relationship between the species using the compound classes as cases and antimicrobial activity as a variable.

The first principal component (PC1) explained 61.9% of the total variance, the second 27.7%. PC1 is generally better correlated with the variables than PC2. This is to be expected because PCs are extracted successively, each one accounting for as much of the remaining variance as possible (Hossain, Patras, Barry-Ryan, Martin-Diana, & Brunton, 2011). These two components have eigenvalues greater than 1. Each principal component (i.e., eigenvector) is an eigenvalue, which gives the amount of variance in the data set that is explained by this principal component and there are many rules for extracting the number of factors (PCs) (Kokota, Matysiaka, Kłs, Kędziab, & Hołderna Kędzi, 2015). According to Kaiser's rule (Kaiser, 1960), the most important are the factors with eigenvalues that exceed one, while according to Morrison (Morrison, 1967), the principal components should account for at least 75% of the variance.

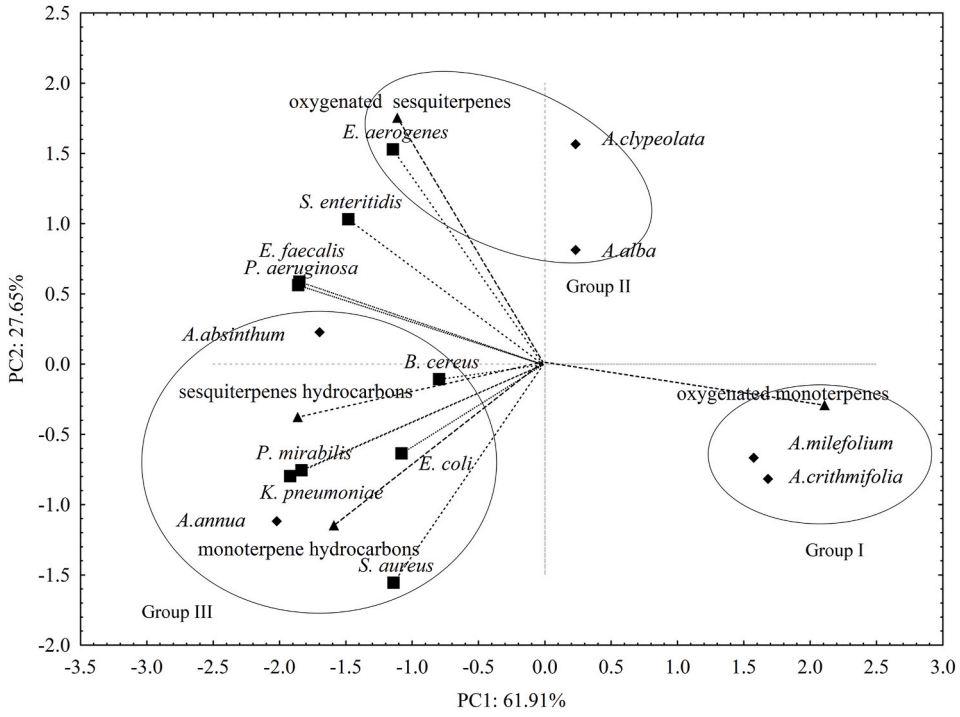

Fig. 3. PCA based on essential oils, their class compounds and antimicrobial activity.

4. Discussions

The distribution of the essential oil components of species from genera *Achillea* and *Artemisa* in our study is similar to previous studies from Serbia. 1,8-cineole, camphor, borneol and *trans*-chrysanthenyl acetate were significant components in selected *Achillea* samples (Palić, Stojanović, & Nasković, 2003; Pljevljakušic, Ristic, & Šavikin, 2017; Simić, Palić, & Randjelović, 2005). Compounds 1,8-cineole and camphor were also dominant in essential oil obtained from Turkish *Achillea* species (Can Başe, 2016). In researched *Artemisa* species from Serbia α-pinene, sabinene, 1,8-cineole, artemisia ketone and camphor were dominant compounds in EOs (Mihajilov-Krstev et al., 2014; Radulović et al., 2013; Đorđević et al., 2013). Different composition of essential oil was found in four *Artemisia* species from Ethiopia. In fact, their EOs mainly dominated by monoterpenes: yogomi alcohol and artemisyl acetate (Asfaw & Demissew, 2015). In most of these studies, oxygenated monoterpenes are the dominant class of compounds.

Relationships of chemical composition and the analyzed species were investigated by hierarchical cluster analysis. The strongest clustering is observed for *A. millefolium* and *A. crithmifolia* EOs, which belong to cluster A. This implies a significant similarity among these samples. GC/MS data showed that these samples have similar chemical compositions. The almost identical contents of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes in both species connects them to the first cluster. However, the low oxygenated sesquiterpenes content separates them from the second cluster.

Cluster B was constituted by four EOs (*A. annua, A. absinthium, A. clypeolata*, and *A. alba*). It is characterized by higher content of oxygenated sesquiterpenes, compared to cluster A. Furthermore, based on the histograms, the difference in the chemical composition between species located in cluster B can be observed. It is divided into two subclusters: B1 and B2. Sub-cluster B_1 represented by EOs of *A. annua* and *A. absinthium* (11.4-Euclidean distances) was highlighted by the content of sesquiterpene hydrocarbons. EOs of *A. clypeolata* and *A. alba* (5.5-Euclidean distances) of the sub-cluster B_2 were highlighted by the highest level of oxygenated sesquiterpenes.

Cluster analysis suggests that there are two main types of EOs: cluster I (EOs of *A. millefolium* and *A.crithmifolia*) characterized by oxygenated monoterpenes as the main constituent but with low content of sesquiterpenes; cluster II (EOs of *A. alba, A. clypeolata, A. annua* and *A. absinthium*) characterized by oxygenated monoterpenes as the main constituent but with sesquiterpenes (above 10%). Similar conclusions were drawn by the Golabadi, Zaghian, and Ercisli (2018) who, using cluster analysis, classified the analyzed species according to the dominant compounds.

Difficulties associated with antibacterial susceptibility testing are that the method used is often the disc-diffusion method. This method is highly dependent on water solubility and the ability of test compound or mixture to diffuse through an agar and inhibits the growth of the test bacteria. Thus, it would be expected that compounds of lower water solubility would show less activity, even if solubility did not affect their activity in other situations. Moreover, the agar disk-diffusion method is not appropriate to determine the minimum inhibitory concentration, as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium (Miladinović et al., 2012).

In this study, the antibacterial activity of six EOs from *Achillea* and *Artemisia* genera were evaluated on nine laboratory control bacterial strains. Based on the results presented in Table 3, we would state subjectively: in overall, researched EOs presented a selective antibacterial activity, having higher activity on gram-positive bacteria than on gram-negative bacteria. Is that so? They would make a subjective mistake without paying attention to *E. faecalis*. According to HCA, bacteria are grouped in two statistically significant clusters (A and B) at $(D_{link}/D_{max}) \times 100 < 50$, showed at dendrogram (Fig. 4). We can see that this bacterium forms a cluster B with the other two most resistant bacteria: *K. pneumoniae* and *P. mirabilis*. Therefore it is important to emphasize the statement that we highlighted in our previous research: to eliminate any kind of subjective analysis, interpretations and discussions of the result presented by tables and/or graphics, the use of chemometric methods is essential (Miladinović, Ilić, Mihajilov-Krstev, Jović, & Marković, 2014).

The capability of *Achillea* and *Artemisia* EOs to inhibit pathogenic bacteria has been well reported in several studies. For instance, *A. clypeolata* EO was reported as having an inhibitory effect on *E. coli*, *K. pneumoniae, P. aeruginosa* and *S. aureus* (Simić et al., 2005). Another study proved the high effectiveness of the *A. crithmifolia* EO to inhibit

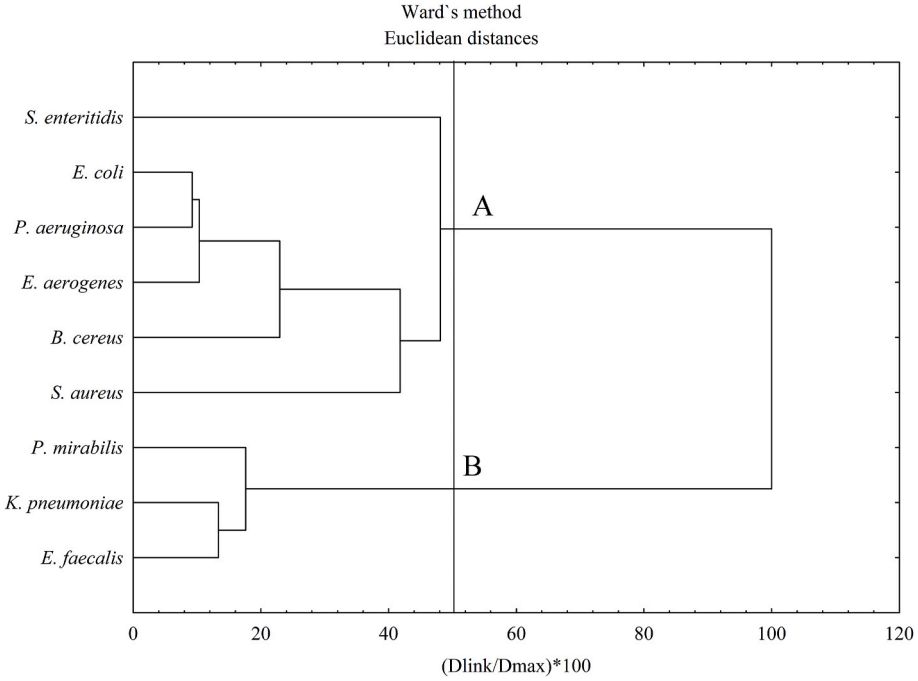

Fig. 4. Cluster analysis diagram of bacteria based on MIC and MBC values of examined EOs.

several strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* (Palić et al., 2003). Also, it has been reaffirmed the important antibacterial activity of wormwood EO (*A. absinthium*) against *S. aureus* (Msaada et al., 2015).

As illustrated in the dendrogram in Fig. 2, EOs of *A. clypeolata* and *A. absinthium* were clustered in the same cluster A with streptomycin and chloramphenicol. The mentioned standard antibiotics have strong bactericidal activity against bacteria and accordingly show the strongest clustering. The results showed that EOs in sub-cluster A_2 (*A. clypeolata* and *A. absinthium*) exhibited similar degrees of inhibition against both Gram-negative bacteria (*S. enteritidis, P. aeruginosa, P. mirabilis* and *K. pneumoniae*) and Gram-positive bacteria (*E. faecalis*), and compared to other EOs show better antibacterial activity. *A. crithmifolia, A. millefolium, A. annua* and *A. alba* EOs located in cluster B are characterized by the lower antibacterial activity. Furthermore, based on the antibacterial activity, the difference between Asteraceae species located in subcluster B1 can be observed. EOs of *A. annua* and *A. alba* have the best potential to inhibit *S. aureus* and *S. enteritidis* bacteria, respectively.

Comparing the dendograms in Figs. 1 and 2, we can see different clustering of essential oil compounds and their antibacterial activity. As we said, strongest clustering is observed for EOs of *A. millefolium* and *A. crithmifolia*, with quite similar chemical composition. On the other hand, *A. millefolium* EO, on the base antibacterial activity, is more similar to that of *A. annua* and *A. alba* EOs. The clustering of the chemical composition of the essential oils was done based on the identified compounds, which in all tested samples belong to the predominantly class of oxygenated monoterpenes. It can therefore be assumed that they are not carriers of the antibacterial activity of the studied essential oils. To make this assumption more verifiable, PCA was applied.

As shown in Fig. 3, the separation was well performed, and several observations can be made. The plot of PC1 against PC2 reveals the presence of three groups. Each group is determined by the species and their class of compounds that cluster them.

PCA technique enabled visualization of complex experimental data and relationships responsible for grouping observed. Considerable variations were noted between different samples of EOs in terms of antibacterial activity and different compound classes. Although cluster analysis suggested that oxygenated monoterpenes were the most abundant compound class in EO samples, they were not selected for the EOs classification based on antibacterial activity.

The PCA separated *A. annua* and *A. absinthum* EOs from other samples, on the negative score value of PC1, whereas other species were positioned on the positive side of PC1. Group I, represented by *A. millefolium* and *A. crithmifolia* EOs. They are located a considerable distance away from all of the other samples of EOs, indicating that their composition and activity differs significantly from the other samples. These EOs in the lower right-hand quadrant of Fig. 3 have the highest positive loadings on PC1 (1.57 and 1.68, respectively). It was expected because these EOs were proved to have the highest content of oxygenated monoterpenes (higher than 80%), which are co-located in this region. On the contrary, *A. millefolium* and *A. crithmifolia* EOs have a low content of oxygenated sesquiterpenes and sesquiterpene hydrocarbons, so they are located diametrically opposite to these compound classes. Also, all bacteria for assessment of antimicrobial activity are grouped to the left side of the plot, located diametrically opposite to group I. This unique location can be pointed out as a reason for the lowest activity against bacteria, suggesting that the dominant EO components may not determine antibacterial activity.

Group II, represented by *A. alba* and *A. clypeolata* EOs, occupied a location between two clusters, located on the positive side of PC2, contains significant content of sesquiterpenes. These species were distinguishable in the PCA and formed a distinct group. The percentage of oxygenated monoterpenes in these species decreased (between 60 and 80%) but the percentage of oxygenated sesquiterpenes is the highest, compared to other species. This may be a reason for better antibacterial activity. EO of *A. alba* showed the highest activity against *S. enteritidis* and *B. cereus* bacteria. *A. clypeolata* EO was found to be the most effective, comparing average MIC and MBC values against all the bacterial species.

Group III, which is formed by EOs of *A. annua* and *A. absinthium*, is positioned to the left side of the plot. This location suggests that these essential oils have relatively high content of sesquiterpenes and monoterpene hydrocarbons which are co-located in this cluster, but with reduced content of oxygenated monoterpenes. They are located diametrically opposite to group II. Golabadi et al. (2018) using the PCA method showed that the position of chemical components and analyzed plants on the graph is related, which confirms our research.

Among the chemical constituents identified in the essential oils, we highlight the minor components (sesquiterpene hydrocarbons and oxygenated sesquiterpenes), for EOs classification based on antibacterial activity. Some studies (Gill, Delaquis, Russo, & Holley, 2002; Mourey & Canillac, 2002) have shown that EOs exhibit stronger antimicrobial activity than that of their major constituents or their mixtures which suggests synergistic effects of the minor components, but also the importance of all components in relation to the biological activity of EOs (Moumni et al., 2020). To evaluate the correlations between the antibacterial activities and the essential oils of the 15 *Eucalyptus* species studied, Elaissi et al. (2012) used all the mean values of the zone diameters inhibition in PCA and HCA analyses.

Viljoen et al. were investigated death kinetics of *Artemisia afra* essential oil and its dominant compounds (1,8-cineole, artemisia ketone, α-thujone and β-thujone) alone and in their various combinations. They found very low antimicrobial activity of all single components and their combinations, so the conclusion was reached that the determined activity must be attributed to minor components and their combination (Viljoen, van Vuuren, Gwebu, Demirci, & Baser, 2006).

It is evident that the carriers of the antibacterial activity of the essential oils are the dominant class of compounds, in most of the studied plant species. However, the importance of minor components of essential oil should not be ignored, as confirmed by this research.

5. Conclusion

This is the first report on a chemometric study of the antibacterial activity of six essential oils from *Achillea* and *Artemisia* species on nine laboratory control bacterial strains, to establish the antibacterial importance of the minor components of essential oil. To classify *Achillea* and *Artemisia* species in the space of studied parameters describing the chemical composition, as well as the antibacterial activity of essential oils, the HCA was applied. In this research, Ward's algorithm was used to produce cluster groups based on Euclidean distance. All researched EOs contained predominantly oxygenated monoterpenes. The strongest clustering is observed for *A. millefolium* and *A. crithmifolia* EOs. GC/MS data showed that these samples have similar chemical compositions, with highest content of oxygenated monoterpenes and lowest content of oxygenated sesquiterpenes and sesquiterpenes hydrocarbons. In PCA analysis these two EOs (group I) are located a considerable distance away from all of the other samples of EOs, indicating that their composition and activity differs significantly from the other samples. The location of these EOs in the lower right-hand quadrant can be explained by the highest positive loadings on PC1 (1.57 and 1.68, respectively). All bacteria for assessment of antimicrobial activity are grouped to the left side of the plot, located diametrically opposite to group I. This unique location can be pointed out as a reason for the lowest activity against bacteria, suggesting that the dominant EO components may not determine antibacterial activity. The advantages of using chemometric methods are confirming and explain experimental results, as well, eliminating any kind of subjective interpretations and discussions. The antibacterial potential of examined essential oils has been identified by chemometric methods, which were suggesting that the minor compounds (in our research oxygenated sesquiterpenes and

sesquiterpenes hydrocarbons) or a combination thereof were possibly responsible for the complete antibacterial activity of EOs.

CRediT authorship contribution statement

Dragoljub L. Miladinović: Conceptualization, Methodology, Writing - review & editing. **Marija V. Dimitrijević:** Formal analysis, Writing - original draft. **Tatjana M. Mihajilov-Krstev:** Investigation. **Marija S. Marković:** Resources. **Vojislav M. Ćirić:** Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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