STUDIA UBB CHEMIA, LXVIII, 1, 2023 (p. 285-301)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2023.1.21

COMPARATIVE CHEMICAL AND ANTIMICROBIAL CHARACTERIZATION OF NON-OZONATED AND OZONATED VEGETABLE OILS

Ana BALEA[a], Irina CIOTLĂUȘ[a,*], MARIA POJAR-FENEȘAN[a], Rahela CARPA[b]

ABSTRACT. In this study, a comparative determination of the quality factors, chemical composition and antimicrobial activity for ozonated and non-ozonated olive, coconut and hemp oils was made. The following quality factors for non-ozonated and ozonated oils were determined: peroxide value, acidity value, iodine value and density. The composition of methyl esters of fatty acids and the final compounds resulting from the ozonation process of the studied vegetable oils was characterized by Gas-Chromatography-Mass Spectrometry (GC-MS). The oil samples showed varying degrees of antibacterial activity against selected pathogens. Ozonated oils act as a matrix capable of releasing active oxygen from ozonides, which have antimicrobial activity. Density, peroxide value and acid value increased in all ozonated oils, while iodine value decreased in all ozonated oils. The gas chromatography showed a change in the degree of unsaturation due to the ozonation process, such that: the total content of unsaturated compounds decreased by 24.58% in ozonated olive oil, by 37.88% in ozonated hemp oil and by 9.14% in ozonated coconut oil. The innovative aspect of the paper consists in the physico-chemical and chromatographic characterization of the ozonated hemp oil and the comparison of the antimicrobial activity of the studied oils.

Keywords: *olive, coconut, hemp, ozonated oil, GC-MS, antimicrobial activity*

[a] *Babeş-Bolyai University, "Raluca-Ripan" Institute for Research in Chemistry, 30 Fântânele str., RO-400294, Cluj-Napoca, Romania.*
[b] *Babeş-Bolyai University, Faculty of Biology and Geology, Molecular Biology and Biotechnology Department, 1 M. Kogalniceanu street, 400084, Cluj-Napoca, Romania.*
** Corresponding author: irina.ciotlaus@ubbcluj.ro*

INTRODUCTION

The characterization of vegetable oils has been the subject of academic studies in recent years. Vegetable oils are complex mixtures of major components, such as triglycerides, partial glycerides and esters of fatty acids, unesterified free fatty acids and minor components such as sterols, hydrocarbons, pigments, phenols, flavonoids or volatile compounds. [1,2]

The ozonation of various vegetable oils has been studied, such as olive oil [3-7], sunflower oil [8-10], soybean oil [11,12] and coconut oil [13,14] under different conditions. Maritza F. Diaz *et al*. [14], studied the antimicrobial activity of coconut ozonated oil obtained by three different ozonation systems, compared to the antimicrobial activity of different antibiotics. All ozonated coconut oils showed antimicrobial activity, and those obtained by ozonating the mixture of oil with water or ethanol showed superior antibiotic activity.

Hemp seeds are a good source of oil, protein and carbohydrates. Hemp seed oil is one of the best sources of essential fatty acids with a perfect 3:1 ratio of omega-3-linolenic acid and omega-6-linoleic acid, suitable for strengthening the immune system [15-16]. It is, also a good source of gamma linoleic acid and is used to lower cholesterol in skin conditions. In Romania, hemp has been cultivated since ancient times as a textile plant as well as for oil seeds. In Romanian folk medicine, hemp seeds have been used to relieve rheumatic pain, to treat venereal diseases, pulmonary congestion, vomiting, intoxication, cough, hemorrhoids, intestinal parasites. [17] Most of this oil has been extensively investigated to determine its chemical composition [18-20] and its therapeutic properties [21], but not much is known about the physical, chemical and structural properties of the ozonated hemp oil, which offer its superior medicinal properties. [22]

Although interest in hemp oil has recently increased, it is surprising that there are few studies focusing on the antimicrobial activity of low THC essential oils, the literature presents several studies on the biological activity of compounds extracted from high THC hemp oils.

Novak *et al*. [23,24], tested the antimicrobial capacity of hemp oil on both Gram-positive and Gram-negative bacteria, with modest results.

A study evaluates the in vitro antimicrobial activity of essential oils extracted from the inflorescence of three legal varieties of hemp (low in THC) on Gram-positive bacteria, including *Clostridium spp. and Enterococcus spp,* Gram-negative bacteria (-) including phytopathogenic bacteria: *Pseudomonas spp. and Pectobacterium spp* and yeasts, related to phytopathogens or human commensals. The results showed that industrial hemp essential oils can significantly inhibit microbial growth, although the number of samples used in that study was quite limited. [25]

A recent study presents the phytochemical characterization of 17 hemp essential oils (EOs) belonging to different varieties, together with the evaluation of their antibacterial activity against some pathogenic and spoilage microorganisms isolated from food and food processing environments. [26]

Ozonated vegetable oils have many interesting applications in the food industry [27], cosmetics and pharmaceuticals [28-29], as well as in medicine. [30-33]

In a pharmacological context, the amount of ozonide is an important measure of the ability of the oil to supply active O_2 and other active species that can be exploited in the treatment of skin diseases. The judicious use of ozone (O3) seems providential because it first eliminates pathogens and then, by releasing oxygen (O2), it activates the proliferation of fibroblasts, hence the formation of the intercellular matrix with consecutive keratinoblast proliferation and successive healing. [31] One of the commonly used methods for ozone therapy is ozonated oils. The most commonly used type of oil is extra virgin olive oil. However, each type of unsaturated oil can be used for ozonation. [5] The ozonation process allows the properties of ozone gas to be combined with those of olive oil; the result is a compound without comparison. [34] Ozonated vegetable oils have been shown to have antibacterial and antifungal properties. [35] These properties are recommended for use in therapeutic and cosmetic purposes. [36]

The natural origin of these oils and their easy use together with their financial accessibility, place them among the best dermatological and cosmetic treatments.

Several methods are available for the characterization of ozonated vegetable oils, for example GC, GC-MS, FT-IR, NMR. [37-40] Ozonolysis is an oxidative reaction between ozone and the carbon-carbon double bond of an unsaturated compound. The reaction of ozone with these vegetable oils takes place almost exclusively at the carbon-carbon double bonds present in unsaturated fatty acids. Analysis of these reactions provide information about changes in functional groups during the ozonation process, according to the well-known Criegee mechanism for the formation of ozonides from alkenes and ozone. [41-42] The ozonolysis process involves a three-stage reaction: (1) formation of the primary ozonide, (2) breakdown of primary ozonide into aldehydes, aldo-acids and carboxylic acids, (3) recombination of carboxylic acid and aldehydes in order to form secondary ozonides. [43]

A comparative determination of the chemical composition and quality factors for ozonated and non-ozonated olive, coconut and hemp oils was made in this paper. Changes in quality factors, chemical composition and antimicrobial activity of already ozonated oils and the raw material (non-ozonated oils) were studied. The monitoring of the ozonation process was

not studied in detail. For non-ozonated and ozonated oils, the physical and chemical parameters were determined: peroxide value, acidity value, iodine value and density. The composition of methyl esters of fatty acids and the final compounds resulting from the ozonation process of the studied vegetable oils was characterized by Gas Chromatography coupled with Mass Spectrometry (GC-MS). The antimicrobial activity of non-ozonated and ozonated oils was tested "in vitro" on Gram-positive microorganisms: *Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis* and Gram-negative microorganisms: *Escherichia coli, Pseudomonas aeruginosa*. The relationship between the physico-chemical properties of oils and their antimicrobial activity has not been reported in the literature. Ozonated hemp oil was studied in comparison with non-ozonated hemp oil to identify physico-chemical and structural differences.

For the physico-chemical and chromatographic validation of ozonated hemp oil, we used standard procedures applied to ozonated vegetable oils.

RESULTS AND DISCUSSION

The physico-chemical characteristics of the oils before and after ozonation are shown in Table 1.

Table 1. Physico-chemical characteristics of the oils

Type of oil	Peroxide value *meqO_2/kg fat*	Acid value *mgKOH/g oil*	Iodine value *gI_2/100g oil*	Density *g/mL oil*
Olive oil	21.6	0.84	97.10	0.831
Ozonated olive oil	108.4	2.24	69.83	0.913
Hemp oil	56	5.88	132.91	0.891
Ozonated hemp oil	228.4	7.84	108.11	0.998
Coconut oil	7.6	0.28	7.02	0.901
Ozonated coconut oil	68	0.67	5.62	1.145

Table 1 shows an increase in values for three characteristics, namely: peroxide value, acid value and density. The increase in these values is due to the higher amount of peroxides due to ozonation, the higher amount of acids released by breaking the triglyceride chains under the action of ozone and oxygen saturation of the double bonds in unsaturated fatty acids. [13,40] The decrease in iodine value is due to the decrease in the degree of unsaturation after ozonation due to the oxidation of unsaturated fatty acids in the oil by the Criegee mechanism. [41] The high value of peroxide also

implies a high viscosity. [37] Bromine iodide reagent has difficult access to the double bond to react. This is a further explanation of why iodine decreases in ozonated oil samples. [22] An increase in acid and peroxide value has been observed in all oils. There is a significant increase in the value of peroxide in the study of coconut oil.

The volatile profile of vegetable oils was characterized by GC-MS. (Table 2).

Before performing GC analysis of olive oil (OO), ozonated olive oil (OOO), Hemp oil (HO), ozonated Hemp oil (OHO), coconut oil (CO) and ozonated coconut oil (OCO)) a trans-esterification (derivatization) reaction was performed. For this purpose, the fatty acid components of oils are more easily converted into volatile derivatives, usually methyl esters. In their free, non-derivatized form, fatty acids from oils can be difficult to analyze because these highly polar compounds tend to form hydrogen bonds, leading to adsorption problems. By reducing their polarity by methylation, they can be analyzed much more easily. This will then allow the column chemistry to make separations by eluting the components by boiling points and also by the degree of unsaturation, the position of unsaturation and even the cis vs. trans configuration of unsaturation.

Methyl esters of saturated and unsaturated fatty acids and ozonation products were detected in the analyzed vegetable oils. GC-MS results are expressed as relative percentage compositions. (Table 2)

During the reaction between ozone and fatty acids in vegetable oils, ozonation products are formed (nonanal, nonanoic acid, methyl-9-oxo-nonanoate, monomethyl nonandioate), according to the Criegee mechanism. [41]

Non-ozonated olive oil (OO) contains mainly methyl esters of fatty acids (75.71%). The main components identified were: methyl oleate (24.75%); methyl palmitate (20.12%), methyl palmitoaleate (10.14%) and squalene (10.63). Ozonated olive oil (OOO) mainly contains: ozonolysis compounds (38.84%), methyl esters of saturated fatty acids (30.74) and methyl esters of unsaturated fatty acids (23.68%). A total of 16 compounds representing 99.96% of the methyl esters of fatty acids were identified in non-ozonated hemp oil (HO). The major components identified were: methyl linoleate (28.91%); methyl palmitate (23.14%), methyl eicosanoate (7.99%); Methyl 12-hydroxy-9Z-octadecenoate (7.74%); Ozonated hemp oil (OHO) contains mainly: ozonolysis compounds (26.59%), methyl esters of saturated fatty acids (37.41%), methyl esters of unsaturated fatty acids (12.61%). Non-ozonated coconut oil (CO) contains 90.36% methylated fatty acid methyl esters and 9.32% unsaturated fatty acids. The ozonated coconut oil (OCO) contains mainly: ozonolysis compounds (14.41%), methyl esters of saturated fatty acids (82.47%) and methyl esters of unsaturated fatty acids (0.18%).

Table 2. Volatile components in vegetable oil

Oil constituent/Type	RT min	LRI	OO %	OOO %	HO %	OHO %	CO %	OCO %
1	2	3	4	5	6	7	8	9
1 Methyl hexanoate (E)	7.61	923	-	-	-	-	4.02	4.15
2 Hexanoic acid (A)	8.85	994	-	-	-	1.21	-	-
3 (E)- 3 -hexenoic acid (A)	8.97	1001	-	-	-	0.76	-	-
4 (E)-4-nonenal (ALD)	10.76	1099	-	-	-	0.93	-	-
5 Nonanal (ALD)	10.99	1112	-	8.05	-	1.58	-	4.40
6 2-Methyl octanoic acid (A)	11.09	1118	-	-	-	0.23	-	-
7 Methyl octanoate (E)	11.22	1125	-	1.86	-	3.12	13.39	12.28
8 (E)-2-Decenal (ALD)	11.95	1166	-	-	-	0.40	-	-
9 Methyl nonanoate (E)	12.97	1225	-	0.17	-	-	-	-
10 2-Octan-1-ol, acetate (E)	13.41	1251	-	-	-	0.44	-	-
11 8-Nonenoic acid (A)	14.09	1291	-	-	-	1.61	-	-
12 Nonanoic acid (A)	14.18	1296	-	9.04	-	0.86	-	3.27
13 Methyl decanoate (E)	14.70	1328	-	-	-	-	12.15	12.04
14 Methyl-10-undecanoate (E)	16.19	1422	-	-	-	1.11	-	-
15 Methyl 9-oxo-nonanoate (E)	16.52	1443		8.98	-	12.81	-	5.64
16 Methyl dodecanoate (E)	17.80	1529	-	0.51	-	-	26.58	21.07
17 Dimethyl nonanedioate (E)	18.13	1552	-	0.29	-	0.71	-	-
18 Monomethyl nonanedioate (E)	18.86	1603	-	7.76	-	6.16	-	2.38
19 Methyl tridecanoate (E)	19.23	1630	-	-	-	-	0.28	0.35
20 Dimethyl undecanedioate (E)	19.49	1649	-	0.50	-	-	-	-
21 13-(Z)-Tetradecenal (ALD)	20.37	1714	-	-	-	0.83	-	-
22 Methyl tetradecanoate (E)	20.63	1734	-	0.35	0.62	4.37	18.06	16.47
23 Methyl 12-oxo-dodecenoate (E)	21.59	1809	-	-	-	1.10	-	-
24 Methyl pentadecanoate (E)	21.82	1831	-	-	0.22	-	-	-
25 Methyl palmitoleate (E)	22.62	1912	10.14	3.27	-	2.78	-	-
26 Methyl palmitate (E)	22.81	1929	20.12	17.32	23.14	15.64	10.64	9.39
27 Linoleic acid (A)	23.54	2016	-	-	-	0.30	-	-
28 Methyl 10(Z)-heptadecenoate (E)	23.59	2023	1.30	2.54	-	-	-	-
29 Methyl heptadecanoate (E)	23.74	2044	-	-	-	0.84	-	0.07
30 Methyl 5-oxo-octadecanoate (E)	24.06	2088	-	-	-	-	-	0.08
31 Methyl γ-linolenate,(Z6,Z9,Z12) E)	24.09	2092	-	-	3.54	3.21	-	-
32 Methyl oleate (E)	24.18	2105	24.75	17.08	-	-	7.88	0.10
33 Methyl stearate (E)	24.75	2192	6.18	2.00	5.37	-	4.78	6.29
34 Methyl linoleate (9Z,12Z) (E)	24.91	2216	0.27	0.29	28.91	6.69	1.07	0.08
35 Methyl linolelaidate, (9E,12E) (E)	24.92	2218	-	-	3.37	-	-	-
36 Methyl-10(Z)-nonadecenoate (E)	24.97	2222	0.36	0.37	-	-	-	-
37 Methyl nonandecanoate (E)	25.16	2255	-	-	-	0.08	-	-
38 Methyl α-linolenate (E)	25.22	2263	-	-	2.11	0.28	-	-
39 α-Fenchene (Ot)	25.28	2272	-	-	-	0.44	-	-
40 1,6-Cyclodecadiene (Ot)	25.38	2278	-	-	-	1.29	-	-

1	2	3	4	5	6	7	8	9
41 Methyl –cis-9,10-epoxy-stearate (E)	25.44	2296	-	1.06	-	-	-	0.42
42 (Z, Z)-9, 12-octadecadienoyl chloride (Ot)	25.51	2306	-	-	-	3.17	-	-
43 Methyl-trans-9,10-epoxy-stearate (E)	25.55	2311	-	2.32	-	-	-	-
44 Methyl-12-OH-9Z-octadecenoate (E)	25.56	2313	-	-	7.74	2.43	-	-
45 Methyl-11(Z)-eicosenoate (E)	25.60	2318	4.67	2.78	4.82	-	0.37	0.73
46 Methyl eicosanoate (E)	25.74	2338	5.21	4.09	7.99	2.53	0.73	-
47 Methyl-15-OH-9,12-octadecadienoate (E)	25.89	2358	-	-	-	0.32	-	-
48 11 -Z-Eicosenoic acid (A)	25.93	23.64	0.87	-	-	-	-	-
49 Methyl 9,10:12,13-diepoxystearate (E)	25.95	2367	-	0.80	-	0.70	-	-
50 Isopropyl -9,10-Epoxy-stearate (E)	26.07	2383	-	0.35	-	-	-	0.03
51 Oxiraneundecanoic acid, 3-pentyl, methyl ester, trans (E)	26.20	2401	-	0.11	-	-	-	-
52 (Z)-13-eicosenoic acid (A)	26.23	2405	-	-	-	2.13	-	-
53 Methyl heneicosanoate (E)	26.43	2430	0.36	0.32	-	-	-	-
54 α-Glyceryl-linolenate (E)	26.54	2444	-	-	-	0.36	-	-
55 Ethyl linoleate (E)	26.64	2457	0.14	0.30	-	0.94	-	-
56 Oxiraneoctanoic acid, 3-octyl, cis	26.70	2465	-	0.08	0.27	0.57	-	-
57 Z,Z-5,16-Octadecadien-1-ol, acetate (E)	26.71	2466	-	-	-	3.06	-	-
58 Glycidyl oleate (E)	26.87	2486	0.28	0.03	-	0.36	-	-
59 Methyl-Z-13-docosenoate (E)	27.09	2531	-	-	0.35	1.41	-	-
60 Methyl docosanoate (E)	27.28	2586	1.79	2.37	7.09	5.59	-	-
61 Tocopheryl acetate (E)	27.80	2682	8.70	-	-	-	-	-
62 Methyl tricosanoate (E)	28.24	2731	0.35	0.35	0.32	0.88	1.05	-
63 Heptanoic acid,docosyl ester (E)	28.52	2756	1.65	0.27	-	-	-	-
64 Glyceryl monooleate (E)	29.16	2812	1.34	-	-	-	-	-
65 Methyl tetracosanoate (E)	29.42	2832	0.78	0.83	3.54	4.00	-	-
66 Squalene (A)	30.72	3008	10.63	3.46	-	1.52	-	-
67 Methyl pentacosanoate (E)	30.81	3015	-	-	-	0.24	-	-
ESTERS (E)			88.39	79.24	99.69	83.72	99.96	99.25
ACIDS (A)			0.87	9.12	0.27	7.44	-	3.27
ALDEHYDES (ALD)			-	8.05	-	3.74	-	4.40
OTHERS (Ot)			10.63	3.46	-	4.90	-	-
Total			**99.89**	**99.87**	**99.96**	**99.8**	**99.96**	**99.92**

RT: retention time; LRI: liniar retention index (on HP-5ms column); olive oil (OO), ozonated olive oil (OOO), hemp oil (HO), ozonated hemp oil (OHO), coconut oil (CO), ozonated coconut oil (OCO).

Changing the degree of unsaturation in the ozonation process

Figure 1 shows that the percentages of oleic acid in olive oil (OO) decrease from 24.75% to 17.08%, in palmitoleic acid decrease from 10.24% to 3.27%, in eicosenoic acid decrease from 5.54% to 2.78% and in squalene,

they decrease from 10.62% to 3.46%, due to the ozonation process. The total content of unsaturated compounds decreased by 24.58%. The percentages of linoleic acid, the main component of ozonated hemp oil (HO) decrease from 28.91% to 6.69% in ozonated hemp oil (OHO). In fresh hemp oil (HO), the total unsaturated fatty acid content decreased by 37.88%. The total unsaturated fatty acid content of coconut oil (CO) decreased from 9.32% to 0.18% in ozonated coconut oil (OCO).

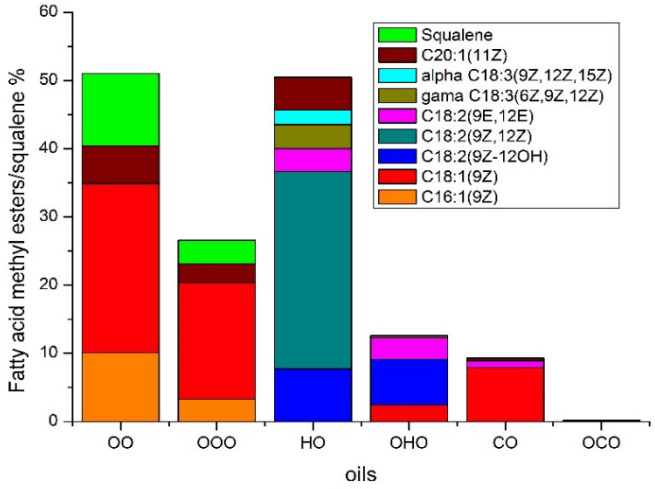
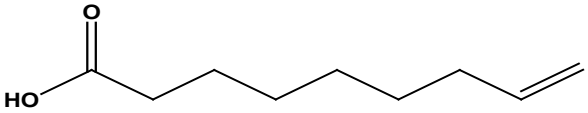
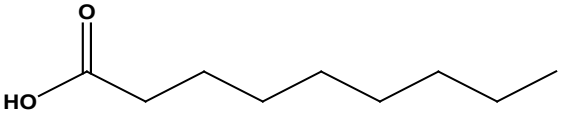
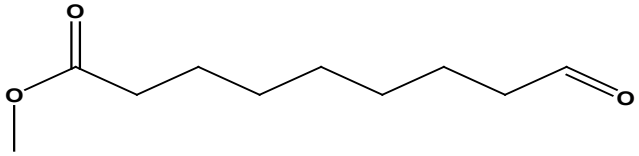
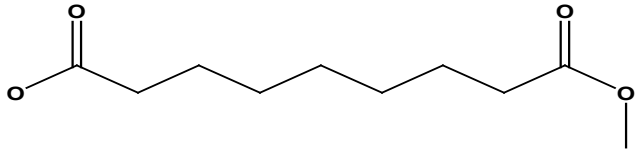
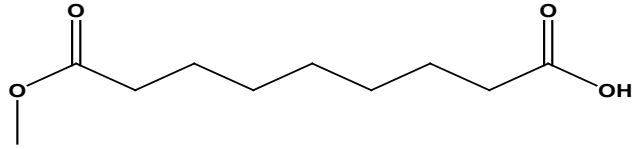
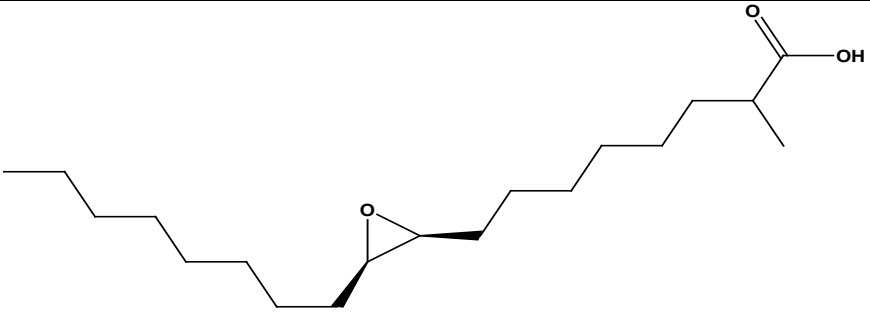


Figure 1. Percentage composition of faty acid unsaturated methyl esters and squalene in oil samples

In the first stage, ozone reacts with the double bonds in vegetable oils to form 1,2,3-trioxolanes or molozonides. Molosonides are unstable, and those synthesized in aprotic environments tend to rearrange into more stable Criegge ozonides (secondary ozonides) through carbonyl oxide (H2COO). In the presence of protic solvents or water, carbonyl oxides can react and form compounds with different functional groups. [41] Because secondary ozonides are composed of high molecular weight and low volatility, they cannot be analyzed by GC-MS. In this case, in our experiments, ozonolysis products resulting from the degradation of the secondary ozonide in the polar reaction environment were highlighted by GC-MS.

The volatile fraction of ozonated oils is composed of saturated aldehydes, aldo-acids, epoxides, carboxylic acids and dicarboxylic acids. Nonanal, nonanoic acid, methyl 9-oxononanoate and monomethyl nonandioate were the main components of the volatile fraction in ozonated vegetable oils. [Table 3].

Table 3. Final compounds resulting from the ozonation process of vegetable oils

	Compounds/Structure
1	 Nonanal
2	 8-Nonenoic acid
3	 Nonanoic acid
4	 Methyl-9-oxo-nonanoate
5	 Dimethyl nonanedioate
6	 Monomethyl nonanedioate
7	 Methyl-cis-9,10-epoxy stearate

The predominant unsaturated fatty acids in OO, HO and CO are oleic and linolenic acids, respectively, from which it is assumed that the new peaks were formed mainly by the reaction of ozone with these fatty acids. In oleic acid, the double bond is present in position C9, the linoleic acid contains two double bonds in positions C9 and C12, while the linolenic acid contains three double bonds in positions C6, C9 and C12. Thus, nonanal, nonanoic acid, 9-oxo-nonanoate were formed by the reaction between ozone and the unsaturated double bond at the C9-C10 position of these acids. [50-52]

Evaluation of antimicrobial activity

The antibacterial activity of ozonated and non-ozonated oils was evaluated by well diffusion methods. Oil samples showed varying degrees of antibacterial activity against the selected pathogens (Figure 2).

Depending on the strain tested and on the type of sample, it can be seen that the inhibition was quite variable but not very high.

The only bacterial strain in which inhibition was completely absent for both categories of oils was the Gram-positive strain *Enterococcus faecalis*.

The test results showed that the diffusion well-variant was more sensitive for ozonated oil samples. Of the 5 bacterial strains tested, only *Pseudomonas aeruginosa* showed inhibition in both types of samples (ozonated and non-ozonated) (Fig. 2).

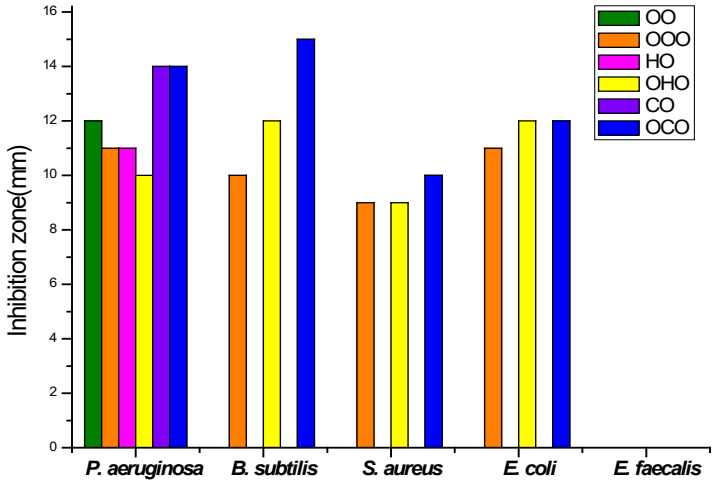


Figure 2. Antimicrobial activity of various oil samples

In the other bacterial strains, the inhibition was recorded only for ozonated oil samples. Of the 3 categories of ozonated oils, coconut oil showed the highest inhibition of all bacterial strains tested.

The antibacterial activity of coconut oil is due to both the medium chain fatty acids in its composition and the ozonides formed by ozonation [53,14]. Coconut oil is a source of beneficial medium chain fatty acids (MCFA), especially lauric acid, capric acid, caprylic acid and caprioic acid [see methyl esters of these acids in Table 2]. As far as neozonated coconut oil is concerned, growth was inhibited only for the *Pseudomonas aeruginosa* strain, while ozonated coconut oil was also active for the other three strains: *S.aureus, E.coli* and *B.subtilis*.

The antibacterial activity of olive oil is due to both the fraction of polyphenols present in the composition[54] and the ozonides formed by ozonation. Ozonated olive oil has antimicrobial activity on the four bacterial strains that we tested, as well as other studies that suggest that ozonated olive oil has antimicrobial activity against both *S.aureus, E.coli, P.aeruginosa* and and *B.subtilis*. [3,38,55]

The antibacterial activity of non-ozonated hemp oil in these organisms can be considered as modest, compared to ozonated oil. In the case of non-ozonated hemp oil, the antibacterial profile is mainly due to the cannabinoids in the composition.[23] Ozonated oil was active on the four strains: *S.aureus, E.coli, P.aeruginosa* and *B.subtilis,* with good results.

To the best of our knowledge, the tests with hemp ozonated oil on the strains studied in this paper are not mentioned in the literature. However, its use “in vivo” against *Microsporum canis* – the main zoonotic pathogenic fungus in veterinary medicine, is mentioned. [56]

CONCLUSIONS

From the physico-chemical analysis, it is observed the change of value of the quality parameters for the ozonated oils compared to the non-ozonated ones, as it follows: the peroxide value, the acid value and the density, all increase and the iodine value decreases.

The gas-chromatographic analysis shows the change in composition and the appearance of new compounds in ozonated oils compared to non-ozonated ones. The ozonation process changes the structure of the oil, so the unsaturations become ozonides, which, due to their instability, break into derivatives with oxygenated functions.

From the evaluation of the antimicrobial activity, in the case of ozonated olive and hemp oils, an efficiency is observed on new strains: *S.aureus, E.coli* and *B.subtilis*, where the non-ozonated oils did not show any type of activity.

This research indicates that at higher levels of peroxide, acidity and density, the antimicrobial activity of olive and hemp oils is higher for *S.aureus, E.coli* and *B.subtilis* and less for *P.aeruginosa*. Coconut oil showed the highest inhibition compared to the other oils studied in the 4 bacterial strains tested.

The innovative aspect consists in the physico-chemical, gas-chromatographic characterization and the antibacterial profile of the ozonated hemp oil. Ozonated hemp oil has been studied in comparison with non-ozonated hemp oil to identify the physical, chemical and structural differences that give its superior properties, which can be exploited in the treatment of skin diseases.

No published results were found for ozonated hemp oil on the correlation between chemical composition and biological activity. This study shows that ozonated hemp oil was active on the four strains with good results: *S.aureus, E.coli, B.subtilis* and *P.aeruginosa.*

The natural origin of these oils and their easy way of use, place them among the best treatments.

EXPERIMENTAL SECTION

Materials

Reagents: Hexane, chloroform, ethanol, ethyl ether, glacial acetic acid, potassium iodine, potassium hydroxide, thiosulfate, starch, phenolphthalein. Reagents/solvents were purchased from Merk, Hohenbrunn-Germany by a local supply company. The oils used for studies in this article were supplied to us by a Romanian manufacturer of ozonated oils. The instrument used for ozonation process was ozone generator (model OzoneFix Business 3) with ozone flow of 3 g O_3/h. All samples was ozonated at a temperature of 25^0C for 6 hours. For this study we used three types of oils: olives, coconut and hemp, non-ozonated and ozonated types, so 6 samples in total. The oils studied oroginate from organic crops and were obtained from the seeds of Hemp (*Cannabis sativa*), Olives (*Olea europaea*) and Coconut (*Cocos nucifera*) by cold pressing.

Physico-Chemical Parameters

a. Peroxide value (PV)

The peroxide value was determined according to SR EN ISO 3960: 2005 - *Animal and vegetable oils and fats*. *Determination of the peroxide value*. [45]

The peroxide value for all determinations is calculated by the equation:

$$\mathrm{PV} = \frac{1000\ (\mathrm{V1} - \mathrm{V0})\ \mathrm{c}}{\mathrm{m}} \quad (1)$$

where V1 is the volume in mL of thiosulphate solution used for titration, V0 is the volume in mL of thiosulphate solution used for titration of a control, c is the mol L^{-1} thiosulphate concentration and m the amount of sample (grams).

The peroxide value, confirms the process of ozonation of oils.

b. Acid value (AV) - represents the amount of potassium hydroxide needed to neutralize the free acids present in the 1g oil sample. It indicates the degree to which the triglycerides in the oil have decomposed to release free fatty acids. The acid value was calculated by the equation:

$$\mathrm{AV} = \frac{M\ x\ C\ x\ V}{m}\ (\mathbf{2})$$

where AV - the acid value expressed as a mass fraction, M is the molar mass, in grams per mole, for KOH, C is the L^{-1} mole concentration of the standard volumetric solution of potassium hydroxide KOH used, V is the volume, in milliliters, of KOH and m is the mass, in grams, of the oil sample.

The Acid value was determined according to SR EN ISO 660:2009- *Animal and vegetable fats and oils. Determination of acid value and acidity*.[46]

c. Iodine value (IV) - Represents the amount of iodine that will react with the double bonds, number of grams of iodine consumed per 100g of fat. A higher iodine value indicates a higher degree of unsaturation. IV is calculated according to the monographs of Pharmacopoeia.

$$\mathrm{IV} = \frac{1{,}256\ (\mathrm{V1} - \mathrm{V2})}{\mathrm{m}}\ (3)$$

where V1 is the volume in mL of thiosulphate solution (0,1 M) used for a blank test, V2 is the volume in mL of thiosulphate solution (0,1 M) used for titration and *m* the amount, in grams, of the substance. The value of acid iodine was determined according to SR EN ISO 3961: 2018 - *Animal and vegetable fats and oils. Determination of iodine value*.[47]

d. Determination of density

The oil density was determined at 20^0 C by weighing an exact volume (2 ml) of oil; the determination was carried out three times for each oil sample.

Determination of Fatty Acid Profile

The Gas-Chromatographic analysis was performed on a Gas-chromatography Mass Spectrometer (GC-MS), purchased from Agilent Technologies, Santa Clara California, USA, by Agilrom Scientific SRL Romania.

a. Sample preparation

The methylation reaction occurs in a cooled methanolic solution of potassium hydroxide, according to the standard procedure. In a test tube with a 5 ml cap, 0.10 g of oil sample was added to a mixed solution of 3 mL of hexane and 500 µL of 2 N mol L^{-1} methanolic KOH. The sample was stirred vigorously for 15 s, allowing the layers to separate until the top solution became clear. The upper layer containing the methyl esters was decanted and dried over MgSO4.

b. Chromatographic conditions

Determinations of methylated fatty acids were performed on an Agilent 7890 & 5975 Series MSD Gas Chromatography Mass Spectrometer (GC-MS) equipped with a HP-5MS chromatographic column (5% phenyl)-methyl polysiloxane, (30 m x 0.25 mm x 0.25 µM) Agilent model, helium carrier gas, injection volume 1 microliter. GC parameters were obtained under the following conditions: helium carrier gas (He 6.0), flow rate 1 ml / min, injector temperature was 260 °C, splitless mode. The column temperature was initially set at 50 ° C, then increased at a rate of 8 ° C / min to 220 ° C / min, then from 220 ˚C to 280 ˚C, increased by 20 ˚C / min, where kept at 280 °C for 5 minutes. MS detector parameters: electron impact mode (EI +), 70 eV, ion source temperature, 230 °C. Mass spectra were recorded in the range of 50-500 a.m.u, in scan mode. All tests were performed in duplicate. Data acquisition and processing were performed using a MSD ChemStation software. An alkane standard (Alkanes Standard Solution C8-C20, C 21 - C40 alkanes Sigma Aldrich) was used to calculate the linear retention index (LRI), and the experimental values were compared to those in the literature, under similar chromatographic conditions. The identification of fatty acid methyl esters was performed by comparing the retention times (RT) in the samples with those of a known standard (Supelco® 37 Component FAME Mix) and by comparing the mass spectra with those in the NIST 14 L database. The composition of methyl esters in vegetable oils was expressed as a percentage of each component in relation to the total area of the chromatogram.

Antibacterial testing

The microorganisms tested in this study were Gram positive: *Enterococcus faecalis* ATCC-29212, *Staphylococcus aureus* ATCC-25923 and *Bacillus subtilis* ATCC-6633 and Gram negative: *Escherichia coli* ATCC-25922 and *Pseudomonas aeruginosa* ATCC-27853 from the Microbiology Laboratory, Faculty of Biology and Geology, UBB, Cluj.[48]

The antimicrobial test method was the diffusimetric one, of the 5 mm diameter, wells cut aseptically in the culture medium (agar well diffusion method) [48]. The wells were then filled with sterile cotton granules. Each granule was loaded with 20 µL of each oil sample. Bacterial suspensions were made from fresh bacterial strains in saline and adjusted to a McFarland turbidity of 0.5 which were inoculated over the entire surface of the agar plates using a sterile cotton swab. The culture medium used to inoculate the bacterial suspensions was Mueller Hinton [49]. After inoculation, the samples were incubated at 37 °C for 24 hours. The diameter of the inhibition zone was then measured for each sample and for each microorganism. At the end of the incubation period, the diameters of the inhibition zones for each oil sample were measured with a ruler.

REFERENCES

1. F.D. Gunstone; *Vegetable oils in food technology: Composition, Properties and Uses*, Blackwell Publishing Ltd, Oxford, UK, **2011**, pp1-352
2. D. Bozdogan-Konuskan; A. Canbas; *Rev. Chim. (Bucharest)*. **2014**, *65*, pp. 788-791
3. M.F. Díaz; R. Hernández; G. Martínez; G. Vidal; M. Gómez; H. Fernándeza; R. Garcés; *J. Braz. Chem. Soc*. **2006**, *1*, 403-416.
4. R. Seda Tigli Aydin; F. Kazanci; *J Am Oil Chem Soc.* **2018**, *95*, pp.1385-1398
5. B. Uysal; *Intercult Ethnopharmacol.* **2014**, *3,* 49-50.
6. M.F.Diaz; J. A. Gavin -Sazatornil; O. Ledea; F. Hernandez; M. Alaiz; R. Garces; *Sci. Eng*. **2005**, *27*, 247- 253.
7. V. Georgiev; M. Anachkov; T. Batakliev; S. Rakovsky; *Bulgarian Chem. Commun.* **2013**, *45*, 203-207.
8. O.E. Ledea-Lozano; L.A. Fernández-García; D. Gil-Ibarra; N. Tenab; R. Garcés; E. Martínez-Force; J.J. Salas; *Grasas Aceites* **2019**, *70*, 1 –12.
9. N.U. Soriano; V.P. Migo; M. Matsumura; *Phys. Lipids*. **2003**, *126*, 133-140.
10. S. Moureu; F. Violleau; D. Ali Haimoud-Lekhal; A. Calmon; *Chem. Phys. Lipids* **2015**, *186*, 79-85.
11. P. Tran; D. Graiver; R. Narayan; *J. Am. Oil Chem. Soc.* **2005**, *82*, 653-659.
12. H.R. Liu; P.J. White; *J. Am. Oil Chem. Soc.* **1992**, *69*, 528-532.
13. H.S. Enjarlis; Y. Anwar; *Int. J. Eng. Technol.* **2018**, *7*, 146-149.

14. M.F. Díaz; N. Núñez; D. Quincose; W. Díaz; F.Hernández; *Ozone: Sci. Eng.* **2005**, *27*, 153-157.
15. S. Shobha-Borhade; *Archives of Applied Science Research*. **2013**, *5*, 5-8.
16. S. Montserrat-De La Paz; F. Marín-Aguilar; M. D. García-Giménez; M. A. Fernández-Arche; *J. Agric. Food Chem*. **2014**, *62,* 1105-1110.
17. S. Manea; *Rev. Hofigal*. **2014**, *41,* 40-41.
18. F. Anwara; S. Latifa; M. Ashraf; *J. Am. Oil Chem. Soc.*, **2006**, *83*, 323-329.
19. S. Sapino; M. E.Carlotti; E. Peira,; Gallarate, M.; *J. Cosmest. Sci.* **2005**, *56*, 227-251.
20. İ. Orhan; Ş. Küsmenoğlu; B. Şener; *J. Fac. Pharnı. Gazi*. **2000**, *17*, 79-81.
21. R. Iseppi; V. Brighenti; M. Licata; A. Lambertini; C.Sabia; P. Messi; F. Pellati Benvenuti; *Molecules*. **2019**, *24*, 2302.
22. N. Rodrigues De Almeida; A. Beatriz; E. José De Arruda; D. Pires De Lima; L.C. Silva De Oliveira; A.C. Michelett; Ozonized Vegetable Oils: Production, Chemical Characterization and Therapeutic Potential in *Vegetable Oil Properties, Uses and Benefits*; Brittany Holt- Nova Editor Science Publishers, **2016**, *Chapter 5*, pp. 129-149.
23. G. Appendino; S.Gibbons; A. Giana; A. Pagani; G. Grassi; M. Stavri; E. Smith; M. M. Rahmanm; *J Nat Prod*. **2008**, *71*,1427-1430.
24. J. Novak; S. Karin Zitterl-Eglseer; G. Deans; C.M. Franz; *Flavou Fragr. J.* **2001**, 16, 259-262.
25. G. Zengin; L. Menghini; A. Di Sotto; R. Mancinelli; F. Sisto; S. Carradori; S.Cesa; C. Fraschetti; A. Filippi; L. Angiolella; M. Locatelli; L. Mannina; C. Ingallina, V. Puca; M. D'Antonio; R. Grande; *Molecules*, **2018**, *23*, 3266.
26. R. Iseppi; V. Brighenti; M. Licata; A. Lambertini; C. Sabia; M. Patrizia; F. Pellati; S. Benvenuti; *Molecules*, **2019**, *24*, 2302.
27. Z. B. Guzel-Seydim; A.K. Greene; A.C. Seydim; *Lebensm. Wiss. Technol.* **2004**, *37,* 453-460.
28. M. Cirlini; A. Caligiani; G. Palla; A. De Ascentiis; P.Tortini; *Ozone: Sci. Eng.* **2012**, *34*, 293-299.
29. N. Bordei-Ionescu; M. Popescu; A. Bratu; D. Istrati; C. Ott; A. Meghea; *Rev. Chim. (Bucharest)*, **2015**, *66*, 1267-1272.
30. H. Kataoka; M. Semma; H. Sakazaki; K. Nakamuro; T. Yamamoto; S. Hirota; K. Tazuya-Murayama; A. Ichikawa; *Ozone Sci. Eng.* **2009**, *31*, 238-246.
31. V. Travagli; I. Zanardi; G. Valacchi; V. Bocci; *Mediators Inflamm*. **2010**, *2010*, 1-9.
32. E. Ugazio; V. Tullio; A. Binello; S. Tagliapietra; F. Dosio; *Molecules*, **2020**, *25*, 334
33. N.S.I Geweely; *Int.J. Agric. Biol.* **2006**, *8*, 670-675.
34. E. Carata; B. A. Tenuzzo; L. Dini; Powerful Properties of Ozonated Extra Virgin Olive Oil, in *Herbal Medicine* Edited by *Philip F. Builders,* IntechOpen, **2018**.
35. M. Montevecchi; A. Dorigo; M. Cricca; L. Checchi; *The New Microbiologica: official journal of the Italian Society for Medical Virology (SIVIM)*. **2013**, *36*, 28 - 302.

36. J. Lu; M. Chen; L.Gao; Q. Cheng; Y. Xiang; J. Huang; K. Wu; *J. Dermatol. Treatment*. **2018**, *29*, 676-681.
37. J. Sadowska; B. Johansson; E. Johannessen; R. Friman; L. Broniarz-Press; J. B. Rosenholm; *Chem. Phys. Lipids.* **2008**, *151*, 85-91.
38. R. Seda Tigh Aydin; F. Kazanci; *J Am Oil Chem Soc*. **2018**, *95*, 1385-1398.
39. P. Guerra-Blanco; T. Poznyak; I. Chairez; M. Brito-Arias; *Eur. J. Lipid Sci. Technol*. **2015**, *117*, 988-998.
40. I. Zanardi; V. Travagli; A. Gabbrielli; L. Chiasserini; V. Bocci; *Lipids*. **2008**, *43*, 877-886.
41. R. Criegee; *Agnew. Chem. Internat.* **1975**, *14*, 745-752.
42. O. Ledea; T. Correa; M. Escobar; A. Rosado; J. Mario; C. Hernandez; D. Jardines; *Ozone Sci. Eng.*, **2001**, *23*, 121-126.
43. J. Zahardis; G. A. Petrucci; *Atm. Chem.Phys.*, **2007**, *7*, 1237-1274.
44. G. Martinez Tellez; O. Ledea Lozano; M. D´laz Gomez; *Ozone: Sci. Eng.,* **2006**, *28*, 181-185.
45. SR EN ISO 3960: **2005** - *Animal and vegetable oils and fats. Determination of the peroxide index*
46. SR EN ISO 660:**2009**- *Animal and vegetable fats and oils. Determination of acid value and acidity*
47. SR EN ISO 3961: **2018** - *Animal and vegetable fats and oils. Determination of iodine value.*
48. R. Carpa; M. Drăgan-Bularda; V. Muntean; *Microbiologie Generală Lucrări Practice*, Ed. Presa Univ. Clujeană, 2014.
49. R. M. Atlas; Handbook of Microbiological Media, 4th edition, CRC Press, New York, 2010.
50. C. M. Paraguassú Cecchi; D. Cesarín-Sobrinho; A. B. Buarque-Ferreira; J. C. Netto-Ferreira; *Catalysts,* **2018**, *8*, 6,1-21
51. A. Soutelo-Maria; J. L. Dubois; J. L. Couturier; G.Cravotto; *Catalysts*, **2018**,*8*, 1-17
52. K. Y. L. Alex; K. C. Chak; *J. Phys. Chem. A*. **2007**, *111*, 6285-6295.
53. F. C. Peedikayi; V. Remy; S. John; T. P Chandru; P. Sreenivasan; G. A. Bijapur; *J. Int Soc Prevent Communit Dent* **2016**, *6*, 447-452.
54. F. Nazzaro; F. Fratianni; R. Cozzolino; A. Martignetti; L. Malorni; G. V. De Feo; A. Cruz; A. Acierno; *Microorganisms*, **2019**, 7, 321.
55. M. Montevecchi; A. Dorigo; M. Cricca & L. Cecchi; *New Microbiologica*, **2013**, *36*, 289-302.
56. G. Appendino; S. Gibbons; A. Giana; A. Pagani; G. Grassi; M. Stavri; E. Smith; M. Mukhlesur Rahman; *J. Nat. Prod*. **2008**, *71*, 1427-1430.