

## COMPARATIVE ANALYSIS OF CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL AND HYDROLATE FROM BLACK PEPPER FRUIT (*PIPER NIGRUM* L.)

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The objective of this study was to analyze the chemical composition of the essential oil (EO) and hydrolate of black pepper fruit, as well as their antioxidant activity. The EO was obtained by Clevenger-type hydrodistillation with hydromodule 1:10 m/v for 240 minutes, and the hydrolate was collected after the hydrodistillation process. The qualitative composition of EO was determined by GC/MS and quantitative by GC/FID method, while the qualitative composition of a hydrolate was determined by HS-SPME-GC/MS and quantitative composition by HS-SPME-GC/FID method. The antioxidant activity was investigated by DPPH assay. Fifty-five compounds were identified from black pepper EO, where the most abundant compounds were (*E*)-caryophyllene (41.6 %), limonene (9.7 %), and sabinene (8.6 %). Twelve compounds were identified from black pepper hydrolate, where the most abundant compounds were  $\alpha$ -terpineol (34.7 %), borneol (17.3 %), and terpinen-4-ol (13.9 %). The hydrolate showed higher antioxidant activity after 20 minutes of incubation with an EC<sub>50</sub> value of 0.993 ± 0.011 mg/cm<sup>3</sup> compared to the EO with an EC<sub>50</sub> value of 67.72 ± 1.871 mg/cm<sup>3</sup>.

According to the results obtained in this study, both the EO and hydrolate are good sources of natural antioxidants with potential uses in the food, organic agriculture, pharmaceutical, and cosmetic industries as a safer alternative to synthetic additives. Furthermore, the possibility of wider uses of the hydrolate should be investigated in more detail. Although researchers interested in hydrolate investigation focus mostly on their antioxidant activity, these "aromatic wastes" could also present promising cosmetic activities.

**Keywords:** black pepper essential oil; hydrolate; aqueous phase of suspension; chemical composition; antioxidant activity

## КОМПАРАТИВНА АНАЛИЗА НА ХЕМИСКИОТ СОСТАВ И АНТИОКСИДАЦИСКАТА АКТИВНОСТ НА ЕСЕНЦИЈАЛНИТЕ МАСЛА И ХИДРОЛАТОТ ОД ПЛОДОТ НА ЦРН ПИПЕР (*PIPER NIGRUM* L.)

Целта на ова истражување е да се анализира хемискиот состав на есенцијалното масло (EO) и хидролатот од плодот на црн пипер, како и нивната антиоксидациска активност. EO беше добиено со хидродестилација од типот на Clevenger со хидромодул 1:10 m/v за 240 минути, по што беше собран хидролатот. Квалитативниот состав на EO беше определен со GC/MS, квантитативниот со GC/FID, додека квалитативниот состав на хидролатот беше определен со HS-SPME-GC/MS, а квантитативниот со HS-SPME-GC/FID. Антиоксидациската активност беше испитана со DPPH-анализа. Од EO на црниот пипер, беа определени 55 соединенија, од кои најзастапени беа (*E*)-кариофилен (41,6%), лимонен (9,7%) и сабинен (8,6%). Во хидролатот беа идентификувани 12 соединенија, од кои најзастапени беа  $\alpha$ -терпинеол (34,7%), борнеол (17,3%) и терпинен-4-ол (13,9%). Хидролатот покажа поголема антиоксидациска активност по инкубација од 20 минути со вредност на EC<sub>50</sub> од 0,993 ± 0,011 mg/cm<sup>3</sup> споредено со EO со вредност од EC<sub>50</sub> 67,72 ± 1,871 mg/cm<sup>3</sup>.

Според добиените резултати од оваа студија, и EO и хидролатот се добри извори на природни антиоксиданси потенцијални за употреба во исхраната, органското земјоделие,

фармацијата и козметичката индустрија како побезбедни замани за синтетичките адитиви. Но сепак е неопходно хидролатот подетално да се испитува. Иако истражувачите најмногу се фокусираат на антиоксидациската активност, овој „ароматичен отпад“ може да има потенцијал за козметичка примена.

**Клучни зборови:** есенцијално масло од црн пипер; хидролат; водна фаза на суспензија; хемиски состав; антиоксидациска активност

## 1. INTRODUCTION

The Piperaceae family has a significant ethnobotanical and ethnopharmaceutical history. The genus *Piper* contains about a thousand plant species and is used as spices and traditional medicines in regions like India, southeast Asia, and Africa. *Piper's* chemical composition has been extensively explored, and phytochemical investigations from across the world have resulted in the isolation of various physiologically active chemicals, such as alkaloids, amides, propenyl phenols, lignans, neolignans, terpenes, and steroids.<sup>1,2</sup> The best-known Piperaceae species is black pepper (*Piper nigrum* L.). It is native to the evergreen woods of southwest India and southeast Asia, and it is also commercially produced in tropical countries, such as Indonesia, Brazil, Thailand, Madagascar, and West Africa. Nowadays, it is widely exported around the world, and in European regions it is mainly used as a spice. Pepper extracts in the form of oleoresins, also containing phenolic compounds, can be found commercially.<sup>3</sup> The black pepper essential oil (EO) is a colorless to yellow-greenish liquid with a spicy aroma and various biological activities, such as antioxidant, antimicrobial, anticancer, anti-inflammatory, etc.<sup>4</sup> The black pepper EO has been well researched, and its chemical composition is quite diverse. Monoterpene hydrocarbons, sesquiterpene hydrocarbons, and small amounts of oxygenated monoterpeneoids and oxygenated sesquiterpeneoids are the most abundant compounds in black pepper EO.<sup>5</sup> The major components identified by GC/MS analysis of various samples of the black pepper fruit EOs are  $\beta$ -caryophyllene, limonene,  $\beta$ -pinene,  $\alpha$ -pinene, sabinene, and 3-carene, as well as camphene.<sup>6,7</sup> The main oxygenated terpenes present in the black pepper EO are caryophyllene oxide, linalool, terpinen-4-ol, and eugenol.<sup>8</sup>

The hydrolate (also known as hydrosol, floral water, aromatic water, or herbal water) is a by-product after the EO distillation process. The hydrolates are colloidal suspensions with specific organoleptic properties and flavors, which may have different biological activities. They are obtained in larger amounts under industrial conditions, such as steam distillation. During industrial

distillation, the water and the EO evaporate, and after condensation of the vapors in contact with cold vessels, the EO and hydrolate separate into two phases inside the collecting vessel.<sup>9</sup> The Clevenger-type hydrodistillation differs from the process of steam distillation in the fact that two hydrolates can be separated. The first is the aqueous part of the distillate, which remains in the measuring tube and is in contact with the EO; the second is the aqueous phase of the suspension, which remains in the flask after distillation and can be separated from plant material by filtration. In this study, the focus is on the second hydrolate (the aqueous phase of the suspension).

The hydrolate mainly contains highly polar (hydrophilic) compounds. The similarity between the EO and the hydrolate composition is mostly determined by the EO's hydrocarbon and oxygenated component content. The degree of similarity between the EO and hydrolate is quite high in materials when oxygenated molecules predominate in the EO. In contrast, when hydrocarbons are the primary constituents of EOs, the hydrolate composition varies significantly from the EO composition.<sup>9</sup> This study could be of great interest because the chemical composition of black pepper hydrolate has not been studied yet.

Considering that today's goal is to reduce waste or make it usable, the objective of this study was to analyze and compare the chemical composition of the EO and hydrolate of black pepper fruit, as well as their antioxidant activity, with the potential use of waste products (hydrolate) as a natural antioxidant.

## 2. EXPERIMENTAL

### 2.1. Material and methods

#### 2.1.1. Plant material

The commercial sample of black pepper fruit (*P. nigrum* L., country of origin: Vietnam, packs Gyros Thessaloniki and top spices; UgriNovski put, part 25-No.32, Zemun, Altina) was used for research. Right before the analysis, the plant material was ground in an electric mill (Braun KSM2 Aromatic Coffee Grinder).

### 2.1.2. Chemicals and reagents

The chemicals used in the study were: ethanol, 96% v/v (Centrochem, Stara Pazova), 1,1-diphenyl-2-picrylhydrazil (DPPH radical), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) (Sigma Chemical Company, St. Louis, USA), anhydrous sodium sulfate (Centrohem, p.a. >99 %). All other chemicals were analytical grade.

### 2.1.3. Production of the essential oil and hydrolate

Disintegrated and homogenized plant material was used for EO isolation by Clevenger-type hydrodistillation with a hydromodule (ratio of plant material:water) of 1:10 m/V for 240 min. The essential oil isolate was separated from the measuring tube after distillation, dried over anhydrous sodium sulfate, and stored in dark bottles in a refrigerator at 4 °C. After the hydrodistillation, the plant material was separated from the aqueous phase of the suspension from the round-bottom flask by filtering with a weak vacuum (filtration on a Büchner funnel). Afterwards, the volume of hydrolate was measured and left in a freezer at 4 °C until further analysis. Before the analysis, the hydrolate was centrifuged for 4 minutes at 4,000 rpm.

### 2.1.4. Analysis of volatile compounds in the hydrolate by headspace-solid phase microextraction (HS-SPME)

In a 10 cm<sup>3</sup> glass bottle, 2 cm<sup>3</sup> of hydrolate was measured and 0.5 g of sodium chloride (NaCl) and a magnetic stir bar were added. The vial was closed with a rubber stopper and wrapped with parafilm. In order to equilibrate the liquid and gaseous phases, the samples were heated for 15 min at a temperature of 50 °C (pre-extraction) while stirring with a magnetic stirrer. An SPME fiber was introduced into the headspace of the sample and volatile compounds were extracted with stirring for 15 minutes at a temperature of 50 °C. Adsorbed compounds were desorbed for 10 minutes by inserting the SPME syringe needle into the split/splitless injector of the gas chromatograph heated to 220 °C in splitless mode, then analyzed using GC/MS and GC/FID methods.

### 2.1.5. GC/MS and GC/FID analysis of black pepper essential oil

Gas chromatography-mass spectrometry (GC/MS) analysis was performed on an Agilent Technologies 7890B gas chromatograph, equipped

with a nonpolar, silica capillary column (HP-5MS; 5 % diphenyl- and 95 % dimethyl-polysiloxane, 30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) and coupled with an inert, selective 5977A mass detector of the same company. Helium was used as the carrier gas at a constant flow rate of 1 cm<sup>3</sup>/min. The oven temperature increased from 60 °C to 246 °C at the rate of 3 °C/min. Temperatures of the mass selective detector (MSD) transfer line, ion source, and quadrupole mass analyzer were set at 300 °C, 230 °C and 150 °C, respectively. The ionization voltage was 70 eV and the mass range was *m/z* 41–415 Da. Gas chromatography-flame ionization detection (GC/FID) analysis was carried out under identical experimental conditions as GC/MS. The flows of the carrier gas (He), make-up gas (N<sub>2</sub>), fuel gas (H<sub>2</sub>), and oxidizing gas (air) were 1, 25, 30, and 400 cm<sup>3</sup>/min, respectively. The temperature of the flame-ionization detector was set at 300 °C.

## 2.2. Data processing

Data processing was performed using MSD ChemStation Data Analysis (revision F.0100.1903) in combination with the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) version 2.70 and National Institute of Standards and Technology Mass Spectrometry Search (NIST MS Search) (version 2.0 g) software (Agilent Technologies, USA). MSD ChemStation Data Analysis was used for automatic and manual integration of peaks on chromatograms and, thus, the quantification of the obtained data. AMDIS enables automatic finding of compounds by deconvolution, i.e., by extracting pure mass spectra from overlapped peaks and comparing them with a reference library of mass spectra with or without retention indices. Finally, the NIST MS Search provides an algorithm for a library search complementary to the probability-based matching (PBM) algorithm of the ChemStation software. Retention indices of the components from the analyzed samples were experimentally determined using a homologous series of *n*-alkanes from C<sub>8</sub>–C<sub>20</sub> as standards. Oil constituent identification was based on the comparison of measured retention indices (RI<sub>exp</sub>) with those available in literature<sup>10</sup> (RI<sub>lit</sub>); mass spectra of the EO's components were compared with the mass spectra from Willey, NIST, and Pesticides retention time lock (RTLPEST) libraries (an acronym MS in the Tables) and wherever possible, by co-injection with an authentic standard (Co-I).

### 2.2.1. Quantitative analysis of black pepper EO

Quantification was done by external standard method using standards in the concentration ranges as follows:  $\beta$ -pinene (0.125–2 mg/cm<sup>3</sup>), 1,8-cineole (0.25–3 mg/cm<sup>3</sup>), limonene (0.5–4 mg/cm<sup>3</sup>), linalool (1.67–15 mg/cm<sup>3</sup>), and  $\gamma$ -terpinene (0.75–5 mg/cm<sup>3</sup>).

The response factor (RF) for each standard used was calculated as follows:

$$RF = \frac{\text{Area}_{\text{std}}}{C_{\text{std}}}$$

where  $\text{Area}_{\text{std}}$  is the peak area of the analytical standard and  $C_{\text{std}}$  is the concentration of the standard used. Then, the mean of the RFs was calculated ( $RF_{\text{mean}}$ ) and used to quantify each of the analyses in the samples using the equation:

$$C_x = \frac{\text{Area}_x}{RF_{\text{mean}}}$$

where  $C_x$  is the concentration of the analyte in the sample,  $\text{Area}_x$  is the peak area of the analyte, and  $RF_{\text{mean}}$  is the mean response factor.<sup>11</sup>

Finally, the content ( $C$ , %) of individual components in the sample was calculated as follows:

$$C (\%) = \frac{C_x}{\sum C_x} \times 100$$

where  $C_x$  is the concentration of an individual component in the sample and  $\sum C_x$  is the total concentration of the components in the sample.

### 2.2.2. Quantitative analysis of hydrolate volatile compounds

A quantitative analysis of volatile compounds was performed using the area normalization method. The relative amount (%) of individual components represents the normalized ratio of the peak area of each of the components and the total peak area of all components in the GC/FID chromatogram. Values were obtained by automatic integration of chromatograms in MSD ChemStation Data Analysis software (Area Percent Report) without any corrections.

### 2.2.3. Antioxidant activity

The antioxidant activity of the EO and hydrolate was determined using the DPPH assay. A series of concentrations of EO (0.2344–30 mg/cm<sup>3</sup>) and hydrolate (0.025–1 mg/cm<sup>3</sup>) dissolved in ethanol were prepared. An ethanolic solution of DPPH radical was added to the prepared solutions as described previously in Stanojević *et al.*<sup>12</sup> This procedure was done in triplicate. The absorption of the samples was measured at 517 nm immediately after adding the DPPH radical, as well as after 20 minutes, for EO and hydrolate.

## 3. RESULTS AND DISCUSSION

The yield of black pepper EO isolated from the fruit was 3.16 cm<sup>3</sup>/100 g of plant material. This result is in agreement with existing research when it comes to the yield of EO from the black pepper fruit, which contains about 1 % – 3 % EO, but some studies have shown values of 6.7 %.<sup>13</sup> The yield of EO can be a result of different factors' integration, such as technique, temperature, contact time, and particle size of the black pepper fruit.<sup>13,14</sup> Dinh and coworkers reported classical hydrodistillation as a better method of EO estimation compared to other techniques.<sup>15</sup>

The GC/MS analysis resulted in the identification of 55 and 12 compounds, representing 99.0 % and 97.7 % of the total EO and hydrolate composition of black pepper, respectively. Compounds are listed according to the order of their elution from the HP-5ms column. All identified compounds and their contents (expressed in %) are given in Tables 1 and 2, while total ion chromatograms (TIC) are given in Figures 1 and 2. All identified components in the EO were classified into the following groups: sesquiterpene hydrocarbons (56.5 %), monoterpene hydrocarbons (33.7 %), oxygenated sesquiterpenes (7.5 %), oxygenated monoterpenes (1.1 %), aromatic compounds (0.2 %) and others, where the most abundant compounds were (*E*)-caryophyllene (41.6 %), limonene (9.7 %), and sabinene (8.6 %).

The structures of the main compounds (components 4, 5, 8, 12, 30, 44, 51, and 52 from Table 1) found in black pepper EO are given in Figure 3.

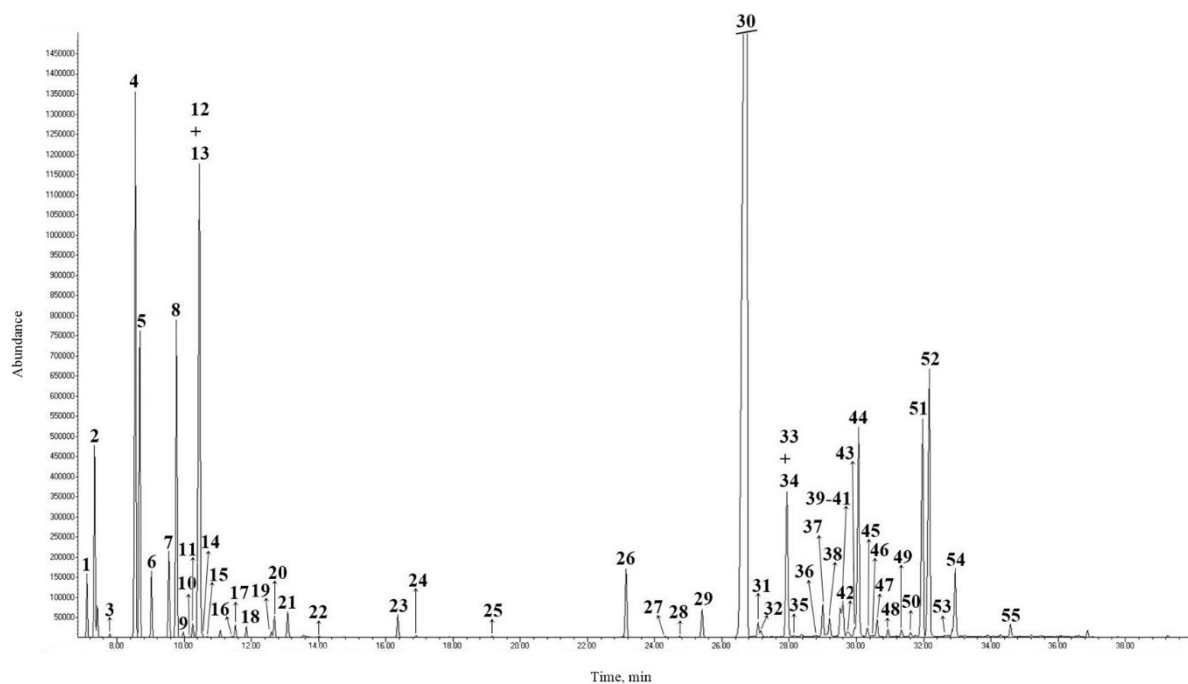


Fig. 1. TIC chromatogram of black pepper essential oil

Table 1

Chemical composition of black pepper essential oil determined by GC/MS and GC/FID analyses

No.	<i>t</i> <sub>ret.</sub> , min	Compound	Molecular formula	RI <sub>exp</sub>	RI <sub>lit</sub>	Method of identification	C*, %
1	7.11	$\alpha$ -Thujene	C <sub>10</sub> H <sub>16</sub>	927	924	RI, MS	0.7
2	7.34	$\alpha$ -Pinene	C <sub>10</sub> H <sub>16</sub>	935	932	RI, MS, Co-I	2.4
3	7.78	Camphene	C <sub>10</sub> H <sub>16</sub>	950	946	RI, MS, Co-I	0.1
<b>4</b>	<b>8.55</b>	<b>Sabinene</b>	<b>C<sub>10</sub>H<sub>16</sub></b>	<b>975</b>	<b>969</b>	<b>RI, MS</b>	<b>8.6</b>
5	8.68	$\beta$ -Pinene	C <sub>10</sub> H <sub>16</sub>	979	974	RI, MS, Co-I	4.4
6	9.03	Myrcene	C <sub>10</sub> H <sub>16</sub>	991	988	RI, MS	1.0
7	9.55	$\alpha$ -Phellandrene	C <sub>10</sub> H <sub>16</sub>	1007	1002	RI, MS	1.1
8	9.78	$\delta$ -3-Carene	C <sub>10</sub> H <sub>16</sub>	1013	1008	RI, MS	4.9
9	9.98	$\alpha$ -Terpinene	C <sub>10</sub> H <sub>16</sub>	1018	1014	RI, MS	0.1
10	10.15	<i>o</i> -Cymene	C <sub>10</sub> H <sub>14</sub>	1023	1020	RI, MS	tr
11	10.25	<i>p</i> -Cymene	C <sub>10</sub> H <sub>14</sub>	1025	1022	RI, MS	0.2
<b>12</b>	<b>10.45</b>	<b>Limonene</b>	<b>C<sub>10</sub>H<sub>16</sub></b>	<b>1031</b>	<b>1024</b>	<b>RI, MS, Co-I</b>	<b>9.7</b>
13	10.46	$\beta$ -Phellandrene	C <sub>10</sub> H <sub>16</sub>	1031	1025	RI, MS	tr
14	10.54	1,8-Cineole	C <sub>10</sub> H <sub>18</sub> O	1033	1026	RI, MS, Co-I	tr
15	10.69	( <i>Z</i> )- $\beta$ -Oocymene	C <sub>10</sub> H <sub>16</sub>	1037	1032	RI, MS	tr
16	11.43	( <i>E</i> )- $\beta$ -Oocymene	C <sub>10</sub> H <sub>16</sub>	1056	1044	RI, MS	tr
17	11.52	$\gamma$ -Terpinene	C <sub>10</sub> H <sub>16</sub>	1059	1054	RI, MS, Co-I	0.2
18	11.85	cis-Sabinene hydrate	C <sub>10</sub> H <sub>18</sub> O	1068	1065	RI, MS	0.2
19	12.59	<i>p</i> -Mentha-2,4(8)-diene	C <sub>10</sub> H <sub>16</sub>	1087	1085	RI, MS	0.1
20	12.68	Terpinolene	C <sub>10</sub> H <sub>16</sub>	1090	1086	RI, MS	0.4
21	13.08	Linalool	C <sub>10</sub> H <sub>18</sub> O	1100	1095	RI, MS, Co-I	0.5
22	14.02	dehydro-Sabina ketone	C <sub>9</sub> H <sub>12</sub> O	1123	1117	RI, MS	tr
23	16.36	Terpinen-4-ol	C <sub>10</sub> H <sub>18</sub> O	1179	1174	RI, MS	0.4
24	16.90	$\alpha$ -Terpineol	C <sub>10</sub> H <sub>18</sub> O	1192	1186	RI, MS, Co-I	tr
25	19.16	Carvone	C <sub>10</sub> H <sub>14</sub> O	1245	1239	RI, MS	tr
26	23.14	$\delta$ -Elemene	C <sub>15</sub> H <sub>24</sub>	1340	1335	RI, MS	1.2
27	24.33	Longicyclene	C <sub>15</sub> H <sub>24</sub>	1369	1371	RI, MS	tr
28	24.75	$\alpha$ -Copaene	C <sub>15</sub> H <sub>24</sub>	1379	1374	RI, MS	tr
29	25.41	$\beta$ -Elemene	C <sub>15</sub> H <sub>24</sub>	1395	1389	RI, MS	0.6

Table 1 continue

30	26.76	(E)-Caryophyllene	C <sub>15</sub> H <sub>24</sub>	1427	1417	RI, MS	41.6
31	27.08	γ-Elemene	C <sub>15</sub> H <sub>24</sub>	1436	1434	RI, MS	0.3
32	27.16	α-trans-Bergamotene	C <sub>15</sub> H <sub>24</sub>	1438	1432	RI, MS	0.1
33	27.93	α-Humulene	C <sub>15</sub> H <sub>24</sub>	1458	1452	RI, MS	2.9
34	27.96	(E)-β-Farnesene	C <sub>15</sub> H <sub>24</sub>	1458	1454	RI, MS	tr
35	28.15	β-Santalene	C <sub>15</sub> H <sub>24</sub>	1463	1457	RI, MS	tr
36	28.80	trans-Cadina-1(6),4-diene	C <sub>15</sub> H <sub>24</sub>	1479	1475	RI, MS	tr
37	29.00	Germacrene D	C <sub>15</sub> H <sub>24</sub>	1484	1484	RI, MS	0.6
38	29.20	β-Selinene	C <sub>15</sub> H <sub>24</sub>	1489	1489	RI, MS	0.3
39	29.52	α-Zingiberene	C <sub>15</sub> H <sub>24</sub>	1497	1493	RI, MS	0.5
40	29.55	α-Selinene	C <sub>15</sub> H <sub>24</sub>	1498	1498	RI, MS	tr
41	29.61	Curzerene	C <sub>15</sub> H <sub>20</sub> O	1500	1499	RI, MS	0.7
42	29.74	α-Murolene	C <sub>15</sub> H <sub>24</sub>	1503	1500	RI, MS	0.1
43	29.95	Germacrene A	C <sub>15</sub> H <sub>24</sub>	1509	1508	RI, MS	0.1
44	30.07	β-Bisabolene	C <sub>15</sub> H <sub>24</sub>	1512	1505	RI, MS	3.5
45	30.32	δ-Amorphene	C <sub>15</sub> H <sub>24</sub>	1519	1511	RI, MS	0.2
46	30.51	Myristicin	C <sub>11</sub> H <sub>12</sub> O <sub>3</sub>	1523	1517	RI, MS	tr
47	30.62	(E)-iso-γ-Bisabolene	C <sub>15</sub> H <sub>24</sub>	1526	1528	RI, MS	0.3
48	30.94	(E)-γ-Bisabolene	C <sub>15</sub> H <sub>24</sub>	1534	1529	RI, MS	0.2
49	31.34	(E)-α-Bisabolene	C <sub>15</sub> H <sub>24</sub>	1545	1540 <sup>a</sup>	RI, MS	0.1
50	31.61	Elemol	C <sub>15</sub> H <sub>26</sub> O	1552	1548	RI, MS	0.1
51	31.97	Germacrene B	C <sub>15</sub> H <sub>24</sub>	1561	1559	RI, MS	4.1
52	32.17	(E)-Nerolidol	C <sub>15</sub> H <sub>26</sub> O	1566	1561	RI, MS	4.8
53	32.62	Himachalene epoxide	C <sub>15</sub> H <sub>22</sub> O	1579	1578	RI, MS	tr
54	32.94	Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	1587	1582	RI, MS	1.4
55	34.57	Muurola-4,10(14)-diene-1-β-ol	C <sub>15</sub> H <sub>24</sub> O	1633	1630	RI, MS	0.3
				<b>Total identified</b>		99.0	
<b>Grouped components (%)</b>							
Monoterpene hydrocarbons (1–9, 12, 13, 15–17, 19, 20)						33.7	
Oxygenated monoterpenes (14, 18, 21, 23–25)						1.1	
Sesquiterpene hydrocarbons (26–40, 42–44, 47–49, 51)						56.5	
Oxygenated sesquiterpenes (41, 45, 50, 52–55)						7.5	
Aromatic compounds (10, 11, 46)						0.2	
Others (22)						tr	

.  $t_{ret}$  – retention time;  $RI_{lit}$  – retention indices from literature;<sup>10</sup>  $RI_{exp}$  – experimentally determined retention indices using a homologous series of *n*-alkanes (C<sub>8</sub>–C<sub>20</sub>) on HP-5MS column. MS – constituent identified by GC co-injection of an authentic sample; (Note: The same notation was used in the second table as well); tr – constituent in traces, < 0.05 %.<sup>16</sup>

C\*: Content (content of each component in the total EO composition), calculated according to the formula:  $C(\%) = \frac{C_x}{\sum C_x} \times 100$

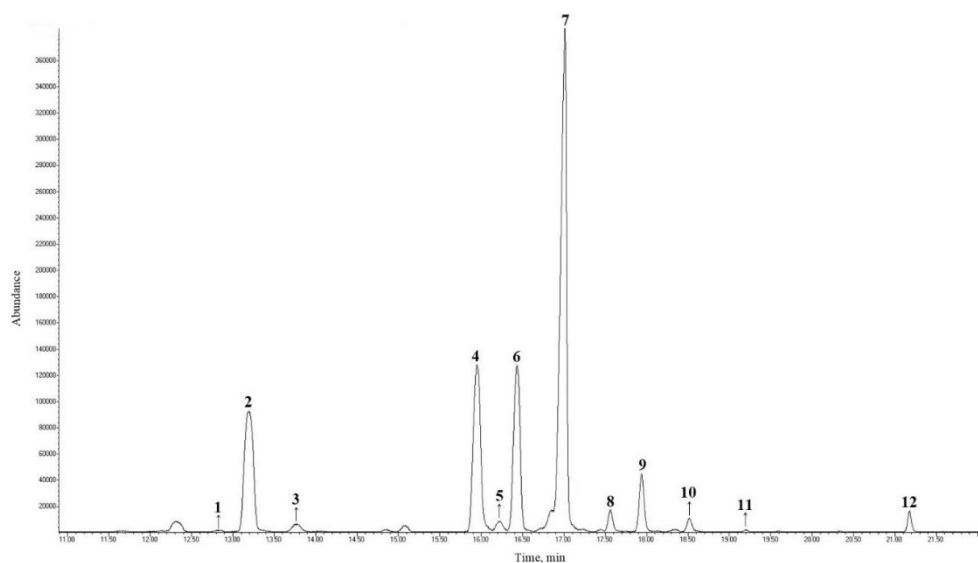


Fig. 2. TIC chromatogram of black pepper hydrolate

Table 2

Chemical composition of volatile compounds in black pepper hydrolate determined by headspace sample-solid phase microextraction (HS-SPME)

No.	$t_{ret.}$ , min	Compound	Molecular formula	RI <sub>exp</sub>	RI <sub>lit</sub>	Identification method	C*, %
1	12.82	<i>p</i> -Cymene	C <sub>10</sub> H <sub>12</sub>	1093	1089	RI, MS	tr
2	13.17	Linalool	C <sub>10</sub> H <sub>18</sub> O	1102	1095	RI, MS	16.3
3	13.75	Phenyl ethylalcohol	C <sub>8</sub> H <sub>10</sub> O	1106	1116	RI, MS	2.4
4	<b>15.94</b>	<b>Borneol</b>	<b>C<sub>10</sub>H<sub>18</sub>O</b>	<b>1169</b>	<b>1165</b>	<b>RI, MS</b>	<b>17.3</b>
5	16.22	Santolanyl acetate	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	1175	1171	RI, MS	1.8
6	<b>16.45</b>	<b>Terpinen-4-ol</b>	<b>C<sub>10</sub>H<sub>18</sub>O</b>	<b>1181</b>	<b>1174</b>	<b>RI, MS</b>	<b>13.9</b>
7	<b>17.01</b>	<b><math>\alpha</math>-Terpineol</b>	<b>C<sub>10</sub>H<sub>18</sub>O</b>	<b>1194</b>	<b>1186</b>	<b>RI, MS</b>	<b>34.7</b>
8	17.56	<i>trans</i> -Dihydrocarvone	C <sub>10</sub> H <sub>16</sub> O	1208	1200	RI, MS	2.6
9	17.95	Linalool formate	C <sub>11</sub> H <sub>18</sub> O <sub>2</sub>	1217	1214	RI, MS	4.5
10	18.52	<i>neiso</i> -Dihydrocarveol	C <sub>10</sub> H <sub>18</sub> O	1230	1226	RI, MS	2.1
11	19.21	Carvone	C <sub>10</sub> H <sub>14</sub> O	1246	1239	RI, MS	tr
12	21.17	Thymol	C <sub>10</sub> H <sub>14</sub> O	1293	1289	RI, MS	2.1
						<b>Total identified</b>	97.7
<b>Grouped components (%)</b>							
Oxygenated monoterpenes (2, 4, 5-11)							93.2
Aromatic compounds (1, 3, 12)							4.5

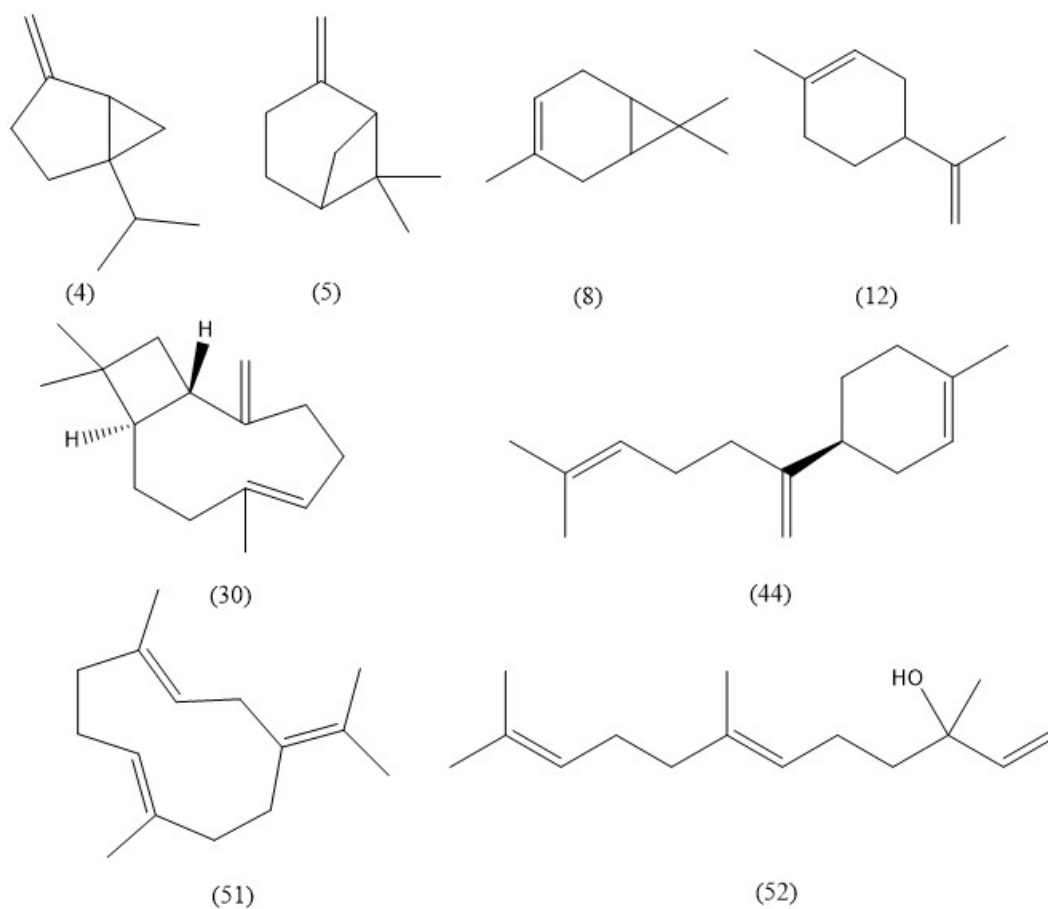


Fig. 3. The main compounds in black pepper essential oil

The EO profile of black pepper was close to the findings of Sruthi *et al.*<sup>17</sup> and Kapoor *et al.*<sup>18</sup> Other important minor components identified in the present EO were germacrene B, (*E*)-nerolidol,  $\delta$ -3-carene,  $\beta$ -bisabolene, and caryophyllene oxide. Contrary to findings from the current study, Jirovetz *et al.* observed that the main compounds from the EO of dried fruits of black pepper from Cameroon were germacrene D (11.01 %), limonene

(10.26 %),  $\beta$ -pinene (10.02 %),  $\alpha$ -phellandrene (8.56 %),  $\beta$ -caryophyllene (7.29 %), and  $\alpha$ -pinene (6.40 %), as well as *cis*- $\beta$ -ocimene (3.19 %).<sup>19</sup> On the other hand, Tran and coworkers found that the main compounds in EO from Vietnam were 3-carene (29.21 %), limonene (20.94 %), and  $\beta$ -caryophyllene (15.05 %).<sup>20</sup> Table 3 shows the chemical profile of black pepper EO, which differs from study to study.<sup>3</sup>

Table 3

*The chemical composition of black pepper essential oil according to other studies<sup>3</sup>*

Component	Percentage	Reference
$\beta$ -Caryophyllene	29.9 %	(Kapoor <i>et al.</i> , 2009) <sup>6</sup>
Limonene	13.2 %	
$\beta$ -Pinene	7.9 %	
Sabinene	5.9 %	
$\alpha$ -Pinene	4.5 %	
Caryophyllene oxide	3.9 %	
Terpinen-4-ol	0.7 %	
$\beta$ -Caryophyllene	19.12 %	(Morshed <i>et al.</i> , 2017) <sup>21</sup>
Limonene	9.74 %	
(+)-Camphene	8.44 %	
$\beta$ -Pinene	8.00 %	
3-Carene	7.08 %	
$\alpha$ -Pinene	6.32 %	
3-Carene	29.21 %	(Tran <i>et al.</i> , 2019) <sup>20</sup>
D-Limonene	20.94 %	
$\beta$ -Caryophyllene	15.05 %	
$\beta$ -Pinene	9.77 %	
$\alpha$ -Pinene	4.69 %	
Caryophyllene oxide	0.89 %	
Linalool	0.42 %	
$\delta$ -3-Carene	18.5 %	(Tchoumboungang <i>et al.</i> , 2009) <sup>22</sup>
Limonene	14.7 %	
$\beta$ -Caryophyllene	12.8 %	
Sabinene	11.2 %	
$\beta$ -Pinene	6.7 %	
$\alpha$ -Pinene	5.6 %	
$\beta$ -Selinene	2.2 %	
Terpinen-4-ol	2.0 %	
T-Cadinol	0.8 %	
Linalool	0.7 %	
$\beta$ -Caryophyllene	23.98 %	(Jeena <i>et al.</i> , 2014) <sup>23</sup>
Limonene	14.36 %	
$\alpha$ -Terpinene	13.26 %	
$\gamma$ -Terpinene	13.26 %	
Caryophyllene oxide	8.68 %	
Naphthalene	4.19 %	
Copaene	3.73 %	
Elemene	3.52 %	
$\beta$ -Caryophyllene	26.2 %	(Vinturelle <i>et al.</i> , 2017) <sup>24</sup>
$\sigma$ -Ocimene	5.8 %	
$\alpha$ -Pinene	5.5 %	
Caryophyllene oxide	4.2 %	
$\alpha$ -Copaene	4.2 %	
$\beta$ -Pinene	4.1 %	
Limonene	2.1 %	



The identified components from the hydrolate of black pepper were classified into two groups: oxygenated monoterpenes (93.2 %) and aromatic compounds (4.5 %), where the most abundant compounds were  $\alpha$ -terpineol (34.7 %), borneol (17.3 %), and terpinen-4-ol (13.9 %). The structure of the main compounds (components 4, 6, and 7 from Table 2) found in black pepper hydrolate are given in Figure 4.

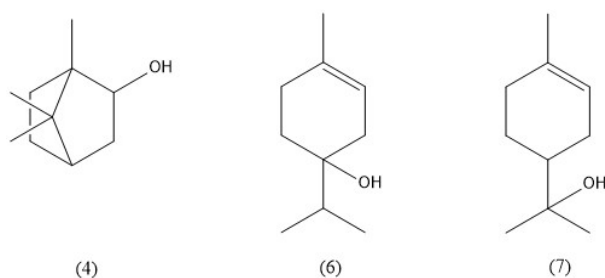


Fig. 4. The main compounds found in black pepper hydrolate

Monoterpene hydrocarbons have an extremely poor solubility in water: at pH = 7, less than  $5 \text{ mg l}^{-1}$  (they are lipophilic compounds). When monoterpene hydrocarbons are the main components of EOs, the compositions of hydrolates differ significantly from the corresponding oils, as is the case with the hydrolates from this study.<sup>25,26</sup> It can be assumed that hydrolate includes some of the water-soluble components of the EO along with other water-soluble plant secondary metabolites.

The percentage of components varies widely depending on the EO isolation technique, the origin and variety of the raw material, and its stage of maturity, as well as the duration and conditions of storage.<sup>3,16,27</sup>

### 3.1. Antioxidant activity

The DPPH radical scavenging activity of the isolated EO and hydrolate of black pepper was shown in Figures 5 and 6, respectively, while the concentrations of black pepper EO and hydrolate required to neutralize 50 % of the initial concentration of DPPH radical ( $EC_{50}$  values) are listed in Table 4. The degree of DPPH radical neutralization depends on oil concentration, as well as the incubation time; it increases with the concentration in-

crease, which is the lowest for non-incubated samples (Figs. 5 and 6). The hydrolate of black pepper showed better antioxidant activity (with the degree of DPPH radical neutralization around 50 %) than the black pepper EO (with the degree of DPPH radical neutralization of about 29 %) after 20 minutes of incubation at concentrations of  $1 \text{ mg/cm}^3$  and  $30 \text{ mg/cm}^3$ , respectively. The samples of the EO and hydrolate that were not incubated showed considerably lower values (around 10.5 % and 13 % at concentrations of  $30 \text{ mg/cm}^3$  and  $1 \text{ mg/cm}^3$ , respectively; Figs. 5 and 6).

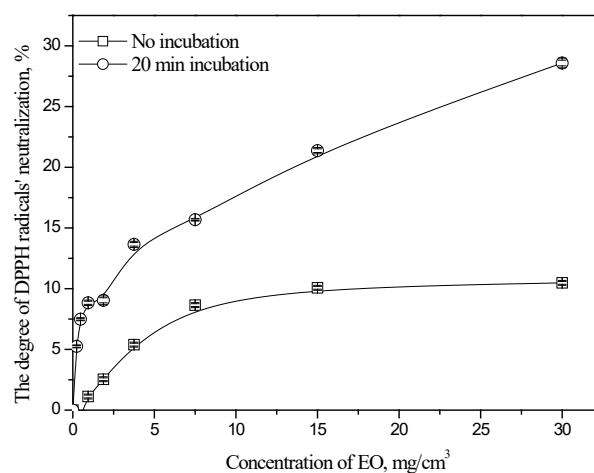


Fig. 5. The degree of DPPH radical neutralization as a function of essential oil concentration ( $\square$ —no incubation,  $\circ$ —20 min incubation)

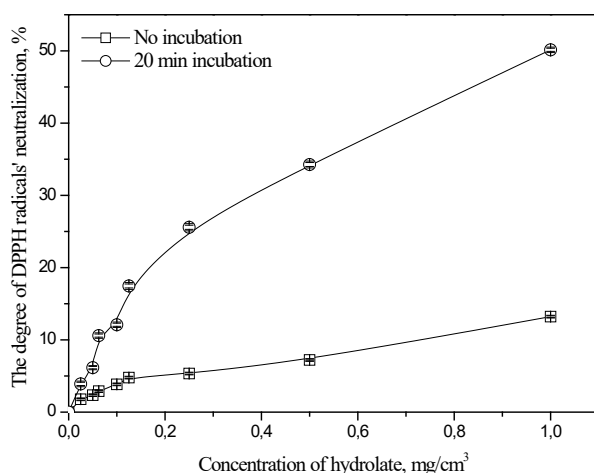


Fig. 6. The degree of DPPH radical neutralization as a function of hydrolate concentration ( $\square$ —no incubation,  $\circ$ —20 min incubation)

Table 4

The  $EC_{50}$  values of the essential oil of black pepper fruit, the hydrolate of black pepper fruit, and synthetic antioxidants

Sample	$EC_{50}$ , mg/cm <sup>3</sup>	
	No incubation	20 min incubation
Black pepper EO	582.01 ± 131.83	67.72 ± 1.871
Black pepper hydrolate	4.450 ± 0.018	0.993 ± 0.011
BHT	–	0.045 ± 0.0001
BHA	0.607 ± 0.004	0.0076 ± 0.00001

BHT – 2,6-ditert-butyl-4-methylphenol (CAS number 128-37-0);  
BHA – 2-tert-butyl-4-methoxyphenol (CAS number 25013-16-5).

The  $EC_{50}$  values (concentration required to obtain a 50 % antioxidant effect) of the EO with no incubation and after 20 minutes of incubation were 582.01 ± 131.83 and 67.72 ± 1.871 mg/cm<sup>3</sup>, respectively. The  $EC_{50}$  values of the hydrolate with no incubation and after 20 minutes of incubation were 4.450 ± 0.018 and 0.993 ± 0.011 mg/cm<sup>3</sup>, respectively. Based on the results obtained, the hydrolate showed better antioxidant activity than the EO; however, both the EO and hydrolate are weaker antioxidants in comparison to synthetic antioxidants BHT and BHA ( $EC_{50}$  values were 0.045 ± 0.0001 and 0.0076 ± 0.00001 mg/cm<sup>3</sup>, respectively, after 20 minutes of incubation). The active components in EOs are responsible for their antioxidant action. They might be present in high amounts, as the major components, or in small amounts. The action is frequently the product of their synergy. Black pepper EO showed moderate activity due to its chemical composition, where the most abundant compounds are sesquiterpene hydrocarbons, which, like their oxygenated derivatives, have very low antioxidant activity. In addition, although monoterpene hydrocarbons can act as active antioxidants, oxygenated monoterpenes are more potent. Oxygenated monoterpenes that are present in the highest percentage ( $\alpha$ -terpineol, borneol, and terpinen-4-ol) and the aromatic compounds like thymol, as well as their synergism, may be responsible for the better antioxidant activity of hydrolate compared to black pepper essential oil. Oxygenated monoterpenes, such as  $\alpha$ -terpineol, linalool, and borneol, individually showed weak to medium antioxidant activity,<sup>28,29</sup> which is much lower compared to the antioxidant activity of the hydrolate. This is probably proof of the synergism of aromatic components (thymol) and oxygenated components.<sup>28</sup> There is agreement with the results of this study and previous research on black pepper EO. Namely, a study by Politeo *et al.*,<sup>7</sup> found that black pepper EO, obtained under similar condi-

tions to this study, showed lower DPPH radical scavenging activity of about 60% and 37% after 60 minutes of incubation at concentrations of 50 mg/cm<sup>3</sup> and 20 mg/cm<sup>3</sup>, respectively.<sup>7</sup> Also, the same study by Politeo and coworkers showed that the most abundant compounds in the EO were caryophyllene (57.6%), sabinene (9.5%), and limonene (8.8%), the same compounds as in the present study.<sup>7</sup> In the study of Misharina,<sup>30</sup> the  $EC_{50}$  value of black pepper EO was 20 mg/cm<sup>3</sup>, and the major components of the EO were  $\alpha$ -pinene and  $\beta$ -pinene, limonene, sabinene, carene, and caryophyllene. The same study showed that black pepper EO was not as efficient as an antiradical compound in a model system containing DPPH radical because terpene hydrocarbons are thought to be weak hydrogen donors for the DPPH radical.<sup>30</sup> The study by Wang *et al.*<sup>14</sup> found that the  $EC_{50}$  value of black pepper EO was 6.348 mg/cm<sup>3</sup> after 30 minutes of incubation, where the most abundant component in the EO, 3-carene, was a significantly stronger antioxidant than caryophyllene.<sup>14</sup> There is no literature data on the antioxidant activity of the hydrolate, which is of great importance for further research, e.g., high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS) analysis and determination of piperine content.

#### 4. CONCLUSION

In the present study, 55 and 12 compounds were identified and quantified by GC/MS and GC/FID analyses of the EO and hydrolate of black pepper, respectively. The most abundant compounds in black pepper EO were (*E*)-caryophyllene, limonene, and sabinene, while the most abundant compounds in the hydrolate were  $\alpha$ -terpineol, borneol, and terpinen-4-ol. The hydrolate of black pepper showed better antioxidant activity than the EO, with an  $EC_{50}$  value of 0.993 mg/cm<sup>3</sup>

after 20 minutes of incubation. The presented results indicate that the EO and hydrolate of black pepper could be used as potential sources of natural antioxidants for the food, pharmaceutical, cosmetic, and chemical industries. Therefore, these isolates from black pepper represent the alternative to synthetic additives that exhibit toxic and carcinogenic effects. The results of the antioxidant activity of waste (hydrolate) in this study suggest not only that hydrolate is a better antioxidant but that more attention should be directed to its use for natural antioxidant isolation and the determination of the other biological activities of the waste.

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