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Biological Profiling of Essential Oils and Hydrolates of *Ocimum basilicum* var. *Genovese* and var. *Minimum* Originated from Serbia

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Abstract: The genus *Ocimum* has many species that are used to treat diverse kinds of illnesses and sicknesses from ancient times. One of them, *Ocimum basilicum* L., commonly known as basil, has a vital role due to its various medicinal goods. It is best known as a plant with pharmacological activities, but also as an antioxidant, antimicrobial, and larvicidal agent. Although it has been traditionally used in Serbia in traditional medicine for centuries as an insecticidal, antibacterial, and antifungal plant as well as a traditional culinary plant, none of the *O. basilicum* varieties have been commercialised until today. There are significant numbers of information across the world that oils and by-products are part of the global market, but no references to the essential oil composition of Serbian plants were found. Therefore, the objective of this work was to evaluate the antioxidant and antimicrobial potentials of essential oil and hydrolate of two different varieties: *O. basilicum* var. *genovese* and *Ocimum. basilicum* var. *minimum* originating from Serbia for further industrial production of antimicrobial- and/or antioxidant-valued products. The results of this study confirm that essential oils of *O. basilicum* var. *genovese* and var. *minimum* represent a significant source of bioactive compounds, especially linalool, with a high rate of biological activities. Similar behaviour is observed for hydrolates, which are the by-product of the essential oil distillation process and can be utilised as bioactive-rich waste in further investigation.

Keywords: basil; alternative crops; antioxidant activity; antibacterial activity; chemical composition; time-kill kinetic study; minimal inhibitory concentration

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1. Introduction

Nowadays, interest in medical and aromatic plants has grown due to the constantly increasing popularity of using natural products. Moreover, it particularly reflects on numerous phytochemicals being produced for their usage in the pharmaceutical, cosmetics, food, and chemical industries. An example of this is the interest in replacing synthetics with more natural and healthy alternatives, such as aromatic compounds from plants. Besides the undoubtedly positive aspect of utilizing natural resources for human consumption, this solution provides a more economically profitable as well as environmentally acceptable production [1]. The Lamiaceae family is one of the most famously used sources of spices worldwide and an excessive source of extracts with strong antibacterial and antioxidant properties [2]. Genus *Ocimum*, known as basil, provides many species within this family. There are large differences in morphology and chemical composition within the *Ocimum* species depending on variety; among them are volatile organic compounds and phenolic profile, as well as leaf and flower size and colour, plant height, flowering period, etc. [3–5]. *Ocimum basilicum* L. is one of 150 species, but it is so widely cultivated, distributed, and

used that it is known as sweet basil, and called the "king of herbs", as it represents an essential oil crop that is cultured commercially worldwide, making it one of the most commonly employed medical plants [2,6].

Ocimum basilicum has also found its application in traditional medicine against wide spectra of diseases and conditions of respiratory (fever and cough, flu, asthma, bronchitis, initial conditions of tuberculosis) and urinary tract (kidney malfunctions), as well as in the prevention of cardiovascular and neurodegenerative disorders [7–9]. Furthermore, it also has hypoglycemic, hypolipidemic, and antipyretic activity, and it is even considered an anticancer agent [10]. On the other hand, *Ocimum* essential oil is frequently used in aromatherapy due to its calming effect on the nervous system, and it also decreases migraines, improves memory, and eliminates mental fatigue and insomnia [11,12]. This essential oil is significant for its antiseptic, antimicrobial, antioxidant, antiviral, and anti-inflammatory effects as well as being a suitable anti-acne agent [5,13]. Moreover, both the essential oil and the plant itself exhibit insecticidal and fungicidal effects [14–16]. However, if the intended use for *O. basilicum* essential oil is as a biopesticide, the dosage of essential oils needs to be put into consideration, as a higher amount may be toxic for some plants [14].

Even though there is a vast range of bioactivity properties that *O. basilicum* is perceived to possess, the ones that are the most investigated at the moment are their antimicrobial and antioxidative properties [17–20]. These properties form the basis of many of the aforementioned applications of *O. basilicum*. Plant-derived essential oils generally demonstrate a broad spectrum of in vitro antimicrobial activities. Meanwhile, hydrolates include a certain number of the same molecules and biological features as the essential oil they were derived from, such as a by-product. Therefore, even as research about hydrolates is still limited, there is an extraordinary potential for usage in various industries, including medicine [6,21].

Ocimum basilicum essential oils consist of about thirty characteristic components, including terpenes (monoterpenes, sesquiterpenes, and their oxidised forms), and they have been reported to exhibit powerful antibacterial activities against both Gram-positive and Gram-negative bacteria. The assumption is that the antimicrobial activity of *O. basilicum* essential oils and hydrolates originates from a varied array of chemical compounds such as estragole, linalool, and eugenol. According to the literature, linalool is suggested to be the main agent responsible for antibacterial activity [22]. Eugenol is known to demonstrate activity against protozoa and fungi, whereas estragole is also described for its antimicrobial and antioxidant activities [23]. Furthermore, *O. basilicum* is considered to be a rich source of phenolic compounds and flavonoids, such as cinnamic, caffeic, sinapic, and ferulic acid, which act as potent antioxidants, free radical scavengers, and metal chelators. It also contains anthocyanins and is an abundant source of acylated and glycosylated anthocyanins [24]. One of the significant antioxidants found in *O. basilicum* is β-carotene, a precursor of vitamin A, which is also important for preventing oxidation of cholesterol in the bloodstream and thus protecting the heart and blood vessels [10]. There is a difference in the chemical composition of *O. basilicum* essential oils depending on the plant variety, harvesting season, soil and climate conditions, growing technology, and postharvest processing, etc. [25–27]. The chemical composition and biological activity of *O. basilicum* essential oils depend on environmental conditions and location, seasonal variation, different agronomic and harvesting techniques, and drying and processing methods [23].

Ocimum basilicum var. *minimum* and *O. basilicum* var. *genovese*, respectively named Greek basil and Genovese basil in Serbia, are grown as the two most common agricultural *O. basilicum* crops and they have been used in traditional medicine for centuries as insecticidal, antibacterial, and antifungal plants. They are used also for specific, traditional foods, but neither of these two varieties have been commercialised. There are significant numbers of information across the world that oils and by-products are part of the market, but no references to the essential oil and hydrolate composition of Serbian plants were found. To determine the potential of the Serbian *Ocimum* specimens growing at the National Institute

of the Republic of Serbia (Institute of Field and Vegetable Crops Novi Sad), this study tested the biological profiles of these two varieties. More precisely, the objective of this study was to explore the differences in in vitro antibacterial and antioxidant properties between both essential oils and hydrolates (as a by-product of the essential oil distillation process) of Serbian *O. basilicum* var. *genovese* and *O. basilicum* var. *minimum*. As the primary prerequisite for further commercialisation of the obtained essential oils as well as the utilisation of hydrolates as by-products, the presented biological profiling offers a scientific-relevant assessment for targeted opportunities using geographically specific plants.

2. Materials and Methods

2.1. Plant Materials

Two varieties of *O. basilicum* were grown in the experimental fields of the Institute of Field and Vegetable Crops Novi Sad (IFVCNS), located in Bački Petrovac, Serbia. The seeds (from the IFVCNS collection) were sown in pots in a greenhouse, in April, and the seedlings were transplanted at the end of May in an experimental plot, on the calcareous gleyed chernozem soil type that was optimally supplied with nutrients. The experiment was designed as a block system, in three replications (7 rows, 20 m long, with a distance between rows of 0.7 m, and the size of one experimental block was approximately 100 m^2). Weather conditions were favourable for plants to grow, and during the vegetation period, only hand weeding and hoeing were performed. Daily values of minimal and maximal temperatures and precipitation during the *O. basilicum* vegetation period (22 May to 31 July 2019) are presented in the Supplement Figure S1. Plants were harvested at the end of July, in the full blooming stage, by cutting the plants at 5 cm above the ground and drying in a flat-bed solar dryer at temperatures less than 40 °C for two days, and the essential oil was extracted via steam distillation. Voucher specimens were confirmed and deposited at the BUNS Herbarium (University of Novi Sad) under numbers 2-1396 (var. *genovese*) and 2-1395 (var. *minimum*).

2.2. Essential Oil and Hydrolate Obtaining Process

The steam distillation of the dried aerial plant parts of *O. basilicum* samples was performed in a semi-industrial distillation unit in the same manner that Aćimović et al. [28] described in the previous work. Briefly, dried biomass was put in the distillation vessel, which was supplied with hot dry steam from a separate steam generator. The obtained oil and condensed water were accumulated in a Florentine flask. After 4 h, the essential oil and the hydrolate were separated: the oil was decanted from the aqueous layer and dried over anhydrous sodium sulfate, while the hydrolate was purified by *filtration using MN 651/120 filter paper*. To prepare a hydrolate sample for the analysis of volatile compounds, hydrolate was extracted by dichloromethane via the Likens-Nickerson apparatus. To simplify the text, the obtained samples are marked in the following text:

- OBGEO for *Ocimum basilicum* var. *genovese* essential oil;
- OBMEO for *Ocimum basilicum* var. *minimum* essential oil;
- OBGH for *Ocimum basilicum* var. *genovese* hydrolate;
- OBMH for *Ocimum basilicum* var. *minimum* hydrolate.

2.3. Analysis of Volatile Compounds

Gas Chromatograph (Agilent *7890A)* with two detectors: flame ionisation (FID) and mass selective (Agilent *5975C)*, and non-polar capillary column HP-5MS (30 m × 0.25 mm ×0.25 μm) were used. Identification of the components was conducted according to their linear retention indices (RI), and comparison with mass spectral libraries (Adams ver. 4, Wiley ver. 5, and NIST ver. 17). The relative abundance of each detected compound was calculated from GC/FID chromatograms as the percentage area of each peak.

2.4. In Vitro Assessment of Antioxidant Activity

Three in vitro antioxidant assays were used to determine the potential antioxidant activity of two variants of OBEO and OBEH. While essential oils were dissolved in methanol at the concentration of 250 mg/mL, as preparation for the performed tests, hydrolates were used after filtration, in their original form. The antioxidant capacity of these samples was assessed by the following methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline- 6 acid (ABTS) and reducing power accordingly. The Trolox equivalents were used to express all of the obtained antioxidant activity results as μmol per 100 mL (μmol TE/100 mL).

The DPPH assay was performed according to Aborus et al. [29]. In DPPH• methanol solution (250 μL, 0.89 mM), 10 μL of the sample was added to a microplate well and left in a dark place for 50 min. Lastly, absorbance was measured at 515 nm, and methanol was used as a blank. For evaluation of the ABTS•+ radical scavenging assay, the method was conducted according to Aborus et al. [29]. Briefly, 250 μL of activated ABTS•+ (with MnO_2) was inserted in the microplate well and measured as the initial absorbance. Then, 2 μL of each sample was added to the plate, and the reaction mix was incubated at 35 °C before the final absorbances were measured. Both initial and final absorbances were measured at 414 nm. Water was used as blank. Reducing power (RP) was determined by the method of Oyaizu [30] adapted for a 96-well microplate. In summary, a 25 μL sample and 25 μL water for the blank test, 25 μL sodium phosphate buffer (pH = 6.6), and 25 μL of 1% potassium iron (III) cyanide were mixed. Then, it was incubated in a water bath for 20 min at 50 °C and after cooling, 25 μL of 10% trichloroacetic acid was added. From this solution, 50 μL was separated and mixed with 50 μL of distilled water and 10 μL of 0.1% iron (III) chloride in the microplate. Finally, absorbances were measured at 700 nm.

2.5. In Vitro Assessment of Antibacterial Activity

2.5.1. Test Microorganisms

For screening the in vitro antibacterial activity of the obtained OBEO and OBH, the panel of six referent bacteria was used. All bacterial strains are obtained from the American Type Culture Collection (Manassas, VA, USA). Briefly, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 35152, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella Typhimurium* ATCC 13311, and *Staphylococcus aureus* ATCC 25923 were used. Prior to use, the cultures were incubated overnight at 37 °C for all strains except for *B. cereus*, whose implied incubation was at 30 °C with a streaking biomass on PCA plates (Plate-Count-Agar, HiMedia, Mumbai, India).

2.5.2. The Disk Diffusion Method

The used disk diffusion method, as the primary step in an antimicrobial assessment, was explained in detail by Aćimović et al. [31]. For the preparation of the stock solution, cells were suspended in a sterile 0.9% NaCl solution and adjusted to a concentration of 6 log CFU/mL (using DensiCheck (BioMérieux, France)). Afterwards, an aliquot of the prepared suspensions for inoculation was homogenised with melted nutrient media and poured into Petri dishes at a ratio of 1:9. After solidification of inoculated nutrient agar, in each Petri dish were applied three sterile cellulose disks. The obtained results were interpreted as follows: sensitive (diameter of inhibition zone above 26 mm), intermediary (inhibition zone of 22–26 mm), and resistance (inhibition zone below 22 mm). The sterile distilled water was used as a negative control, while the positive control was tetracycline (30 μg/disk) (Sigma-Aldrich, St. Louis, MO, USA). As an additional control, 5% DMSO was tested to check the possibility to use it as a solvent for further testing of antimicrobial activity.

2.5.3. Determination of Minimal Inhibitory Concentration

For all sensitive bacteria, a minimal inhibitory concentration was determined following the procedure described by Micić et al. [32]. An aliquot of initial bacterial suspension (6 log

CFU/mL) was mixed Müller-Hinton broth (HiMedia, Mumbai, India) in a ratio of 1:9. For obtaining the final mixture volume in each well (n = 3), 100 µL of the inoculated Müller-Hinton broth medium was mixed with 100 µL of oil or hydrolate samples. More precisely, the final concentration in each well was 200 µL (100 µL of inoculated nutrient medium and 100 µL of tested sample). In each test, the microtiter plates were a positive control (inoculated media without plant sample) and negative control (100 µL of non-inoculated medium mixed with 100 µL of plant samples). All test plates were incubated for 24 h at 37 °C or 30 °C (*Bacillus* strain). The decreasing concentrations of the plant samples (100–0.39%) were prepared using 5% DMSO solution in a ratio of 1:1 in the case of essential oil, or distilled water in the case of hydrolate.

The pharmacodynamics potential of antimicrobial activity for sensitive bacteria was monitored by the time-kill kinetics [28]. The bacterial cells in the concentration of 6 log CFU/mL were exposed to MIC concentrations and its double values (2xMIC) during different contact times during incubation (0 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, and 24 h) at 37 °C or 30 °C (*Bacillus* strain). An inoculated medium without the plant sample was a positive control, while a non-inoculated medium was a sterility control.

2.6. Statistical Analysis

In this investigation, the kinetics modelling was explored, as indicated by the four-parameter sigmoidal numerical model, which is suitable for biological systems and described in detail by Romano et al. [33]. The expected data are in the form of an S-shaped curve model and might be presented in the form of Equation (1), where y(t) represents the number of viable cells during contact time with the extract output value. The regression coefficients reflect the minimum and maximum of experimentally obtained values (*a* and *d*, respectively), the point between these two, viz. the inflection point (*c*), and the steepness of the inflection point c (*b*).

$$\mathrm{y(t)} = d + \frac{a-d}{1+\left(\frac{t}{c}\right)^b}, \tag{1}$$

The adequacy of the developed models was tested using the coefficient of determination (r^2), reduced chi-square (χ^2), mean bias error (MBE), root mean square error (RMSE), and mean percentage error (MPE). These parameters can be calculated using Equations (2)–(5).

$$\chi^2 = \frac{\sum_{i=1}^{N}\left(x_{\mathrm{exp},i} - x_{pre,i}\right)^2}{N-n}, \tag{2}$$

$$\mathrm{RMSE} = \left[\frac{1}{N}\cdot\sum_{i=1}^{N}\left(x_{pre,i} - x_{\mathrm{exp},i}\right)^2\right]^{1/2}, \tag{3}$$

$$\mathrm{MBE} = \frac{1}{N}\cdot\sum_{i=1}^{N}\left(x_{pre,i} - x_{\mathrm{exp},i}\right), \tag{4}$$

$$\mathrm{MPE} = \frac{100}{N}\cdot\sum_{i=1}^{N}\left(\frac{\left|x_{pre,i} - x_{\mathrm{exp},i}\right|}{x_{\mathrm{exp},i}}\right) \tag{5}$$

where $x_{\mathrm{exp},i}$ stands for the experimental values and $x_{pre,i}$ are the predicted values obtained by calculating from the model for these measurements. N and n are the numbers of observations and constants, respectively.

Analysis of variance (ANOVA) for the comparison of the sample means was used to analyse variations in observed parameters among the samples. All data were processed statistically using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Volatile Compounds of Essential Oils and Hydrolates

The chemical compositions of essential oils and hydrolates of two different *O. basilicum* varieties are presented in Table 1. The main component found in both essential oils and hydrolates is linalool. OBGEO consists mostly of linalool (40.5%), followed by significantly smaller amounts of volatile compounds such as epi-α-cadinol (6.1%), germacrene D, and β-elemene (5.3%), γ-cadinene (5.2%), etc. On the other hand, hydrolates from the same variety contain a fewer number of compounds, with by far the highest percentage of linalool (75.5%), containing also 5.5% of 1.8-cineol and 5.4% of terpinen-4-ol. Similarly, the composition results of both OBMEO and OBMH show 71.5% and 77.1% of linalool, respectively. Some other significant compounds found in OBMH include eugenol (7.7%) and 1,8-cineole (5.9%). Results from Table 1 demonstrate a much closer similarity between essential oil and hydrolates' composition of *O. basilicum* var. *minimum* than *O. basilicum* var. *genovese*. This similarity mainly varies with the relation of hydrocarbons and oxygenated compounds in the essential oil, with a higher similarity found when oxygenated compounds are the main component in an essential oil [34].

Table 1. Chemical composition of *O. basilicum* essential oils and hydrolates.

No	Compound	RI	OBGEO	OBGH	OBMEO	OBMH
1.	3E-Hexenol	851	-	tr	-	0.5
2.	*n*-Hexanol	864	-	-	-	0.1
3.	α-Pinene	931	0.2	-	0.2	-
4.	Camphene	946	0.1	-	0.1	-
5.	Sabinene	970	0.1	-	0.2	-
6.	β-Pinene	974	0.2	-	0.4	-
7.	1-Octen-3-ol	977	-	tr	-	0.1
8.	Myrcene	988	0.1	tr	0.3	-
9.	α-Terpinene	1014	0.1	tr	-	-
10.	*p*-Cymene	1022	0.6	-	0.2	-
11.	Limonene	1025	0.4	-	0.2	-
12.	1,8-Cineole	1028	2.4	5.5	3.4	5.9
13.	*trans*-β-Ocimene	1044	0.1	-	0.2	-
14.	γ-Terpinene	1055	0.2	0.1	0.1	-
15.	*cis*-Sabinene hydrate (IPP vs OH)	1063	0.3	tr	-	-
16.	*cis*-Linalool oxide (furanoid)	1069	0.1	1.5	0.1	0.7
17.	Terpinolene	1086	0.1	-	0.1	-
18.	*trans*-Linalool oxide (furanoid)	1088	-	1.3	-	0.8
19.	Linalool	1100	40.5	75.5	71.5	77.1
20.	*cis*-*p*-Menth-2-en-1-ol	1121	-	0.1	-	-
21.	*trans*-Pinocarveol	1137	-	0.1	-	-
22.	Camphor	1141	1.1	2.7	0.6	1.2
23.	Borneol	1163	0.1	0.2	tr	0.1
24.	δ-Terpineol	1163	-	0.1	-	0.2
25.	*cis*-Linalool oxide (pyanoid)	1171	-	0.2	-	0.2
26.	*trans*-Linalool oxide (pyanoid)	1172	-	-	-	0.2
27.	Terpinen-4-ol	1174	1.4	5.4	0.3	1.3
28.	α-Terpineol	1188	0.3	1.1	0.3	1.5
29.	Estragole	1196	3.2	0.5	3.1	0.5
30.	Verbenone	1207	-	0.1	-	-
31.	Geraniol	1252	0.1	0.3	-	0.6
32.	Chavicol	1252	-	0.1	-	-
33.	Linalool acetate	1253	0.1	-	0.4	-
34.	Bornyl acetate	1284	1.5	0.1	0.5	tr

Table 1. *Cont.*

No	Compound	RI	OBGEO	OBGH	OBMEO	OBMH
35.	Thymol	1291	0.6	0.5	0.4	-
36.	Carvacrol	1301	0.1	0.1	-	0.3
37.	δ-Elemene	1335	0.4	-	0.2	-
38.	α-Cubebene	1347	0.2	-	0.1	-
39.	Eugenol	1356	0.3	2.7	1.2	7.7
40.	α-Copaene	1373	0.5	-	0.3	-
41.	β-Bourbonene	1382	1.1	-	0.4	-
42.	β-Cubebene	1387	0.3	-	0.2	-
43.	β-Elemene	1390	5.3	-	1.9	-
44.	*trans*-Caryophyllene	1417	1.4	-	0.6	-
45.	β-Copaene	1427	0.3	-	0.1	-
46.	β-Gurjunene	1430	0.5	-	0.2	-
47.	*trans*-α-Bergamotene	1434	3.1	-	1.7	-
48.	α-Guaiadiene	1441	0.1	-	0.8	-
49.	α-Humulene	1452	2.2	-	0.6	-
50.	*trans*-β-Farnesene	1455	0.3	-	0.1	-
51.	*epi*-Bicyclosesquiphellandrene	1461	1.0	-	0.3	-
52.	Germacrene D	1480	5.3	-	3.0	-
53.	Bicyclogermacrene	1496	0.7	-	0.5	-
54.	α-Bulnesene	1505	3.4	-	1.2	-
55.	γ-Cadinene	1513	5.2	-	1.6	-
56.	*trans*-Calamenene	1521	0.6	-	0.2	-
57.	δ-Cadinene	1522	0.2	-	0.1	-
58.	α-Cadinene	1536	0.1	-	tr	-
59.	*trans*-Nerolidol	1562	0.3	-	-	-
60.	Maaliol	1567	-	0.1	-	-
61.	Spathulenol	1575	0.6	tr	0.1	-
62.	Caryophyllene oxide	1581	0.3	-	-	-
63.	Humulene epoxide II	1606	0.4	-	-	-
64.	1,10-di-*epi*-Cubenol	1612	1.1	tr	-	-
65.	*epi*-α-Cadinol (=τ-cadinol)	1639	6.1	-	1.0	0.1
66.	10-*epi*-α-Cadinol	1641	-	0.2	-	-
67.	β-Eudesmol	1648	0.3	-	-	-
68.	α-Cadinol	1653	0.4	tr	-	-

RI—Retention Index on C8-C36 alkanes on HP-5MS capillary column; tr—trace (lower than 0.1%).

Even though the varieties investigated in this research are not the same, the results of chemical composition are fairly similar, especially in comparison with the OBMH. Many studies related to an investigation of the chemical profiles of *O. basilicum* essential oil and their hydrolates can be a base for a better understanding of the unique profile of the selected plant materials in this study. The variety in the chemical profile of the samples is reflected in geographical origin and habitat, growing/harvesting season, plant maturity, cultivation, and weather conditions, etc. [8,34–43]. Edris [35] reported that the main components of essential oil and hydrolates cultivated in Egypt were linalool (59.2% and 65.9%, respectively), eugenol (7.0% and 26.1%, respectively) and 1.8-cineol (5.1% and 5.9%, respectively). Smigielski et al. [36] stated methyl eugenol, linalool, eugenol, and 1.8-cineole as the main components in essential oil and hydrolate of *O. basilicum* cultivated in Poland with a significantly smaller percentage of linalool (9.7% and 10.2%, respectively). *O. basilicum* samples collected from four different seasons during 2005 had a weather-dependant chemical composition of essential oils with linalool, epi-α-cadinol, α-bergamotene, and γ-cadinene as the main compounds in different concentrations [37]. The *O. basilicum* essential oil cultivated in India contained methyl eugenol (39.3%) and estragole (38.3%), with a high percentage the methyl eugenol/estragole chemotype essential oil compared to other countries [38]. In the case of *O. basilicum* var. *minimum*, linalool (44%) and 1,8-cineole (15,5%) were reported as the two main compounds of Brazilian samples of essential oil [39], which represent a lower concentration of linalool, but a higher concentration of 1,8-cineole, as compared to this study. The essential oil of the same variety cultivated in the São Tomé and Príncipe island region contained linalool, eugenol, and *trans*-α-bergamotene in the percentage of 52.7, 9.1, and 7.8, respectively [40].

On the other hand, the chemical composition of *O. basilicum* var. *genovese* can also be summarised through geographically different results: linalool (65.38%), τ-cadinol (8.2%), and eugenol (5.26%) were confirmed for the sample from Romania [41], and the samples from Italy contained linalool, 1,8-cineole, and *trans*-α-bergamotene in concentrations of 77.09, 11.85, and 3.31%, respectively [42], while Fedoul et al. [8] reported that the sample from Algeria had linalool (41.3%), β-acorenol (10.3%), and spathulenol (6.0%) as three dominant constituents. Comparing this study with others related to *O. basilicum* samples from Serbia, Ilić et al. [43] analysed the sample from the south part of Serbia in 2018, reporting the same three main constituents: linalool (15.4%), α-bergamotene (9.25%), and 1,8-cineole (6.2%), as in this study; however, the mentioned percentages are quite different from ones presented in this study. Therefore, comprehensive profiling during a few seasons and different regions can offer answers to these differences.

Based on the results in Table 1, the PCA biplot diagram was formed, describing the relations between essential oil and hydrolate compounds of *O. basilicum* var. *genovese* and var. *minimum*.

The presentation of the chemical composition by the PCA biplot has enabled a better perception of similarities and differences among tested samples. The first PC explained 68.48%, and the second 17.11% of the total variance within the experimental data. The separation between samples could be recognised in Figure 1, where essential oil samples are grouped on the left side of the graphic, while the hydrolate samples have the opposite place on the same graphic. The obtained points, which are geometrically close to each other, specify the similarity of outlines. The direction of vectors defines the variable in the factor space (the indication of trend), while vector length is related to the square of the correlation values between the fitting value for the variable and the variable itself. The angles between corresponding variables indicate the degree of their correlation (small angles corresponding to high correlations).

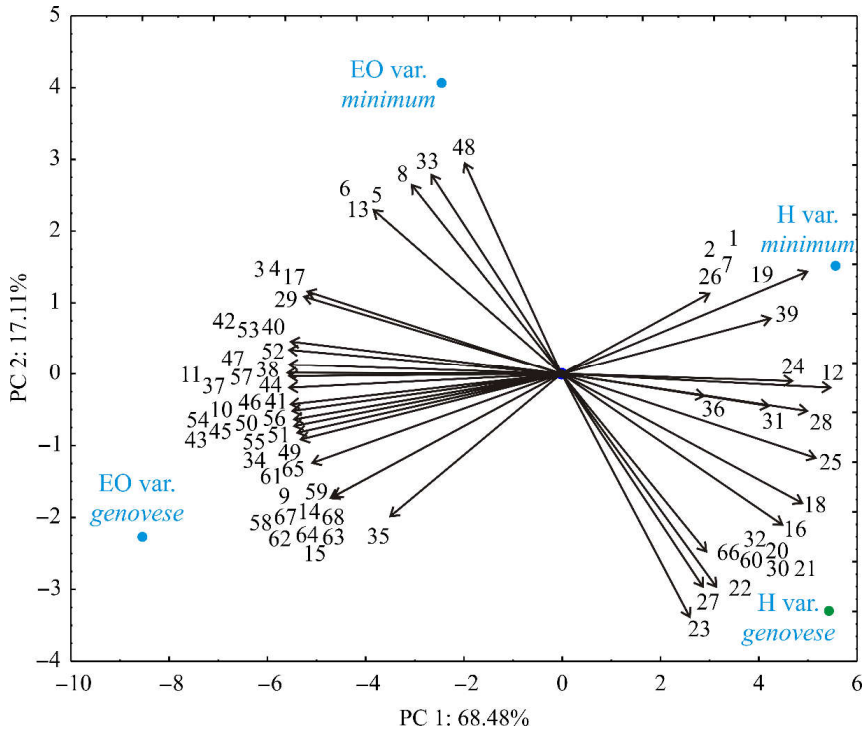

Figure 1. The PCA biplot diagram describes the relations between essential oil and hydrolate compounds of *O. basilicum* var. *genovese* and var. *minimum* (number codes in this figure present the compounds listed in Table 1), EO—essential oil; H—hydrolate.

3.2. In vitro Assessment of Antioxidant Activity

The results of in vitro antioxidant activity of *O. basilicum* var. *genovese* and *O. basilicum* var. *minimum* essential oils and hydrolates are presented in Table 2.

Table 2. In vitro antioxidant activity of *O. basilicum* essential oils and hydrolates.

Antioxidant Activity (μmol TE/mL)	Samples			
	OBGEO	OBMEO	OBGH	OBMH
DPPH•	41.77 ± 1.79	43.25 ± 2.43	2.10 ± 1.47	0.94 ± 0.49
ABTS•+	478.065 ± 27.30	508.36 ± 31.03	2.91 ± 0.36	1.51 ± 0.17
RP	21.0 ± 1.51	22.71 ± 0.97	0.76 ± 0.07	0.48 ± 0.02

Results are expressed as mean ± standard deviation. DPPH•—2,2-diphenyl-1-picrylhydrazyl; ABTS•+—2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid and RP—reducing power; g-genovese, m-minimum.

All three antioxidative tests demonstrate that there are no significant differences between the two investigated variants of *O. basilicum*. Respectively, both essential oils in the concentrations of 250 mg/mL demonstrated the highest scavenging activity against ABTS•+ (478.065 and 508.36 μmol TE/mL), followed by DPPH• (41.77 and 43.23 μmol TE/mL). Their reducing power was lower with 21.0 and 22.71 μmol TE/mL values. The OBMEO exhibits slightly stronger activity than one from var. *genovese;* however, based on the results, both can be considered as proper antioxidant agents. In comparison to the scientific relevant results, it can be observed that Danesi et al. [44] have demonstrated that their OBGEO sample had total antioxidant activity according to the ABTS•+ assay of 590 μmol TE/mL, which is in line with the gained result in this study (478.065 μmolTE/mL). The DPPH test was also used in some other studies, where the reported antioxidant activity of OBGEO was 1.24 ± 0.08 μg/mL [45], with 6.13 ± 4.8 μg/mL harvested in a vegetative state and 133.33 ± 23.09 μg/mL in flowering stage [44]. Kwee and Niemeyer [46] reported the antioxidant activity determined by DPPH for the dried plant OBMEO of 13.27 ± 1.45 mmol/100 g DW. Similarly, Ademiluyi et al. [47] researched the activity of *O. basilicum* essential oil and its ABTS•+ scavenging ability, and the reducing power was 13.35 ± 1.1 mmol TEAC/g and 62.23± 3.8 mg AAE/g, respectively. Good antioxidant properties of *O. basilicum* essential oils derive from a large number of phenolic compounds, and they are dependent on both the amount of eugenol and the time of harvest [36]. On the other hand, *O. basilicum* hydrolate has energizing and relaxing properties, and as such, it finds its use in aromatherapy and cosmetics. On the other hand, according to the literature, there are no recordings of its antioxidative activity.

In this study, as can be observed in Table 2, hydrolates OBGH and OBMH have demonstrated scavenging activity against ABTS•+ (2.10 and 0.94 μmolTE/mL, respectively) and DPPH (2.91 and 1.51 μmolTE/mL, respectively), although with significantly lower values than essential oils. The reducing power of OBGH and OBMH (0.76 and 0.48 μmolTE/mL, respectively) demonstrates small to almost no antioxidative effect. Interestingly, as opposed to the obtained results for essential oils, OBGH showcases stronger activity than OBMH. Data about the antioxidant activity of *O. basilicum* hydrolates is still very limited and not researched enough. Aazza et al. [48] investigated eight different plant-based hydrolates from Morroco using the ABTS•+ test to determine potential antioxidant activity, but *O. basilicum* hydrolate demonstrated no activity.

3.3. In vitro Assessment of Antibacterial Activity

As shown in Table 3, it was found that the antibacterial potential of *O. basilicum* varied between investigated essential oils, which is a consequence of different extracted main substituents (Table 1). On the other hand, tested hydrolates of both varieties did not demonstrate antibacterial activity, suggesting that residual bioactive compounds are not in sufficient concentration for microbial inhibition. In summary, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella Typhimurium* were more sensitive to the effect of tested essential oil

samples, especially in the case of OBMEO. Both tested essential oils of *O. basilicum* had relatively high antimicrobial activities against food-borne bacteria, which is very important in view of the addition of antimicrobial substances to food, since most consumers want high-quality food without preservatives [49].

Table 3. Antimicrobial potential of *O. basilicum* essential oils and hydrolates.

	Bacterial Strain	Inhibition Zones * (mm)						
		OBGEO	OBMEO	OBGH	OBMH	Water	Tetracycline	5% DMSO
Gram-negative bacteria	*E. coli* ATCC 25922	38.00 ± 0.00	40.00 ± 0.00	nd	nd	nd	15.00 ± 0.00	nd
	P. aureginosa ATCC 27853	12.00 ± 0.00	12.00 ± 0.00	nd	nd	nd	11.00 ± 0.00	nd
	S. Typhimurium ATCC 13311	32.00 ± 0.00	40.00 ± 0.00	nd	nd	nd	21.00 ± 1.00	nd
Gram-positive bacteria	*B. cereus* ATCC 11778	14.67 ± 1.15	nd	nd	nd	nd	17 ± 0.00	nd
	L. monocytogenes ATCC 35152	19.00± 1.00	nd*	nd	nd	nd	23.33 ± 0.58	nd
	S. aureus ATCC 25923	35.67± 0.58	38.00 ± 2.00	nd	nd	nd	28.33 ± 0.58	nd

* Mean value diameter of the zone including disc (6 mm) standard deviation; nd—not detected.

In the case of OBGEO, the lower antibacterial behaviour against *E. coli* (inhibition zone of 12 mm) and *S. aureus* (inhibition zone of 12 mm) was reported by Steifan et al. [41], who tested plant origin from Romania, while there was minimal activity against *S. aureus* (inhibition zone of 10 mm) and *L. monocytogenes* (inhibition zone of 11 mm), which Caravić-Stanko et al. [50] reported for oil from Croatia, indicating variations based on geographical origin. Although different studies were dealing with the antimicrobial activity of *O. basilicum*, data about the antibacterial activity of OBMEO are limited.

Minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit microbial growth after overnight incubation [51]. Therefore, MICs of both essential oils were determined for all sensitive bacteria (Table 4). The dissolution of essential oil was conducted using 5% DMSO solution, and distilled water for hydrolate. According to Table 3, 5% DMSO solution and distilled water did not affect antimicrobial activity against tested microorganisms, since the inhibition zone did not exist for any strain. It is in correlation with the study by Joshi [20] and Avetisyan et al. [52], who also dissolved *O. basilicum* essential oil in DMSO solution (up to 10%) without an impact on antimicrobial testing. It could be observed that all oil samples exhibited satisfying antimicrobial activity due to low MICs (<25% of initial concentration), but OBMEO has lower MIC values for the same bacteria. According to the present results in Table 4, it can be concluded that the tested cocci bacterium is the most sensitive bacteria due to the lowest average MIC values.

Table 4. Minimal inhibitory concentration (MIC) of *O. basilicum* essential oils and hydrolates.

Bacterial Type	MIC (%*)			
	OBGEO	OBMEO	OBGH	OBMH
Pseudomonas aureginosa ATCC 27853	>100	>100	>100	
Escherichia coli ATCC 25922	6.25	3.125		
Staphylococcus aureus ATCC 25923	1.56	0.78		
Listeria monocytogenes ATCC 35152	>100	>100		
Salmonella Typhimurium ATCC 13311	25.0	12.5		
Bacillus cereus ATCC 11778	>100	>100		

* Initial concentration (pure oil or hydrolate sample) is defined as 100%.

A higher concentration of linalool, which is detected in the essential oil of *O. basilicum* var. minimum compared with var. *genovese* (Table 1), can be a reason for lower MIC values in the case of all sensitive bacteria. This fact is supported by the previously mentioned literature data that linalool is one of the main substances in *O. basilicum* responsible for antibacterial activity [22].

Using a time-kill kinetics study, presented in Figure 2 and Tables 5 and 6, kinetic modelling was performed for a time-relative response of microbial growth inhibition during 24 h for all sensitive bacteria to OBGEO and OBMEO. The control samples (inoculated medium without the plant samples) were presented in Supplement Figure S1, while the corresponding kinetics parameters are given in Supplement Tables S1 and S2. The growth kinetics follow increasing trends, which were expected, due to the fact that inhibition of biomass production did not exist in the used nutrient medium.

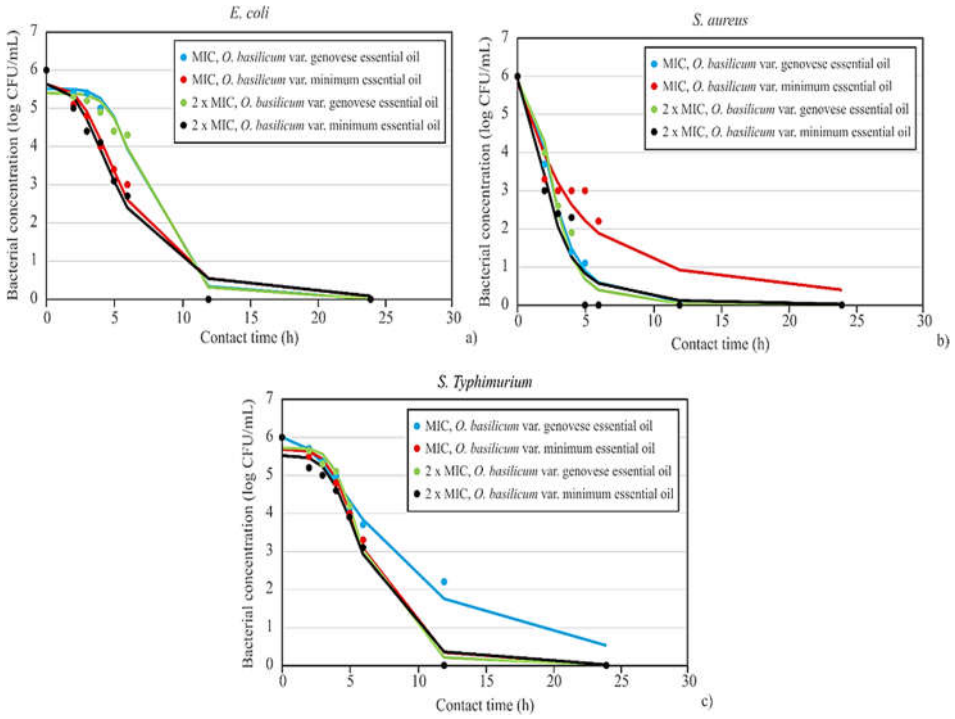

Figure 2. Kinetics during contact time essential oil samples with (**a**) *E. coli*; (**b**) *S. aureus*; (**c**) *S. Typhimurium*.

Table 5. Regression coefficients for time-kill kinetics study of *O. basilicum* samples.

Bacteria		***E. coli***		***S. aureus***		***S. Typhimurium***	
Sample		**OBGEO**	**OBMH**	**OBGEO**	**OBMH**	**OBGEO**	**OBMH**
MIC	*d*	0.00	0.00	0.00	0.00	0.00	0.00
	a	5.51	5.63	5.91	5.83	6.00	5.68
	b	5.29	3.00	2.71	1.35	2.10	4.20
	c	7.14	5.70	2.67	3.47	7.88	6.24
2 × MIC	*d*	0.00	0.00	0.00	0.00	0.00	0.00
	a	5.40	5.64	5.90	5.93	5.72	5.52
	b	5.47	2.80	3.28	2.30	4.88	4.02
	c	7.22	5.38	2.69	2.28	6.16	6.18

Table 6. The 'goodness of fit' tests for prediction models.

Parameters	χ^2	RMSE	MBE	MPE	r^2	Skew	Kurt	SD	Var.
				MIC					
E. coli									
OBGEO	0.10	0.30	−0.03	4.17	0.98	0.79	−0.55	0.32	0.10
OBMEO	0.11	0.30	−0.05	4.18	0.98	0.17	−0.42	0.32	0.10
S. aureus									
OBGEO	0.11	0.32	−0.05	6.36	0.98	−0.06	0.51	0.33	0.11
OBMEO	0.34	0.54	−0.07	10.20	0.92	−0.09	−0.97	0.58	0.33
S. Typhimurium									
OBGEO	0.08	0.27	−0.03	4.26	0.98	−0.14	1.02	0.29	0.08
OBMEO	0.05	0.20	−0.04	2.68	0.99	0.60	0.06	0.21	0.05
				2x MIC					
E. coli									
OBGEO	0.12	0.32	−0.03	4.59	0.98	1.13	0.33	0.34	0.12
OBMEO	0.11	0.31	−0.05	4.40	0.98	−0.13	−1.25	0.32	0.10
S. aureus									
OBGEO	0.16	0.38	−0.06	6.12	0.97	0.22	0.57	0.40	0.16
OBMEO	0.34	0.55	−0.06	9.22	0.93	0.74	0.84	0.58	0.34
S. Typhimurium									
OBGEO	0.03	0.16	−0.02	1.54	1.00	0.35	0.90	0.16	0.03
OBMEO	0.08	0.26	−0.04	3.29	0.99	0.93	0.75	0.27	0.07

* χ^2—reduced chi-square; RMSE—root mean square error; MBE—mean bias error; MPE—mean percentage error; r^2—coefficient of determination; Skew—skewedness; Kurt—kurtosis; SD—standard deviation; Var—variance.

The viability of *E. coli* and *S. aureus* at MIC values of OBGEO was completely reduced over the first 12 h, while in the case of *S. Typhimurium*, it was necessary to double the time for the same effect. On the other hand, 12 h-contact time between all sensitive bacteria and OBMEO was enough for killing all bacterial cells.

Using a double MIC value (2x MIC), a shorter contact time was established in the case of *S. aureus*, where the necessary contact between cells and OBGEO or OBMEO was reduced by 7 h, and in the case of *S. Typhimurium*, cells and OBMEO was reduced by 12 h. The only exception is the case of *E. coli*; there was no decrease in the contact time with both tested essential oils required for complete growth inhibition. Table 5 summarises regression coefficients of kinetic models for time-kill kinetics study for MIC and 2xMIC values during contact time of all sensitive bacteria with OBGEO and OBMEO, which deeper explains the trends (the speed and intensity) of the examined kinetics pathways.

The goodness of fit, between experimental measurements and model calculated results, were shown in Table 6. The quality of the model fit was tested and the residual analysis of the developed model was presented in Table 6. The presented four-parameter sigmoidal mathematical model appears to be simple, robust, and accurate (all coefficients of determination were between 0.916 and 0.996). Mathematical models had an insignificant lack of fit tests, which means that all the models represented the data satisfactorily. A high r^2 is indicative that the variation was accounted for and that the data fitted adequately to the proposed model.

4. Conclusions

The results of this study confirm that essential oils of *O. basilicum* var. *genovese* and var. *minimum* represent a significant source of bioactive compounds with a high rate of biological activities. Similar behaviour is observed for hydrolates, which are by-products of the essential oil distillation process and can be utilised as bioactive-rich waste

in further investigations. Generally, the results imply that the most dominant compound in all samples was linalool, but with significant differences in tested oils. *O. basilicum* var. *minimum* had higher linalool content. In hydrolates' samples, antimicrobial testing resulted in minimal inhibition activity, which is the opposite to the results of oil. The obtained biocide effect of both tested oils was observed for the three most important food-borne bacteria: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella Typhimurium* ATCC 13311. The minimal inhibitory concentration for *O. basilicum* var. *genovese* is in a range between 1.56 and 25% of the initial essential oil sample, while the range for *O. basilicum* var. *minimum* is doubly lower: between 0.78 and 12.5%. In particular, the lower values of MICs (0.78–6.5%) for *S. aureus* and *E. coli* are one of the highlights of this study, because these bacteria are of medical importance, and preventing them in food-related and medical-related situations is crucial. The perspective of the application of *O. basilicum* essential oil and hydrolate as natural additives in food products such as ready-to-eat meals will be determined in the following studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10091893/s1. Supplement Figure S1. Weather conditions during the Ocimum basilicum vegetation period (from May 22 to 31 July 2019); Supplement Figure S2. Kinetics during contact time of control samples with *E. coli*; *S. aureus*; and *S. Typhimurium*; Supplement Table S1. Regression coefficients for time-kill kinetics study of control samples with *E. coli*; *S. aureus*; and *S. Typhimurium*; Supplement Table S2. The 'goodness of fit' tests for prediction models of control samples.

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