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Variation of *Salvia officinalis* L. Essential Oil and Hydrolate Composition and Their Antimicrobial Activity

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Abstract: This study aimed to investigate the chemical composition of steam distillate essential oil and corresponding hydrolate obtained from *S. officinalis* grown in Serbia, as well as the influence of weather conditions (temperature and precipitations) on their chemical profiles. Furthermore, their antimicrobial activity was investigated in vitro. The main compounds in essential oil were *cis*-thujone, followed by camphor, *trans*-thujone, and 1,8-cineole, while hydrolate was slightly different from the essential oil, with camphor, *cis*-thujone, and 1,8-cineole as the main compounds. Among the eight respiratory-associated microorganisms, *Klebsiella oxytoca* was the most sensitive to the tested EOs (minimum inhibitory concentration (MIC)/minimal bactericidal/fungicidal concentration (MBC/MFC) were 14.20 and 28.4 μL mL^{-1}, respectively). MIC and MBC values of other tested bacteria ranged between 28.40 and 227.25 μL mL^{-1} while for *Candida albicans* MIC/MFC ranged from 28.40/56.81 to 56.81–113.63 μL mL^{-1}. Antibiotic susceptibility patterns for the analyzed eight respiratory-associated microorganisms showed an intermediate level of resistance to commonly used antibiotics such as ampicillin, levofloxacin, and ciprofloxacin. As a preliminary approach to the antimicrobial profiling of the tested EO, the obtained results revealed that the tested samples possess remarkable antibacterial activities and could be used to develop pharmaceutical formulations as an alternative to conventional antibiotic therapy.

Keywords: *Salvia officinalis*; essential oil; hydrolate; weather conditions; antimicrobial activity; in vitro

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1. Introduction

Antimicrobial resistance (AMR) poses a major threat to human health worldwide. Over several decades, to varying degrees, bacteria causing common or severe infections have developed resistance to each new antibiotic coming to market. Faced with this reality, the need for action to avert a developing global crisis in health care is imperative. Aiming to solve problems of antibiotic resistance in bacteria, many attempts have been made to investigate the potential role of essential oil and its active compounds. Many essential oils have been reviewed to possess different biological properties such as anti-inflammatory, sedative, digestive, antimicrobial, antiviral, antioxidant as well as cytotoxic activities [1–4].

Salvia officinalis L. (Sage) is a Balkan–Apennine endemic species spread worldwide [5]. Dalmatian sage, *S. officinalis* subsp. *officinalis* [6] is worth mentioning due to its high economic value. Today, it is cultivated in many European countries [7]; however, the largest share of the world sage market belongs to plants gathered from the wild or cultivated in Albania [8] and Croatia [9], followed by Turkey, Italy, Greece, France, and Spain [6]. In

Serbian flora, wild *S. officinalis*, a typical Mediterranean species, could be found only in Sićevo Gorge (Southeast Serbia) [10]. Based on morphological features, this population is defined as *S. officinalis* subsp. *multiflora* Gajić [11]. Studies of *S. officinalis* essential oil from Serbia analyzed this population [12–15] or commercially cultivated plants [16–19]. However, they all belong to chemotypes with dominant *cis*-thujone and camphor [20].

The essential oil of *S. officinalis* is known for its chemical composition, which has beneficial properties. Therefore, *S. officinalis* is a recognized herb in traditional medicine [21]. Owing to the chemical composition of sage essential oil, which provides a variety of medicinal properties, including antibacterial, antiviral, antifungal, and antioxidant actions [22–27]. Up to now, many research studies have been conducted to document the traditional uses of *S. officinalis* and to find new biological effects of sage. Even though the chemical composition of *S. officinalis* EOs has already been documented, the composition of EOs is quite complex and depends on the plant part, harvesting period, season, genetic variety, climate, and meteorological conditions [27,28]. Recently, conducted research studies have supported the antimicrobial effects of *S. officinalis*. The essential oil and ethanolic extract of *S. officinalis* show strong bactericidal and bacteriostatic effects against both Gram-positive and Gram-negative bacteria [21,24,29]. Additionally, *S. officinalis* has been reported to induce antifungal effects. Antifungal activity has been reported against *Candida glabrata, Candida albicans, Candida krusei, and Candida parapsilosis* [30].

The essential oils of the *Salvia* species contain various bioactive compounds such as terpenoids, steroids, flavonoids, and polyphenols, among others. The antimicrobial efficacy of *S. officinalis* is mainly attributed to terpens and terpenoids. It is well known that pure EOs typically have stronger antibacterial activity than single major components and their combinations indicate that the minor components are crucial to the synergistic activity though antagonistic and additive effects have also been noted [31]. It has been confirmed that camphor, thujone, and 1,8-cineole have antibacterial effects against *Aeromonas hydrophila*, *Aeromonas sobria*, *B. megatherium*, *B. subtilis*, *B. cereus*, and *Klebsiella oxytoca* [32]. Moreover, some compounds such as oleanolic and ursolic acid, two triterpenoids of *S. officinalis*, showed inhibitory action on the growth of multidrug-resistant bacteria [33].

In recent years, there has been an increasing trend of growing typical Mediterranean plants such as immortelle and lavender in Serbia [1,34]; growing *S. officinalis* is noted as well, but in significantly minor areas. The aim of this study was to investigate the chemical composition of steam distillate essential oil obtained from *S. officinalis* grown in Serbia, as well as the influence of weather conditions on their chemical profiles. Furthermore, general interest in finding new ways of valorizing processing by-products is an increasing new global trend [35,36]. For this purpose, the *S. officinalis* hydrolate as a by-product of the corresponding essential oil was also investigated by GC-MS to determine its chemical composition. Additionally, a preliminary approach to determination of the antimicrobial activity of both essential oil and hydrolate was investigated *in vitro*.

2. Materials and Methods

2.1. Plant Material

Salvia officinalis variety "Primorska" was established in spring 2017. Sage seedlings produced in the greenhouse (height 10 ± 2 cm) were transplanted in the first decade of May, in 70 cm rows with 30 cm intra-row planting space. After seed planting, irrigation was performed. Plants were grown without fertilization, while weed control was performed manually (weeding and hoeing).

2.2. Field Location

The study was carried out on the experimental plot of the Institute of Field and Vegetable Crops Novi Sad (Department for Alternative crops located in Bački Petrovac, detailed information is given in Table 1 over three successive growing years: 2018/19, 2019/20 and 2020/21.

Table 1. Characteristics of the experimental site conditions.

Criteria	Value
Altitude	79 m asl
Longitude	45°21′ E
Latitude	19°35′ N
Soil texture	Chernozem calcereous galeyic type
Soil pH	7.33–7.77 (alkaline soil)
Calcium carbonate	4.92% (moderate $CaCO_3$ content)
Humus	2.57% (low humus content)
Total Nitrogen	0.13% (moderate nitrogen content)
Phosphorus	75.0 mg/100 g (very high content of P_2O_5)
Potassium	37.74 mg/100 g (high content of K_2O)

2.3. Weather Conditions

Changes in climatic conditions in Serbia show a significant increase in temperature (increase in averaged temperature) and change in precipitation patterns (decrease in summer precipitation). These changes significantly influence agricultural production [37]. Assortment and agro-technology for some important crops, such as maize, soybean, sunflower and wheat, must be modified to achieve cost-effective production. Contrastingly, some medicinal and aromatic plants originating from the Mediterranean found optimal growing conditions in Serbia. Average monthly temperatures and precipitations for three successive investigated years are shown in Table 2.

Table 2. Average monthly temperatures and precipitations for three investigated years (2018/19, 2019/20, 2020/21).

	2018/19		2019/20		2020/21	
	T	P	T	P	T	P
IX	18.6	42.7	18.0	64.3	19.3	19.0
X	14.5	5.1	13.6	26.0	12.3	79.5
XI	7.8	23.6	11.1	58.9	6.5	17.2
XII	1.6	36.1	4.4	48.7	4.9	36.4
I	−0.1	47.9	0.2	10.1	2.9	68.0
II	4.0	14.1	6.0	42.9	5.0	52.3
III	9.3	14.4	7.7	57.3	6.4	32.0
IV	13.3	66.3	13.2	16.5	10.0	47.5
V	15.2	137.7	16.4	64.7	16.9	42.2
VI	23.5	83.8	20.4	122.2	23.8	14.2
VII	22.8	34.5	22.8	42.9	25.5	93.9
VIII	24.0	55.8	23.5	126.8	22.5	141.3
Average	12.9	562.0	13.1	681.3	13.0	643.5

2.4. Microbial Isolates and Growth Conditions

The eight clinical isolates used in this study were selected from a strain repository of the Department for Microbiological Diagnostics of the Institute for Pulmonary Diseases of Vojvodina. Isolates were obtained from wound swabs: *Staphylococcus aureus* (8684), *Enterobacter cloacae* (8923), *Pseudomonas aeruginosa* (8762), *Candida albicans* (8937), *Klebsiella oxytoca* (8929), *Escherichia coli* (8965) and blood cultures: *Staphylococcus aureus* (H2846), *Klebsiella pneumoniae* (H2807) of hospitalized patients.

Wound swabs are seeded on Blood agar, McConkey agar (MC, Himedia, Mumbai, India), Sabouraud dextrose agar (SDA, Himedia, Mumbai, India), and Thioglycollate broth (TB, Himedia, Mumbai, India), incubated for 48 h on 37 °C. The incubation of blood cultures was performed using the automated BacT/Alert system (BacT/Alert 3D, Biomerieux, Marcy-l'Étoile, France). Positive blood cultures were inoculated on Blood agar and McConkey agar. After overnight incubation at 37 °C for bacterial strains and 25 °C for the fungal strain, macroscopic examinations were performed. Final identification of

isolates and antimicrobial susceptibility testing was performed using the Vitek 2 Compact automated system (BioMérieux, Marcy-l'Étoile, France). The interpretation of antimicrobial susceptibility testing was performed according to EUCAST [38].

2.5. Antimicrobial Activity

All microorganisms were cultured on non-selective agar and incubated at 37 °C for 24 h. Afterwards, a single bacterial colony was picked up from the agar and then incubated in Nutrient Broth (NB, Himedia, Mumbai, India) at 37 °C, for 24 h. The strain *C. albicans* was cultured on Sabourad dextrose Broth (SDB, Himedia, Mumbai, India) at 25 °C, for 24 h.

After centrifugation at 10,000× *g* for 5 min., obtained pellets were resuspended in Peptone salt solution the density of the suspensions used for tests was adjusted to 0.5 Mc Farland units (~1.5 × 10^8 CFU mL^{-1}) using a densitometer DEN-1 (Biosan, Riga, Latvia). The efficacy of EOs on microorganisms was determined according to the CLSI [39] with slight modifications [40].

Mueller–Hinton broth (MHB, HiMedia, Mumbai, India) supplemented with 0.05% Tween 80 (Polyoxyethylenesorbitan monooleate, HiMedia, Mumbai, India) filled into each test well (100 µL). In the case of EOs, the first well was filed with 100 µL of EO. The mentioned surfactant served as a solvent for the essential oil samples. Afterwards, serial doubling dilutions of the tested EOs were prepared in a 96-well microtiter plate well (Sigma-Aldrich, St. Louis, Missouri, United States) over the range from 454.4 to 0.22 µL mL^{-1}. At the end, from the last test well, 100 µL was removed. Subsequently, 10 µL suspensions were added to each test well. The final volume in each well was 110 µL mL^{-1} and the final microbial concentration was 10^6 CFU mL^{-1}. The plate was incubated for 24 h at 37 °C, while *C. albicans* plates were incubated at 25 °C.

Mueller–Hinton broth (MHB, HiMedia, Mumbai, India) without Tween 80 filled into each test well (100 µL). In the case of hydrolates, the first well was filed with 200 µL of hydrolates. Afterwards, serial doubling dilutions of the tested hydrolates were prepared in a 96-well microtiter plate (Sigma-Aldrich, St. Louis, Missouri, United States) over the range from 606.0 to 0.295 µL mL^{-1}. In the end, from the last test well, 200 µL was removed. Subsequently, 10 µL suspensions were added to each test well. The final volume in each well was 110 µL/mL and the final microbial concentration was 10^6 CFU mL^{-1}. The plate was incubated for 24 h at 37 °C, while *C. albicans* plates were incubated at 25 °C.

In all tests, growth control (MHB + test organism), sterility control I (MHB + test oil), and sterility control II (MHB) were included. Microbial growth was determined by adding 10 µL of 0.01% resazurin (HiMedia, Mumbai, India) aqueous solution. The plates were further incubated at 37 °C for 24 h in darkness. A change of color from blue (oxidized-resazurin remained unchanged) to pink (reduced) indicated the growth of bacteria. Referring to the results of the minimal inhibitory concentration (MIC), the wells showing a complete absence of growth were identified and 100 µL of the solutions from each well were transferred to Mueller–Hinton Agar (MHA, Himedia, Mumbai, India) and incubated at 37 °C for 24 h. In the case of *C. albicans*, growth was identified on Sabourad dextrose Agar (SDA Himedia, Mumbai, India) at 25 °C, for 24 h.

The minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) were defined as the lowest concentration of the EOs/Hydrolates at which 99.9% of the inoculated microorganisms were killed.

3. Results and Discussion

3.1. Chemical Composition

The chemical compositions of the essential oils of *S. officinalis* obtained in 2019, 2020, and 2021 are presented in Table 3, while the raw data are included in Supplement Figure S1a–c. According to the obtained results, the main compounds in *S. officinalis* essential oil were *cis*-thujone with 23.5% on average for three years (content ranged between 19.9 and 29.0%), camphor with 17.7% (15.8–19.6%), *trans*-thujone 12.9% (12.3–13.3%) and 1,8-cineole 10.0% (8.8–11.3%). The *cis*-thujone and *trans*-thujone, and their relationship,

were often familiar as two compounds depicting chemotypes of *S. officinalis*, as well as camphor combined with other compounds, but the obtained results suggest a mixture of the mentioned isomers and camphor as the most dominant constituent of the same samples in all three tested growing years. A much closer similarity between essential oil obtained in 2019 and 2021 can also be observed. This similarity might be explained based on a relation of hydrocarbons and oxygenated compounds in the essential oil, with a higher similarity, found when oxygenated compounds are the main component in the oil sample. It is worth emphasizing that the main constituent, *cis*-thujone, represents a monoterpene ketone that is usually found in different plants such as *Salvia officinalis*, *S. sclarea*, *Tanacetum vulgare*, *Thuja occidentalis* etc. Although ketones are generally non-toxic, thujone is the most toxic one, and its presence in food and beverages has been managed by different regulations at the continent level [41].

Table 3. *Salvia officinalis* essential oil chemical composition.

No.	Compound	RI	2019	2020	2021
1	*cis*-Salvene^{NMT}	846	0.5	0.1	0.3
2	*trans*-Salvene^{NMT}	856	0.1	nd	nd
3	Tricyclene^{MT}	920	0.2	0.1	0.2
4	α-Thujene^{MT}	924	0.2	0.1	0.2
5	α-Pinene^{MT}	931	4.3	2.8	4.3
6	Camphene^{MT}	946	6.2	4.7	6.3
7	Sabinene^{MT}	970	0.1	0.1	0.1
8	β-Pinene^{MT}	974	2.2	2.4	3.9
9	Myrcene^{MT}	988	1.2	0.8	1.0
10	α-Phellandrene^{MT}	1004	0.1	tr	0.1
11	α-Terpinene^{MT}	1014	0.2	0.2	0.2
12	*p*-Cymene^{MT}	1022	0.7	0.2	0.4
13	Limonene^{MT}	1025	3.1	2.7	3.1
14	1,8-Cineole^{OMT}	1028	8.8	9.8	11.3
15	*cis*-β-Ocimene^{MT}	1034	nd	0.1	nd
16	γ-Terpinene^{MT}	1055	0.4	0.4	0.4
17	Terpinolene^{MT}	1086	0.2	0.3	0.3
18	Linalool^{OMT}	1099	0.3	0.4	0.5
19	*cis*-Thujone^{OMT}	1106	29.0	21.7	19.9
20	*trans*-Thujone^{OMT}	1115	12.3	13.2	13.3
21	*iso*-3-Thujanol^{OMT}	1132	0.1	0.2	nd
22	*trans*-Sabinol (OH vs. IPP)^{OMT}	1138	0.1	nd	nd
23	Camphor^{OMT}	1143	17.7	19.6	15.8
24	Isoborneol^{OMT}	1151	nd	tr	nd
25	*trans*-Pinocamphone^{OMT}	1158	tr	0.1	nd
26	Borneol^{OMT}	1163	1.6	3.5	3.6
27	*cis*-Pinocamphone^{OMT}	1168	nd	0.2	nd
28	Terpinen-4-ol^{OMT}	1174	0.3	nd	nd
29	α-Terpineol^{OMT}	1188	0.1	0.1	nd
30	Myrtenol^{OMT}	1194	0.1	0.1	nd
31	Neral^{OMT}	1239	0.1	nd	nd
32	Geranial^{OMT}	1269	0.1	nd	nd
33	Bornyl acetate^{OMT}	1284	0.6	1.6	1.1
34	*trans*-Sabinyl acetate (Ac vs. IPP)^{OMT}	1291	0.2	nd	nd
35	α-CopaeneST	1369	nd	nd	0.1
36	β-BourboneneST	1379	nd	tr	nd
37	*trans*-CaryophylleneST	1417	2.4	5.5	4.2
38	α-HumuleneST	1452	3.2	4.8	4.0
39	*allo*-AromadendreneST	1459	0.1	0.1	0.1

Table 3. *Cont.*

No.	Compound	RI	2019	2020	2021
40	γ-MuuroleneST	1472	nd	0.1	nd
41	ViridifloreneST	1494	0.1	0.1	nd
42	δ-CadineneST	1518	nd	0.1	0.1
43	Caryophyllene oxide^{OST}	1581	0.2	0.3	nd
44	Viridiflorol^{OST}	1589	1.6	nd	nd
45	Humulene epoxide II^{OST}	1606	0.4	nd	0.4
46	13-epi-Manool^{OST}	2054	nd	nd	0.3
	Normonoterpene hydrocarbons (NMT)		0.6	0.1	0.3
	Monoterpene hydrocarbons (MT)		19.1	14.9	20.5
	Oxygenated monoterpenes (OMT)		71.4	70.5	65.5
	Sesquiterpene hydrocarbons (ST)		5.8	10.7	8.5
	Oxygenatedsesquiterpens (OST)		2.2	0.3	0.7
	Total identified		99.1	96.5	95.5

nd—not detected.

To the authors' knowledge, many relevant scientific papers deal with geographically different *S. officinalis* samples, but not with growing-year-dependencies. Comparing the average value of the chemical composition of the samples in this work with other *S. officinalis* samples, it can be noted that the main constituents are not correlated with samples from different parts of the Balkan Peninsula and other parts of southeastern Europe summarized [42]. The main similarity is the ever-present camphor and some isomers of thujone but in completely different concentrations. Other constituents, especially in minor quantities, can be completely different at the geographical region level. Each *S. officinalis* essential oil sample can be described through diverse chemotypes, but they mutually correspond only partly with the findings in this study. Russo et al. [43] emphasized the fact that inconsistency in EOs constituents of *S. officinalis* is contingent on environmental factors such as altitude, water availability, and pedoclimatic conditions.

For easier understanding and presenting the differences and similarities of the tested essential oil samples, the calculated correlations are illustrated in Figure 1 using the "corrplot" function, using the "circle" method, from R Studio 1.4.1106 program. The size and the circle's color rely on the correlation coefficients; if the color is blue, the positive correlation was conducted; on the contrary, the red color symbolizes the negative correlation. Furthermore, the circle's dimensions are increased with the correlation coefficient's absolute value.

Thoroughly illustrating the structure of the experimental data would deliver a more profound explanation of relations between diverse samples of *S. officinalis* from 2019, 2020, and 2021; PCA was used, and the received results are shown in Figure 2. The PCA of the relations between volatile compounds of *S. officinalis* essential oil explained that the first two principal components summarized 100% of the total variance in the 46 parameters (volatile compounds). The first PC explained 67.43% and the second 32.57% of the total variance between the experimental data. The parting within samples could be seen from the PCA figure, where the volatile compounds of *S. officinalis* essential oil during 2019 are grouped on the left, 2020 on the top, and 2021 on the bottom side of the graphic.

Given that the samples from different geographical areas differ significantly, a comparison of samples from this work with samples from the same geographical area, more precisely from the Republic of Serbia, is given in Table 4, as well as their compatibility with the ISO 9909 standard [44], limited amounts of toxic thujones, and other compounds.

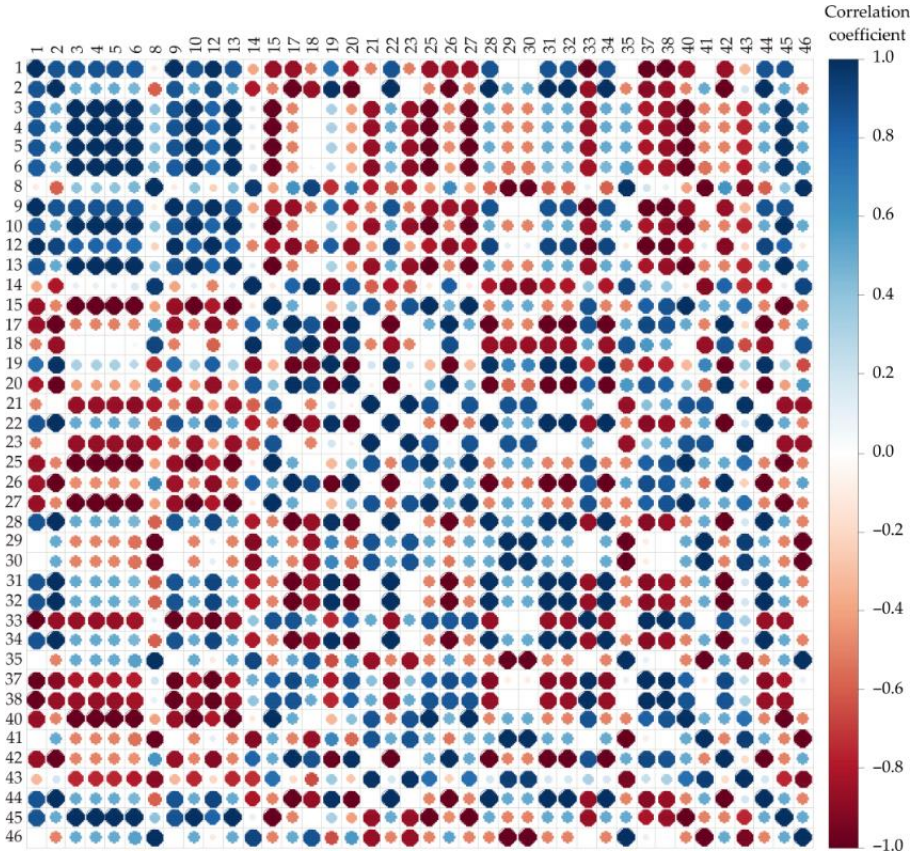

Figure 1. Correlation between volatile compounds of *S. officinalis* essential oil (the compounds were codded according to Table 3).

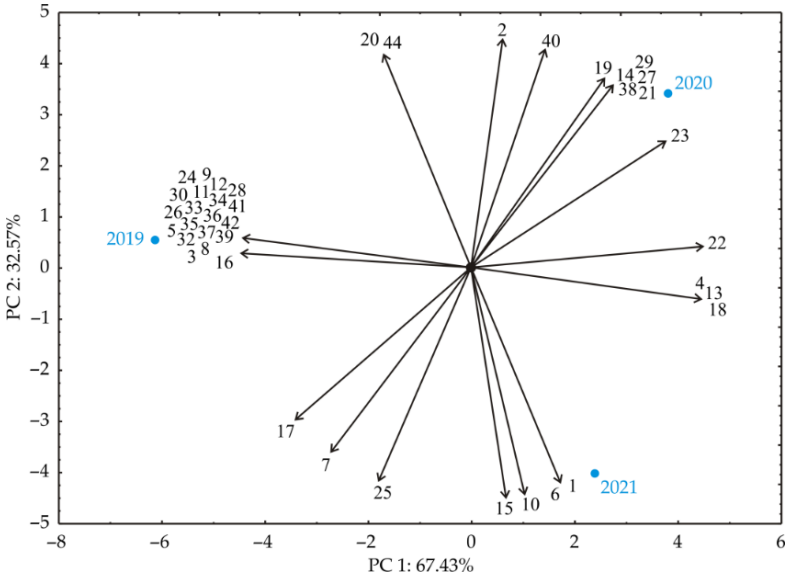

Figure 2. The PCA biplot diagram describing the relations between volatile compounds of *Salvia officinalis* essential oil.

Table 4. Chemical composition of *S. officinalis* essential oil samples from Serbia and limited values according to ISO 9909 standard.

	References	α-Pinene	Camphene	Limonene	1,8-Cineole (Eucalyptol)	Linalool + Linalyl Acetate	*cis*-Thujone (α-Thujone)	*trans*-Thujone (β-Thujone)	Camphor	Bornyl Acetate	α-Humulene
Wild groving	[12]	3.5	3.1	0.0	9.8	0.0	24.9	8.1	16.0	2.7	0.0
	[14]	3.7	1.9	0.9	16.2	tr	22.1	2.9	5.4	0.3	11.0
	[14]	0.8	tr	0.8	12.6	tr	22.1	3.0	4.4	0.3	12.7
	[13]	3.2	2.4	0.0	16.7	0.0	19.5	3.8	9.7	2.9	7.6
	[13]	3.0	5.3	0.0	6.4	0.0	19.9	3.5	24.8	4.9	4.0
	[15]	3.2	1.6	tr	16.4	4.3	19.1	2.3	2.5	0.3	8.7
Cultivated	[16]	5.1	3.7	1.2	14.4	0.0	37.5	4.7	13.8	0.4	5.0
	[17]	4.3	8.5	8.3	5.8	0.0	13.3	1.7	32.7	1.4	3.4
	[18]	3.0	4.6	4.4	11.5	0.3	27.1	12.3	19.3	0.5	2.4
	[19]	0.0	0.0	1.2	12.1	0.0	35.5	6.6	20.7	2.4	1.7
	TS19	4.3	6.2	3.1	8.8	0.3	29.0	12.3	17.7	0.6	3.2
	TS20	2.8	4.7	2.7	9.8	0.4	21.7	13.2	19.6	1.6	4.8
	TS21	0.2	6.3	3.1	11.3	0.5	19.9	13.3	15.8	1.1	4.0
	[44]	1.0–6.5	1.5–7.0	0.5–3.0	5.5–13.0	≤1.0	18.0–43.0	3.0–8.5	4.5–24.5	≤2.5	≤12.5

tr—in traces; grey fields represent values which satisfied ISO 9909 standard.

Additionally, as can be seen from Table 4, α-pinene content in *S. officinalis* samples from Serbia ranges between 0.0 and 5.1%. However, the ISO 9909 standard specifies content of this compound from 1.0 to 6.5%. Further, camphene content ranged between 0.0 and 8.5%, while recommend values according to the ISO 9909 are between 1.5 and 7.0%. Limonene content in *S. officinalis* from Serbia ranged from 0.0 to 8.3%, while the percentages limited by the ISO 9909 standard are between 0.5 and 3.0%. The 1,8-cineole ranges between 5.8 and 16.7%, while limits are within 5.5 and 13.0%. The mixture of linalool and linalyl acetate in Serbian samples is between 0.0 and 4.3%, while the limit value is 1.0%. Cis-thujone varied between 19.1 and 37.5%, while the ISO 9909 standard limits this compound between 18.0 and 43.0%. However, trans-thujone varied between 1.7 and 13.3%, while between 3.0 and 8.5% is allowed. Camphor content is between 2.5 and 32.7%, while the ISO 9909 standard recommended between 4.5 and 24.5%. Bornyl acetate content in *S. officinalis* samples from Serbia is between 0.3 and 4.9%, while recommended values are below 2.5%. The last limited compound according to the ISO 9909 standard is α-humulene (≤12.5%), whose values ranged between 0.0 and 12.7%. As can be seen from the table, no one sample of *S. officinalis* essential oil satisfied ISO 9909 standard criteria for all ten compounds. According to the results, it can be seen that the tested samples (TS 19, TS 20, and TS 21) stand out with a higher amount of trans-thujone than all other samples and the recommended standard value. For all other constituents, it can be concluded that Serbian samples are quite different, and defining the chemical composition each time is necessary and needs to be part of a standardized protocol for the further use of essential oil of this plant.

The same methodology for chemical characterization was used for hydrolate of *S. officinalis* from all tested growing years. The hydrolate represents a by-product of the essential oil production process through a distillation process and lagging in large quantities. Therefore, the potential utilization of this water rich in phytochemicals can ensure a greener pathway in essential oil production. Based on the results shown in Table 5 (the raw data were included in Supplement Figure S1d–f), the main compounds in *S. officinalis* hydrolate were camphor with 44.9% on average for three years (content ranged between 42.4 and 49.6%), followed by *cis*-thujone with 15.7% (13.4–19.8%) and 1,8-cineole with 15.5% (12.3–20.5%). It can be observed that camphor is the dominant constituent of hydrolates, followed by *cis*-thujone, while the opposite situation was determined in the case of tested essential oils (Table 3).

Table 5. *Salvia officinalis* hydrolate chemical composition.

No.	Compound	RI	2019	2020	2021
1	3-Hexanol^{O}	795	0	0	0.1
2	Isovaleric acid^{O}	828	0.1	0.2	0
3	Furfural^{O}	830	0.1	0	0
4	*cis*-3-Hexenol^{O}	847	0	0.3	0.3
5	*trans*-3-Hexenol^{O}	850	0.1	0	0
6	cis-2-Hexenol^{O}	861	0	0	0.1
7	1-Octen-3-ol^{O}	975	0.3	0.2	0.3
8	6-methyl-5-Hepten-2-one^{O}	985	0.4	0	0
9	*p*-Cymene^{MT}	1023	0.1	0	0
10	1,8-Cineole^{OMT}	1031	13.7	12.3	20.5
11	Benzyl alcohol^{O}	1036	0.1	0	0
12	Benzene acetaldehyde^{O}	1042	0.2	0.1	0.1
13	*cis*-Linalool oxide (furanoid)^{OMT}	1071	0.3	0.4	0.4
14	*trans*-Linalool oxide (furanoid)^{OMT}	1089	0.3	0.4	0.3
15	Linalool^{OMT}	1103	0.5	0.4	0.8
16	*cis*-Thujone^{OMT}	1108	19.8	13.4	13.9
17	*trans*-Thujone^{OMT}	1117	7.1	5.7	6.8
18	*iso*-3-Thujanol^{OMT}	1140	0.3	0.4	0.4
19	Camphor^{OMT}	1150	42.8	49.6	42.4
20	Isoborneol^{OMT}	1159	0.1	0.1	0
21	*trans*-Pinocamphone^{OMT}	1160	0.1	0.2	0.1
22	Borneol^{OMT}	1167	4.7	10.5	9.1
23	*cis*-Pinocamphone^{OMT}	1169	0	0.3	0.1
24	*trans*-Linalool oxide (pyranoid)^{OMT}	1174	0.1	0	0
25	Terpinen-4-ol^{OMT}	1177	1.2	0.9	1.3
26	*p*-Cymen-8-ol^{OMT}	1185	0.4	0	0
27	*α*-Terpineol^{OMT}	1191	0.4	0.5	0.4
28	*p*-Mentha-1,5-dien-8-ol^{OMT}	1192	0.2	0	0
29	Myrtenol^{OMT}	1197	0.2	0.3	0.2
30	*exo*-2-Hydroxycineole^{OMT}	1210	0.1	0	0
31	*trans*-Carveol^{OMT}	1219	0.2	0.2	0.2
32	*cis*-*p*-mentha-1(7),8-dien-2-ol^{OMT}	1228	0.1	0	0
33	*cis*-Carveol^{OMT}	1230	0.1	0	0
34	Neral^{OMT}	1241	0.1	0	0
35	Carvone^{OMT}	1244	0.1	0	0
36	Geraniol^{OMT}	1254	0.1	0	0
37	Geranial^{OMT}	1271	0.3	0	0
38	Bornyl acetate^{OMT}	1286	0.1	0.2	0.1
39	Thymol^{OMT}	1293	0.1	0	0
40	Carvacrol^{OMT}	1302	0.1	0.3	0
41	*p-vinyl*-Guaiacol^{O}	1314	0.1	0	0
42	Piperitenone^{OMT}	1342	0.1	0	0
43	Eugenol^{PP}	1358	0.1	0.1	0,1
44	Humulene epoxide II^{OST}	1609	0.1	0.1	0
	Monoterpene hydrocarbons (MT)		0.1	tr	/
	Oxygenated monoterpenes (OMT)		93.7	96.1	97.0
	Oxygenatedsesquiterpens (OST)		0.1	0.1	/
	Phenylpropanoids (PP)		0.1	0.1	0.1
	Other (O)		1.4	0.8	0.9
	Total identified		95.4	97.1	98.0

tr—trace (less than 0.05%).

For easier understanding and presenting the differences and similarities of the tested hydrolate samples, the correlation analysis was performed to analyze the similarities between volatile compounds of *S. officinalis* hydrolate, and the results are displayed in Figure 3.

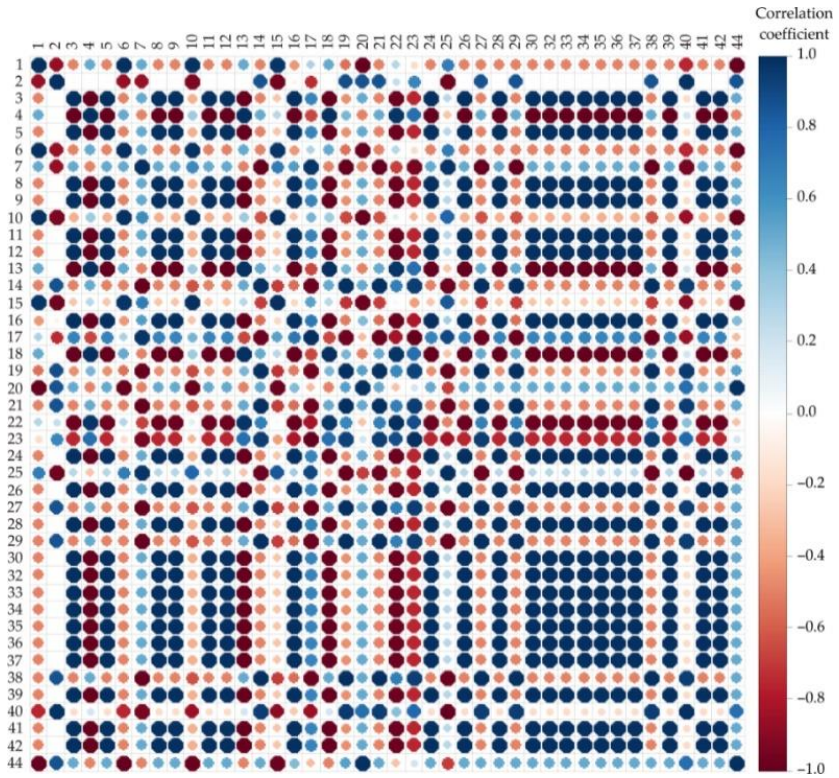

Figure 3. Correlation between volatile compounds of *S. officinalis* hydrolate (the compounds were codded according to Table 5).

The PCA of the relations between the volatile compounds of *S. officinalis* hydrolate explained that the first two principal components summarized 100% of the total variance in the 44 parameters (volatile compounds). The first PC explained 59.18% and the second 40.82% of the total variance between the experimental data. The parting within samples could be seen from the PCA Figure 4, where the volatile compounds of *S. officinalis* hydrolate during 2019 are assembled on the left, 2020 on the right, and 2021 on the bottom side of the graphic.

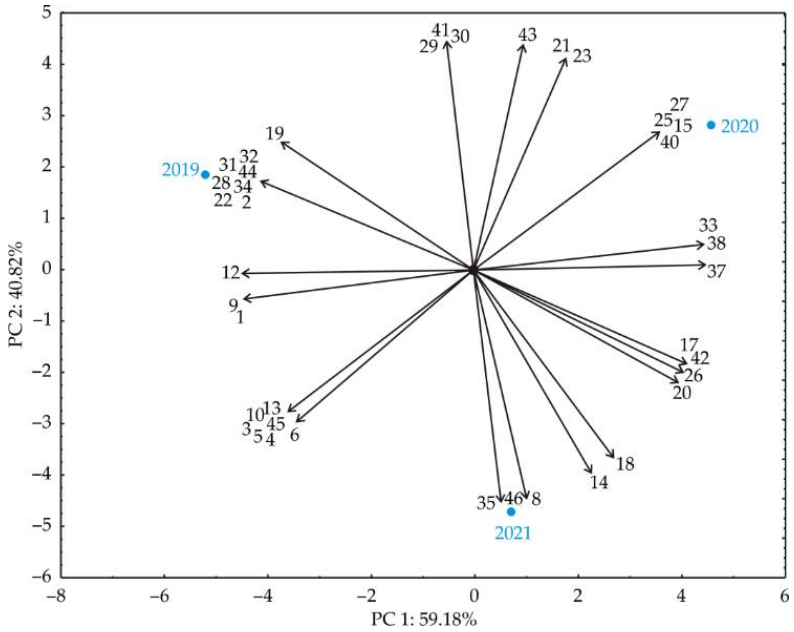

Figure 4. The PCA biplot diagram describing the relations between volatile compounds of *S. officinalis* hydrolate.

There are only two previous studies including a comparative analysis of *S. officinalis* essential oil and corresponding hydrolate [45,46], while one focuses only on hydrolate [47]. The hydrolate composition was rather different from the essential oil [46]. The main compounds in both, as in our study, are 1,8-cineole (10.0–30.4% in EO and 15.5–61.4% in H), *cis*-thujone (9.7–23.5% in EO and 8.4–15.7% in H) and camphor (17.1–19.9% in EO and 22.5–44.9% in H) (Table 6). As can be seen, the content of the first two compounds (1,8-cineole and *cis*-thujone) was higher in essential oil than in hydrolate, while the content of camphor was higher in hydrolate in comparison to essential oil. The highest content of camphor in hydrolate in comparison to essential oil could be explained by the fact that camphor is highly soluble in distillation water [46].

Table 6. Essential oil and hydrolate composition according to this study and literature.

No.	Compound	This Study		[46]		[48]		[47]
		EO	H	EO	H	EO	H	H
1	α-Pinene	2.4	nd	2.8	tr	6.0	nd	nd
2	Camphene	5.7	nd	3.52	tr	7.3	nd	nd
3	β-Pinene	2.8	nd	2.5	tr	3.8	nd	nd
4	Myrcene	1.0	nd	1.2	tr	nd	nd	nd
5	*p*-Cymene	0.4	nd	0.5	tr	1.1	nd	nd
6	Limonene	3.0	nd	1.8	tr	nd	nd	nd
7	1,8-Cineole	10.0	15.5	17.9	24.0	30.4	61.4	24.0
8	γ-Terpinene	0.4	nd	0.3	tr	0.3	nd	nd
9	Linalool + linalyl acetate	0.4	0.6	1.8	2.7	nd	nd	0.1
10	*cis*-Thujone	23.5	15.7	20.1	15.5	9.7	8.4	3.6
11	*trans*-Thujone	12.9	6.5	9.1	4.4	nd	3.4	12.9
12	Camphor	17.7	44.9	19.9	43.4	17.1	22.5	51.0
13	Borneol	2.9	8.1	4.6	7.7	1.6	nd	2.2
14	α-Terpineol	0.1	0.4	0.2	tr	0.3	nd	2.2
15	Myrtenol	0.1	0.2	0.5	0.6	nd	nd	nd
16	Bornyl acetate	1.1	0.1	1.3	tr	1.1	1.4	nd
17	*trans*-Caryophyllene	4.0	nd	4.4	tr	3.6	nd	nd
18	α-Humulene	4.0	nd	1.8	tr	2.5	nd	nd
19	Caryophyllene oxide	0.2	nd	0.9	tr	0.5	nd	nd
20	Viridiflorol	0.5	nd	2.2	tr	0.6	nd	nd
	Total tujones	36.4	22.2	29.2	19.9	9.7	11.8	16.5
	Total	93.2	93.1	96.62	98.3	91.7	97.1	96.0

EO—essential oil; H—hydrolate; nd—not detected; tr—trace (less than 0.05%).

According to this study and the literature, the correlation analysis results about the similarities between volatile compounds of essential oil and hydrolate composition were graphically presented in Figure 5.

According to this study and the literature, the PCA of the relations between volatile compounds of essential oil and hydrolate composition (Figure 6) explained that the first two principal components summarized 71.6% of the total variance in the 20 parameters (volatile compounds). The first PC explained 49.43% and the second 22.17% of the total variance between the experimental data. The parting within samples could be seen from the PCA figure, where the volatile compounds of *S. officinalis* essential oil are arranged on the left side, while the volatile compounds of *S. officinalis* hydrolate are arranged on the side of the figure.

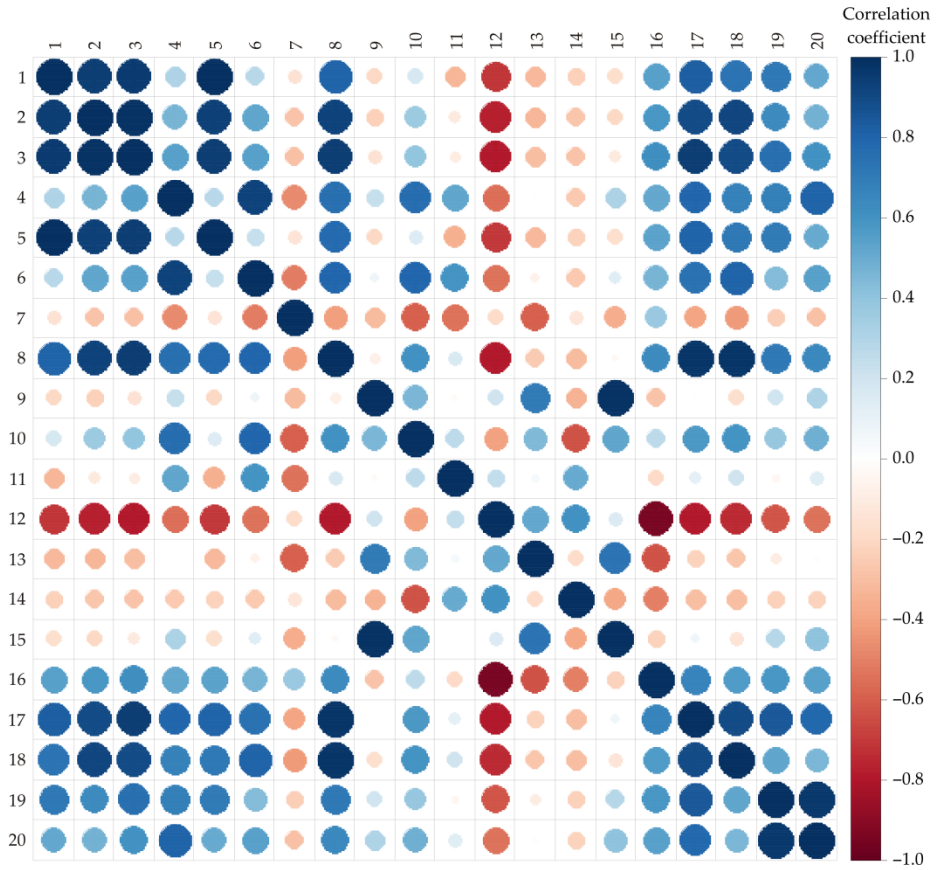

Figure 5. Correlation between volatile compounds of essential oil and hydrolate composition according to this study and literature (the compounds were codded according to Table 6).

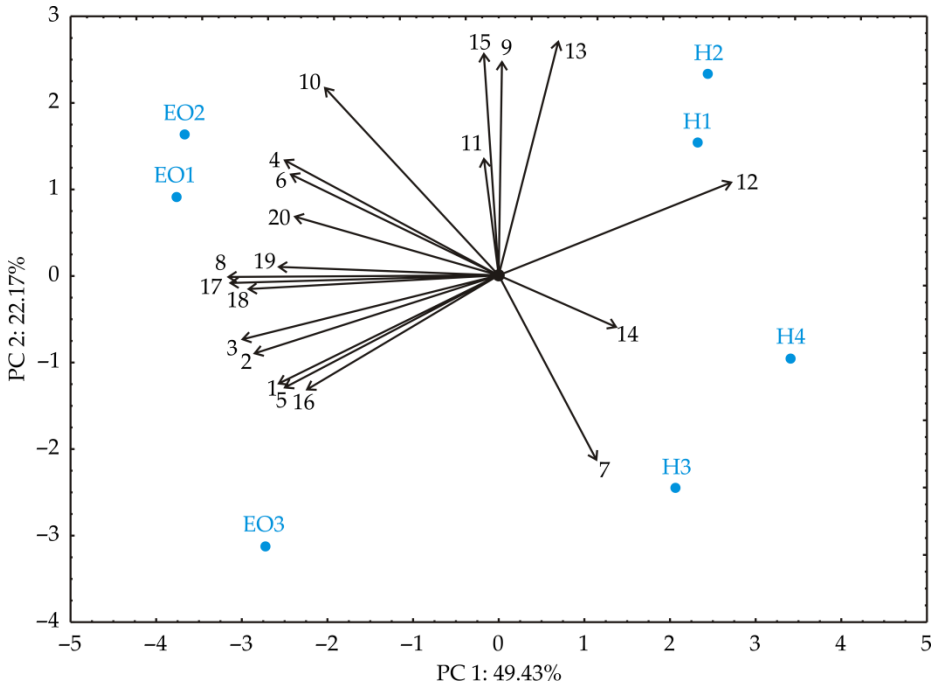

Figure 6. The PCA biplot diagram describing the relations between volatile compounds of essential oil and hydrolate composition according to this study and the literature.

3.2. Antibiotic Susceptibility Testing of Wound-Associated Microorganisms

As mentioned, antimicrobial resistance is a foremost threat to human health. Different bacteria and fungi, causing infections in human and animal populations, have developed resistance to each new antibiotic coming to market [2]. Therefore, testing all clinical-relevant isolates on different well-known antibiotics, but also finding effective alternatives for the antibiotic need to be imperative to this and the next human generation. In this study, antibiotic susceptibility patterns for the analyzed eight respiratory-associated bacteria are shown in Table 7. Antibiotics included in the testing are ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefuroxime (axetil or sodium) (CXM), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), gentamicin (GEN), amikacin (AMK), tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LVX), cotrimoxazole (CMX), ertapenem (ERT), imipenem (IMI) meropenem (MEM), erythromycin (ERY), clindamycin (CLY), tetracycline (TET), linezolid (LZD), vancomycin (VAN), tigecycline (TGC). It can be seen that *S. aureus* strains are most sensitive to the greatest number of the tested antibiotics, while *P. aeruginosa*, *E. coli*, and *Klebsiella* representatives are resistant to some of the most commonly used antibiotics in clinical respiratory infections.

Table 7. Antibiotic susceptibility patterns for respiratory-associated bacteria (S—sensitive, I—intermediate, R—resistant).

Number	Bacterial Isolate	AMP	AMC	TZP	CXM	CTX	CRO	CAZ	FEP	GEN	AMK	TOB	CIP
1	*S. aureus* H2846	S	S	S	S	S	S	/	S	S	S	S	I
2	*S. aureus* 8684	S	S	S	S	S	S	/	S	S	S	S	I
3	*E. cloacae* 8923	R	R	S	S	S	S	S	S	S	S	S	S
4	*E. coli* 8965	S	S	S	S	S	S	S	S	S	S	S	R
5	*P. aeruginosa* 8762	/	/	R	/	/	/	R	R	/	S	S	I
6	*K. oxytoca* 8929	R	S	S	S	S	S	S	S	S	S	S	S
7	*K. pneumoniae H2807*	R	S	S	S	S	S	S	S	S	S	S	S
Number	Bacterial isolate	LVX	CMX	ERT	IMI	MEM	ERY	CLY	TET	LZD	VAN	TGC	
1	*S. aureus* H2846	I	S	S	S	S	S	S	S	S	S	S	
2	*S. aureus* 8684	I	S	S	S	S	S	S	S	S	S	S	
3	*E. cloacae* 8923	S	S	S	S	S	/	/	/	/	/	/	
4	*E. coli* 8965	R	S	S	S	S	/	/	/	/	/	/	
5	*P. aeruginosa* 8762	I	/	/	I	S	/	/	/	/	/	/	
6	*K. oxytoca* 8929	S	R	S	S	S	/	/	/	/	/	/	
7	*K. pneumoniae H2807*	S	S	S	S	S	/	/	/	/	/	/	

ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefuroxime (axetil or sodium) (CXM), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), gentamicin (GEN), amikacin (AMK), tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LVX), cotrimoxazole (CMX), ertapenem (ERT), imipenem (IMI) meropenem (MEM), erythromycin (ERY), clindamycin (CLY), tetracycline (TET), linezolid (LZD), vancomycin (VAN), tigecycline (TGC).

The graphical presentation of correspondence analysis for the experimental results of antibiotic susceptibility test presented in Table 7 is illustrated in Figure 7. Microorganisms are numbered according to Table 7 (*C. albicans* was omitted because is not a bacterial strain). Significant correspondence was detected among the considered categories (total inertia was 0.301; χ^2 was 101.13; df = 132; $p < 0.00097$). The first two dimensions account for 94.22% of the total inertia, using a considerably satisfactory quota of the raw information. From Figure 7, it can be seen that the most effective antibiotics on *S. aureus* H2846 and *S. aureus* 8684 were vancomycin, linezolid, tigecycline, tetracycline, erythromycin and clindamycin. *E. coli* 8965 was the most sensitive to ceftriaxone, amoxicillin-clavulanic acid, cefotaxime, cotrimoxazole, ertapenem, cotrimoxazole, gentamicin and ampicillin. *E. cloacae* 8923, *K. oxytoca* 8929 and *K. pneumoniae* H2807 were most sensitive to piperacillin-tazobactam, cefepime, gentamicin, cefotaxime, imipenem, and ceftazidime. Finally, *P. aeruginosa* 8762 was the most sensitive to meropenem, amikacin, and tobramycin.

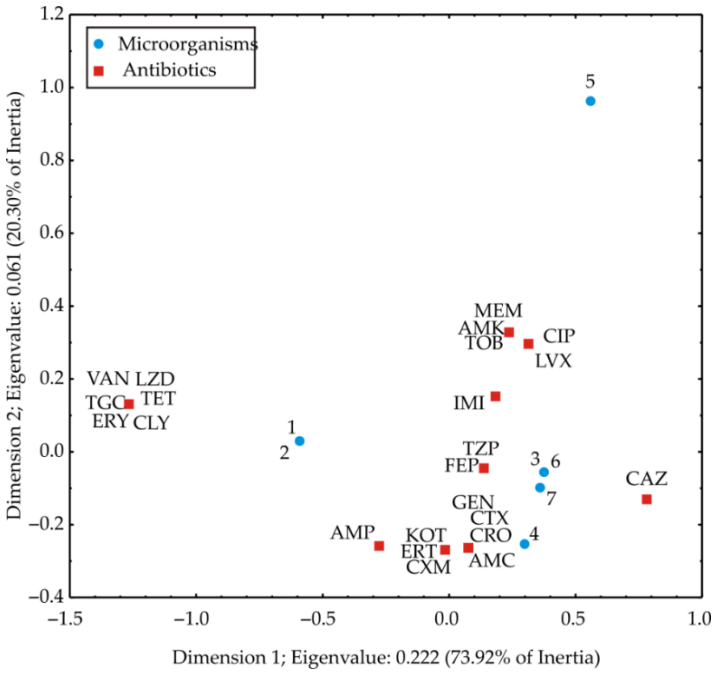

Figure 7. Correspondence plot diagram for antibiotic susceptibility patterns for respiratory-associated bacteria.

3.3. Antimicrobial Activity

As a preliminary approach and primary step in the antimicrobial profiling of the tested samples, minimal inhibitory and biocidal concentration was determined. The obtained minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) values of the investigated EOs and hydrolates are summarized in Table 8. As can be seen, *S. officinalis* oil showed the greatest effectiveness compared to hydrolates. Generally, EOs of *S. officinalis* showed remarkable antimicrobial activity against seven of eight tested microorganisms. MIC and MBC of sensitive bacteria ranged between 28.40 and 227.25 μL mL^{-1} while for *C. albicans* MIC/MFC ranged from 28.40/56.81 to 56.81–113.63 μL mL^{-1}.

S. officinalis oil from 2019 and 2020 showed equal MIC against gram-positive bacteria (*S. aureus* H2846 and *S. aureus* 8684) and gram-negative bacteria (*E. coli*, *E. cloacae*, *K. pneumonia*, and *K. oxytoca*) MIC = 56.81 μL mL^{-1} with the exception of strain *P. aeruginosa* (MIC/MBC= >454.50 μL mL^{-1}). A high level of intrinsic resistance of *P. aeruginosa* to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell, and production of antibiotic-inactivating enzymes such as β-lactamases, has been shown through many studies [48].

Further, the strongest activity shows essential oil distilled from plants grown in 2021, against *K. oxytoca* (the lowest concentrations for MIC and MBC, 14.20 and 28.4 μL mL^{-1}, respectively). According to Table 3, this sample has the highest concentration of oxygenated monoterpenes (among them 1,8-cineole, linalool, *trans*-thujone, and borneol), and their synergistic activity could be responsible for the effect. On the other side, hydrolates were inactive against all tested pathogens with MIC/MBC higher than 606 μL mL^{-1} (Table 8).

Antibacterial activity of *S. officinalis* can be attributed primarily to the presence of camphor, *cis*-Thujone, *trans*- Thujone, and 1,8-cineole but not to other compounds with lower amounts [26]. In other research, the presence of the components 1,8-cineole, thujone, and camphor has also been related to the antimicrobial activity of sage essential oil [32,49]. Results obtained in this study were confirmed by Delamare et al. [32] who showed that the antimicrobial activities of *S. officinalis* against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus* were attributed to high concentrations of thujone, 1,8-cineole and camphor [45]. In the same way, Hammer et al. demonstrated that camphor and 1,8-cineole were the main components

responsible for the antibacterial activity against *B. subtilis*, *E. coli*, and *S. aureus* [50]. Dorman and Deans [51] stated that the minor components of essential oil, such as borneol, possess antimicrobial activities.

Table 8. Minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) for *S. officinalis* essential oil and hydrolate.

Strains	Tested Concentration (μL mL^{-1})	Essential Oils			Hydrolates		
		2019	2020	2021	2019	2020	2021
S. aureus H2846	MIC MBC	56.81	56.81	56.81	>606.00 *	>606.00	>606.00
S. aureus 8684	MIC MBC	113.63 227.25	56.81 113.63	28.40 56.81	>606.00	>606.00	>606.00
E. cloacae 8923	MIC MBC	56.81	28.40	28.40	>606.00	>606.00	>606.00
E. coli 8965	MIC MBC	56.81	56.81	28.40	>606.00	>606.00	>606.00
P. aeruginosa 8762	MIC MBC	>454.50 *	>454.5	>454.50	>606.00	>606.00	>606.00
K. oxytoca 8929	MIC MBC	56.81	56.81	14.20 28.40	>606.00	>606.00	>606.00
K. pneumoniae H2807	MIC MBC	56.81	56.81	28.40 56.81	>606.00	>606.00	>606.00
C. albicans 8937	MIC MFC	56.81 113.63	56.81 113.63	28.40 56.81	>606.00	>606.00	>606.00

* Meaning that MIC and MBC were higher than the highest used concentration in the test.

4. Conclusions

Essential oils are traditionally used as antibacterial and antifungal agents in natural medicine. The increasing interest of modern society and the pharmaceutical industry in medicinal plants makes scientific studies aimed at confirming these effects and founding new therapeutic agents crucial. The obtained results revealed that the main compounds in *S. officinalis* essential oil were *cis*-thujone with 23.5% on average for three years (content ranged between 19.9 and 29.0%), camphor with 17.7% (15.8–19.6%), *trans*-thujone 12.9% (12.3–13.3%) and 1,8-cineole 10.0% (8.8–11.3%, while the main compounds in *S. officinalis* hydrolate were camphor with 44.9% on average for three years (content ranged between 42.4 and 49.6%), followed by *cis*-thujone with 15.7% (13.4–19.8%) and 1,8-cineole with 15.5% (12.3–20.5%). Furthermore, this research demonstrated that the tested *S. officinalis* L. essential oil possesses excellent antibacterial activities and could be potentially used to create pharmaceutical formulations as an alternative to established antibiotic therapy. On the other hand, more antimicrobial assays should be performed to confirm the role of *Salvia officinalis* L. in antimicrobial effects such as anti-adherence and anti-biofilm assay as well as gene expression studies, as further steps in the examination of this plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10081608/s1, Supplement Figure S1. The chemical profile of *S. officinalis*: (a) EO from 2019; (b) EO from 2020; (c) EO from 2021; (d) hydrolate from 2019; (e) hydrolate from 2020; (f) hydrolate from 2021.

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