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OZONATION OF VEGETABLE OILS AND STUDY ON THEIR PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERISTICS

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ABSTRACT: Free ozone offers significant benefits in biological applications due to its efficacy as a disinfectant, but toxicity and instability are associated with it. Hence, producing ozonated vegetable oil (OVO) has been explored as a potential solution, yielding stable ozonation by-products with medical potential, such as antimicrobial activity. Several studies have explored OVO's characteristics and biological effects, including olive oil, sunflower oil, and canola oil. However, optimizing ozonation conditions is still lacking, with many other types of vegetable oils yet to be studied. This research comprises three phases: i) ozonation of selected oils: red palm oil (RPO), rice bran oil (RBO), peanut oil (PO), and virgin coconut oil (VCO), ii) screening for the most effective OVO against three bacteria (*Staphylococcus aureus, Bacillus subtilis,* and *Escherichia coli*), and iii) physicochemical testing. Results show increased peroxide and acidity values in most OVO and a decrease in iodine value compared to untreated oil. Ozonated virgin coconut oil (OVCO) exhibits the highest antibacterial activity by showing a zone of inhibition of 11.3 mm and 84.35% killing rate at 30 minutes incubation time, particularly against *S. aureus*. Further optimization using Design Expert®6.0.8 software identifies the most effective ozonation conditions for OVCO, achieving a peak killing rate of 100% against *S. aureus* with 360 mins of ozone exposure and ozone flow rates of 1 l/min. Kinetic studies confirm rapid bacterial eradication, with over 90% of *S. aureus* killed by OVCO within 2 mins. Moreover, OVCO proved to be non-toxic to human foreskin fibroblast (HFF1) cells, maintaining 80% viability even after exposure to 1 mg/ml OVCO treated with ozone for 120 and 240 mins. These findings underscore the promising medical potential of OVCO, particularly in treating skin diseases.

ABSTRAK: Ozon bebas menawarkan manfaat signifikan dalam aplikasi biologi disebabkan keberkesanannya sebagai bahan pembasmi kuman, namun ia turut dikaitkan dengan ketoksikan dan ketidakstabilan. Oleh itu, penghasilan minyak sayuran berozon (OVO) telah diteroka sebagai potensi penyelesaian, menghasilkan hasil sampingan ozonasi yang stabil dengan potensi perubatan seperti aktiviti antimikrob. Beberapa kajian telah meneliti ciri-ciri dan kesan biologi OVO termasuk minyak zaitun, minyak bunga matahari, dan minyak kanola. Namun, proses pengoptimuman keadaan ozonasi masih belum lengkap dan banyak lagi jenis minyak sayuran belum dikaji. Kajian ini terdiri daripada tiga fasa: i) ozonasi minyak terpilih iaitu minyak sawit merah (RPO), minyak dedak padi (RBO), minyak kacang tanah (PO), dan minyak kelapa dara (VCO), ii) saringan keberkesanan OVO terhadap tiga jenis bakteria (*Staphilokokus aureus*, *Basillus subtilis*, dan *Escherichia coli*), dan iii) ujian fisikokimia. Keputusan menunjukkan peningkatan nilai peroksida dan keasidan dalam kebanyakan OVO serta penurunan nilai iodin berbanding minyak yang tidak dirawat. Minyak kelapa dara berozon (OVCO) menunjukkan aktiviti antibakteria tertinggi dengan zon perencatan berdiameter 11.3 mm dan kadar pembunuhan bakteria sebanyak 84.35% dalam masa inkubasi

30 minit, khususnya terhadap *S. aureus*. Pengoptimuman lanjut menggunakan perisian Design Expert®6.0.8 mengenal pasti keadaan ozonasi paling berkesan bagi OVCO, dengan pencapaian kadar pembunuhan maksimum 100% terhadap *S. aureus* pada pendedahan ozon selama 360 minit dan kadar aliran ozon 1 l/min. Kajian kinetik mengesahkan penghapusan bakteria yang pantas, dengan lebih 90% *S. aureus* dibunuh oleh OVCO dalam masa 2 minit. Tambahan, OVCO terbukti tidak toksik terhadap sel fibroblas kulit manusia (HFF1), dengan mengekalkan 80% daya hidup walaupun selepas pendedahan kepada 1 mg/ml OVCO yang dirawat ozon selama 120 dan 240 minit. Penemuan ini menekankan potensi perubatan OVCO, khususnya dalam merawat penyakit kulit.

KEYWORDS: *Ozonated vegetable oil, Cytotoxicity, Antibacterial, Killing rate*

1. INTRODUCTION

Ozone is acknowledged for its powerful disinfectant properties in therapeutic and biological applications, but its free form is harmful to living cells and the lungs when inhaled [1]. To overcome this limitation, ozonated vegetable oil (OVO) has emerged as an alternative, containing therapeutic by-products resulting from the alteration of unsaturated fatty acids through ozonation to produce active oxygen and other active substances such as ozonides, aldehydes, and peroxides. Moreover, unlike gaseous ozone, OVO offers enhanced stability and efficacy [2]. OVO contains a series of ozonation by-products that remain active for years when properly stored [3]. OVO possesses diverse biological properties, functioning as bactericidal and antifungal agents, and facilitating wound healing. Thus, it is a potential substitute for traditional antibiotics and antifungals, particularly in dermatology, dental care, ophthalmology, and gynaecology.

Vegetable oils are rich in saturated fatty acids, unsaturated fatty acids, antioxidants, fat-soluble vitamins, and various minor components. These oils have low allergenic potential, are non-toxic, and are utilized in fuel, food, and functional products due to their natural therapeutic properties [4]. Considering the benefits associated with vegetable oils, numerous studies have been undertaken to explore the attributes and potential biological activities of diverse ozonated vegetable oils, encompassing olive oil, grapeseed oil [2], sunflower oil [5], and soybean oil [2]. Studies have demonstrated that ozonation significantly enhances the antibacterial properties of vegetable oils [6]. These studies also highlight variations in physicochemical properties, such as density, viscosity, and ozonation by-products, which arise due to differences in oil composition, including the levels of saturated and unsaturated fatty acids and the degree of ozonation applied [7]. However, despite these findings, a comprehensive comparison of various types of OVO under standardized ozonation conditions has not yet been conducted. This gap limits the ability to determine which vegetable oils yield the most effective antibacterial properties under similar conditions. With many vegetable oils having unique compositions and origins, it is crucial to study their ozonated properties systematically. This would facilitate the identification of the most potent OVO with antibacterial properties, providing valuable insights for therapeutic and industrial applications. Additionally, while the benefits of ozonation are well-documented, there has been limited focus on optimizing ozonation parameters to maximize antibacterial activity. Another key gap in current research is the limited investigation of potential toxicity linked to prolonged ozonation treatment times. Addressing these gaps is essential to ensure the practical and safe application of OVOs in the medical and pharmaceutical fields.

Thus, this study aims to address these gaps by investigating the physicochemical parameters (peroxide values, acidity values, iodine values, and functional groups) and

antibacterial properties of several other types of OVO including red palm oil (RPO), rice bran oil (RBO), peanut oil (PO), and virgin coconut oil (VCO). OVO with the best antibacterial activity against several different bacterial strains will be screened first before determining the optimal ozonation conditions to achieve optimal antibacterial activity. The time-dependent effect of the optimized OVO against the bacteria, as well as the toxicity effect of the OVO against normal skin cells, was also investigated in this study. The comprehensive analysis obtained from this study will contribute to understanding OVO's properties and investigate its reasonable toxicity towards normal skin cell lines to support its utilization as a sustainable and effective alternative for combating bacterial infections.

2. MATERIALS AND METHODOLOGY

2.1. Chemical and Materials

Vegetable oils (RPO, RBO, PO, and VCO) were obtained from a local supermarket, and a NANO ozone generator from Absolute Ozone for ozonation was used in the ozonation process. To analyze the OVO, potassium hydroxide (0.1 M), sodium thiosulfate ($Na_2S_2O_3$), sodium hydroxide (NaOH), Wijs solution, phenolphthalein, chloroform ($CHCl_3$), and deionized water were obtained from Chemiz and Sigma. The bacteria used in the study are *E. coli*, *B. subtilis,* and *S. aureus* obtained from INHART, IIUM.

2.2. Ozonation of Vegetable Oils for Screening Process

The experiment started with 20 ml of each vegetable oil being ozonated in a Dresher bottle (Quickfit®) connected to an ozone generator. Ozone is produced by the silent discharge of oxygen (purity of 99.9%) from the ozone generator with a 30 mg/l ozone concentration. The oils were exposed to the ozone at room temperature for 120 minutes with a 1.5 l/min ozone flow rate.

2.3. Characterization of OVO

2.3.1. Density Value and Color Observation

Density represents the ratio between the mass of a substance and its volume at a determined temperature, expressed in grams per milliliter (g/ml). The calculation involved determining the ratio of the mass of a given volume of the ozonated vegetable oil at 20°C to the mass of an equivalent volume of water at 4°C. All measurements were performed in triplicate [6]. Color observation was conducted using visual inspection. Equal amounts of untreated vegetable oil and OVO were placed in separate beakers. The samples were examined under consistent lighting conditions, and any color differences were observed.

2.3.2. Peroxide Value

The peroxide value (PV) is a number that quantifies the number of peroxides present in 1000 g of a substance in 1 ml equivalents of active oxygen. In a 250 ml conical flask, 5 g of the OVO was weighed and mixed with 30 ml of acetic acid/chloroform [18 ml Acetic Acid: 12 ml chloroform]. Then, 0.5 ml of saturated potassium iodide solution was added once the material was completely dissolved. The solution was stirred and left for 1 min. Subsequently, 30 ml of distilled water was added to the solution, followed by titration with sodium thiosulfate solution (0.05 M) and 1 ml of starch solution as an indicator. The steps were repeated without oil for the blank sample. Each sample was analyzed in triplicate. Subsequently, the peroxide value was calculated using Eq. (1).

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$$PV = 1000\ (V_1 - V_0).\frac{c}{w} \quad (1)$$

where PV is the peroxide value, V_1 is the volume of the thiosulfate solution used for the titration, V_0 is the volume of the thiosulphate solution used to carry out a blank sample, c is the sodium thiosulfate normality, and w is the mass of the oil sample (g).

2.3.3. Acidity Value

Acidity value (AV) is the amount of sodium hydroxide needed to neutralize the free acids in 1.0 grams of the substance. It acts as an indicator of the oil's oxidation, reflecting how much the triglycerides of the OVO have broken down to produce free fatty acids. The AV of the oil samples was determined using ethanol titration with phenolphthalein as an indicator.

The experiment began by dissolving 1 g of each oil in 15 mL of ethanol in a measuring flask. This resulted in a solution titrated with sodium hydroxide (NaOH), using phenolphthalein as an indicator [6]. Three replications were made for each of the samples. Eq. (2) was used to calculate the acid value.

$$AV = \frac{McV}{w} \quad (2)$$

where AV is the acid value (mg NaOH/g fat), M is the molar mass of NaOH (g/mol), c is the concentration of NaOH (M), V is the volume of NaOH utilized (ml), and w is the mass of the oil sample (g).

2.3.4. Iodine Value

The iodine values (IV) can be used to determine the presence of double bonds in vegetable oils. It measures the amount of iodine that can react with the oils' carbon-carbon double bonds, indicating their unsaturation rate. When the ozonation process is successful, the iodine value of the vegetable oil decreases.

The experiment started by dissolving 2.5 g of the OVO sample in 12.5 ml of carbon tetrachloride (CCl4), then 12.5 ml of Wijs solution was added to the mixture along with 1 ml of potassium iodide, and the mixture was kept in the dark for approximately 1 h to complete the reaction. Subsequently, 50 ml of deionized water was added and was titrated with 0.05 M sodium thiosulfate ($Na_2S_2O_3$) until reaching the endpoint (turning into a milky white color solution). The same procedure above was repeated for a blank sample without the OVO. Each sample was analyzed in triplicate. Subsequently, the peroxide value was calculated using Eq. (3).

$$IV = \frac{12.69(V_1 - V_0)N}{w} \quad (3)$$

where IV is the iodine value, V_1 is the volume of the thiosulfate solution used for the titration, V_0 is the volume of the thiosulphate solution used to carry out a blank sample, N is the sodium thiosulfate normality, and w is the mass of the oil sample (g).

2.4. Screening of OVO for Antibacterial Activity

2.4.1. Seed Culture Antibacterial Testing

A single colony of bacteria was taken from the culture plate using an inoculum loop and dipped into 10 mL sterile LB broth in a 50 mL centrifuge tube. The cultures were incubated overnight at 37°C with 150 rpm in the incubator shaker. After overnight incubation, the cultures were collected and centrifuged at 3500 rpm for 5 minutes. The supernatant was then discarded,

and the pellet was dissolved in PBS. The optical density (OD) of the solution was adjusted to a specific value of 0.1 using a spectrophotometer.

2.4.2. Antibacterial Testing

The study used two methods to investigate the antibacterial properties of the untreated vegetable oil and OVO: the agar diffusion method and the plating method. The agar diffusion method is a common technique to study the antibacterial properties of OVO. The procedure described involves mixing 0.5 ml of DMSO with 4 ml of oil in a 15 ml centrifuge tube, incubating the mixture for 30 minutes at 37°C, and then spreading 0.1 ml of the seed culture sample on LB agar. Discs are impregnated with 0.04 ml of the incubated solutions and dropped on the agar plate containing the seed culture. Each sample was analyzed in triplicate. The agar plates were incubated overnight at 37°C, and the inhibition diameter was calculated to determine the antibacterial activity of the OVO.

For the plating method, 0.5 ml DMSO with 4 ml oil and 0.5 ml seed culture is added to a 15 ml centrifuge tube. The samples were vortexed and incubated for 30 minutes at 37°C. Afterward, 0.1 ml of the sample was plated onto LB agar and continued incubating overnight at 37°C. Three replications were made for each of the samples. Then, the number of colonies was calculated using Eq. (4) to obtain the percentage of the killing rate as follows:

$$Killing\ Rate\ (\%) = \frac{(control\ colony\ number - tested\ colony\ number)}{control\ colony\ number} \times 100 \quad (4)$$

2.5. Optimization of the ozonation condition for antibacterial activity

The optimization study used Design Expert® 6.0.8 software, employing the Response Surface Method (RSM) with a Face-Centered Central Composite Design (FCCD). The best OVO found after the screening process on the efficacy of antibacterial properties using the plating method was chosen for the optimization study. In this experiment, two independent variables were selected: ozone exposure time (120 mins to 360 mins) and ozone flow rate (1 l/min to 3 l/min). The range was selected based on a previous study [2][3].

2.6. Kinetic Study

A kinetic study on the antibacterial activity was conducted using the best OVO from the optimization study at 120 mins and 360 mins ozone exposure time with a flow rate of 1 l/min. The procedure was the same as the plating method. The cultures were incubated separately at various incubation periods before being plated onto LB agar and incubated overnight at 37°C. Then, the number of colonies was calculated to obtain the percentage of the killing rate using Eq. (4). All samples were performed in triplicate. Graphs of the percentage killing rate of the OVO against time were plotted to obtain the kinetic pattern of antibacterial activity over time.

2.7. Cytotoxicity Test

Cell viability of HFF1 (normal fibroblast skin cells) upon exposure to the OVO was assessed by performing the MTT assay. Cells were cultivated in 96-well plates at 50,000 cells per well. Subsequently, the cells were treated with variable concentrations of OVO produced at different ozonation times. This treatment spanned a period of 48 h within a 5% CO_2 incubator, maintaining a temperature of 37°C. After the treatment period, the cells were rinsed and exposed to 0.01 ml of MTT solution, following which the plates were incubated at 37°C in a dark environment for 4 h. Once formazan crystals had formed, 0.1 ml of DMSO was added, and the absorbance was quantified at 570 nm utilizing a microtiter plate reader [7].

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3. RESULTS AND DISCUSSIONS

3.1. Physical Characterization of Ozonated Vegetable Oils Illustrations

Physical characterization studies the changes in density and color of vegetable oils before and after ozonation. Table 1 shows that all vegetable oils showed a slight increment in their density after the ozonation process. ORPO has the highest density at 0.962 g/ml, reflecting an 8.09% increase, followed by OVCO at 0.945 g/ml, with a 3.85% increase. OPO has a density of 0.935 g/ml, showing a 1.63% increase, while RBO has the lowest increase of 1.13%, with a density of 0.895 g/ml. A higher percentage of density increment for ORPO is primarily due to its high levels of unsaturated fatty acids (50%), such as oleic and linoleic acids, as well as reactive components like carotenoids and tocopherols. These substances undergo significant ozonation, resulting in the creation of heavier oxidation products and polymerized structures. Conversely, RBO with the lowest increment at only 1.13%, likely has its unique antioxidant profile, including oryzanol and tocotrienols, which helps stabilize the oil during ozonation and reduces the formation of denser oxidation products [8].

The ozonation of vegetable oils transforms their original color, yellow, to a lighter yellowish and eventually a colorless appearance for RBO, PO, and VCO. In contrast, untreated RPO has a distinctive red color because it contains high carotene and low free fatty acid levels. It shows a significant change in color to deep yellow, losing its original color as ozonation time increases. Additionally, RPO is known for its balanced fatty acid composition, consisting of 50% saturated fats (palmitic and stearic acids), 40% monounsaturated fats (oleic acid), and 10% polyunsaturated fats (linoleic acid). This particular composition allows it to remain semi-solid after ozonation [1]. Compared to untreated vegetable oil, OVO is more transparent and has a thicker texture with a tendency to solidify.

3.2. Chemical Characterization of Ozonated Vegetable Oils

Chemical characterization of oils involves assessing key parameters like peroxide, acidity, and iodine values to evaluate the oil changes after ozone exposure. The ozonation process can modify the composition of linoleic acid in unsaturated fatty acids by breaking double bonds, forming various degradation products such as ozonides, peroxide, and aldehydes with different chemical properties [9].

Table 2 presents untreated and ozonated oils' peroxide, acid, and iodine values. RPO, RBO, and PO exhibit higher peroxide values after ozonation than VCO, which has a much lower peroxide value. This is due to their higher unsaturated fatty acid content, approximately 50% for RPO, 70% for RBO, and 80% for PO. Unsaturated fatty acids are more reactive with ozone, forming hydroperoxides, peroxides, diperoxides, and polyperoxides [10].

Despite its relatively low level of unsaturated fatty acids (9%), OVCO shows a significant increase in peroxide value from 3.78 meq/kg to 26.31 meq/kg, a rise of 596.03%. This notable increase is likely due to the high concentration of lauric acid (40%-60%) in VCO, which is reactive with ozone. The ozonation process generates various reactive intermediates that contribute to increased peroxide values. A higher peroxide number indicates more ozone reacted with fatty acids, reflecting the effectiveness of the ozonation process. Measuring peroxide levels is crucial, as peroxides are indicators of oxidation and contribute to the biological activity of oils [10,11].

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Table 1. Physical properties of the vegetable oil

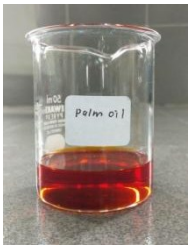
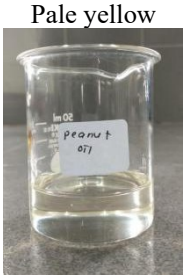
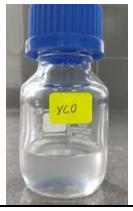
Types of Oil	Color Changes		Density (g/ml)	
	Before	After	Before	After
RPO	Red 	Deep Yellow 	0.890	0.962
RBO	Yellow 	Yellow 	0.885	0.895
PO	Pale yellow 	Clear Off white 	0.920	0.935
VCO	Off white 	Clear Off white 	0.910	0.945

Table 2. Chemical characterization of untreated vegetable oils and OVO

Types of Oil	Peroxide Value		Acidity Value		Iodine Value	
	Untreated vegetable oils	OVO	Untreated vegetable oils	OVO	Untreated vegetable oils	OVO
RPO	38.34	116.16	2.39	3.52	3.74	1.69
RBO	34.87	71.52	0.75	2.13	2.06	1.82
PO	13.91	63.01	1.09	7.86	3.52	1.38
VCO	3.78	26.31	0.30	0.91	1.37	0.32

The acid value measures the amount of free fatty acid in oil, reflecting its degree of oxidative degradation. Table 2 shows that the acid values of oils generally increase after ozonation, signifying higher acidity. OPO exhibits the most significant rise in acid value, increasing from 1.09 mg NaOH/g to 7.86 mg NaOH/g, representing a 621.1% increase. This substantial increase is likely due to the breakdown of triglycerides into free fatty acids during ozonation, a process accelerated by the high reactivity of ozone with double bonds in unsaturated fatty acids, such as oleic acid or linoleic acid [12]. This reaction forms peroxides,

aldehydes, and carboxylic acids, ultimately contributing to higher acidity levels. In contrast, RPO shows the smallest increase in acid value, rising by only 47.28% from 2.39 mg NaOH/g to 3.52 mg NaOH/g. This is likely due to antioxidants like tocopherols and carotenoids, which can neutralize free radicals and reduce the formation of acidic compounds during ozonation [1].

The iodine value measures the degree of unsaturation in oils, indicating the number of double bonds available to react with iodine. Ozonation reduces iodine values across all oil types due to ozone's cleavage of double bonds [4]. Thus, all types of OVO have decreased iodine values as depicted in Table 2, which suggests that ozonation succeeds in attacking and lowering the double bond on the vegetable oils. OVCO experiences the highest reduction in iodine value by 76%, attributed to its high saturation and susceptibility to ozone. Conversely, ORBO demonstrates the lowest reduction in iodine value due to its unique antioxidant content, including γ-oryzanol, tocopherols, and tocotrienols. These antioxidants shield the double bonds in unsaturated fatty acids from oxidation by neutralizing reactive intermediates, thereby preserving a higher degree of unsaturation [13].

3.3. FT-IR Spectroscopy

The FT-IR spectroscopy is used to highlight differences in the functional groups before and after the oil ozonation, particularly the decrease of the bands corresponding to both C = C and =C–H stretching and the increase of the band corresponding to ozonide C-O and peroxides stretching [14].

From Figure 1 (a-c), the conversion of double bonds to ozonides was observed in ORPO, ORBO and OPO as shown by the expected decreases in the characteristic wave numbers for C=C double bond stretching (1500 cm^{-1}) and =C–H stretching (3000 cm^{-1}), along with an increase in the ozonide C-O stretching signal (1100 cm^{-1}), which aligns with Criegee's mechanism during ozonation [5]. Additionally, ozone reacts with the linoleic and oleic acids in all vegetable oils under study, producing peroxides at 2920 cm^{-1} [15]. The FT-IR spectrum of each OVO consistently shows a reduction in the frequency of the characteristic wave associated with double bonds. However, in OVCO, the presence of ozonides is not visible and remains undetected, though a peak at C-H is observed (Fig.1d). This absence of detectable ozonides may be due to the lower amount of unsaturated fatty acids in OVCO [12], which leads to ozone reacting more with saturated fatty acids like lauric acid. This reaction could result in the formation of fewer aldehydes. In the IR spectra of an aldehyde, a peak typically appears around 2720 cm^{-1}. It often manifests as a shoulder peak adjacent to the alkyl C–H stretching vibrations compared to more unsaturated oils. The resulting ozonide intermediates may be unstable or transient, quickly decomposing or reacting with other constituents in the oil, which could explain their undetectable levels. The chemical changes are further supported by the data in Table 2, where OVCO exhibits the lowest number of peroxide values compared to other OVOs.

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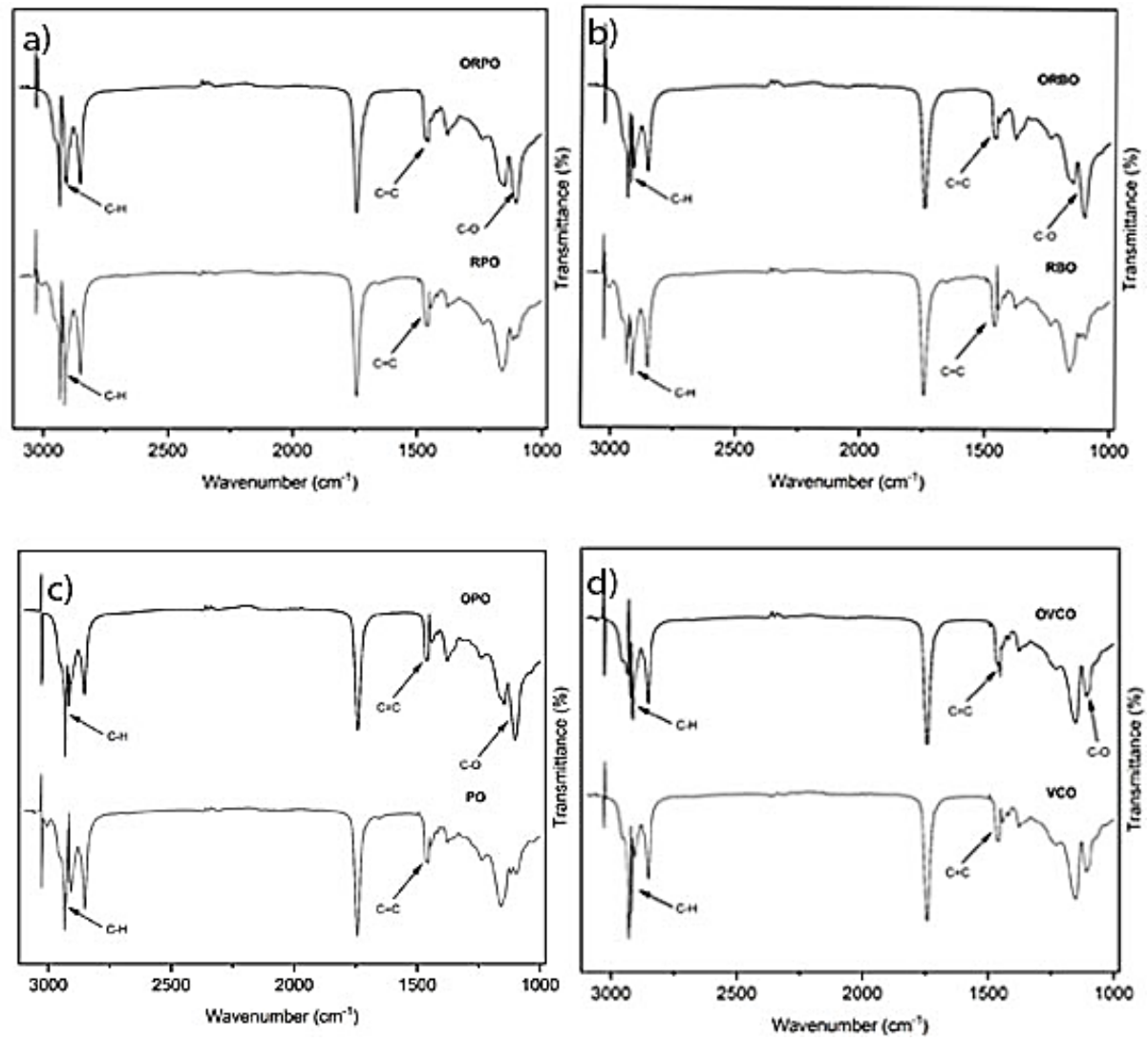

Figure 1. FT-IR Spectroscopy of the untreated and OVO; a) RPO, b) RBO, c) PO, d) VCO

3.4. Antibacterial screening of different types of OVO

OVO exhibits significant activity against the tested bacterial strains, as its ozonation by-products (such as peroxides and ozonides) contribute to its ability to destroy bacterial membranes [16]. In agar diffusion, the size of the zone of inhibition diameter in the antibacterial studies indicated that OVO has significant activity against the bacteria tested under study, as shown in Table 3. Gram-positive bacteria: *B. subtilis* and *S. aureus* are the strains most sensitive to OVO and have a larger inhibitory zone diameter than *E. coli*. The thick peptidoglycan layer in *B. subtilis* and *S. aureus* makes them more susceptible to certain antibacterial substances like OVO. The high peroxide value of OVO is the main reason for its high antibacterial activity. The previous study's findings [17] confirm this hypothesis, as they discovered a link between the peroxide value of OVO and its antibacterial efficacy against gram-positive bacteria.

In the zone of inhibition test, it was observed that both ORPO and OVCO exhibited significant antibacterial activity compared to the other types of OVO studied. The inhibition zone diameters for ORPO against E. coli, B. subtilis, and S. aureus are 3.7, 8.0, and 10.2 mm, respectively. Meanwhile, for OVCO, inhibition zone diameters are 6.6, 8.4, and 11.3 mm,

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respectively, the highest among the other tested OVOs. This finding shows that peroxide is not the only ozonation by-product toxic to the bacteria, as OVCO has the lowest peroxide value. A plating method was used to confirm the result further and identify which specific bacteria were most sensitive to OVCO and ORPO.

Table 3. Antibacterial activities of vegetable oils using the zone of inhibition test

Types of Oil	Zone of Inhibition Diameter (mm)		
	Bacteria		
	E. coli	*B. subtilis*	*S. aureus*
RPO	4.5	6.0	5.0
ORPO	3.7	8.0	10.2
RBO	5.4	5.0	5.0
ORBO	3.9	6.1	9.7
PO	3.8	4.2	6.0
OPO	5.2	7.3	9.5
VCO	5.5	7.0	5.5
OVCO	6.6	8.4	11.3

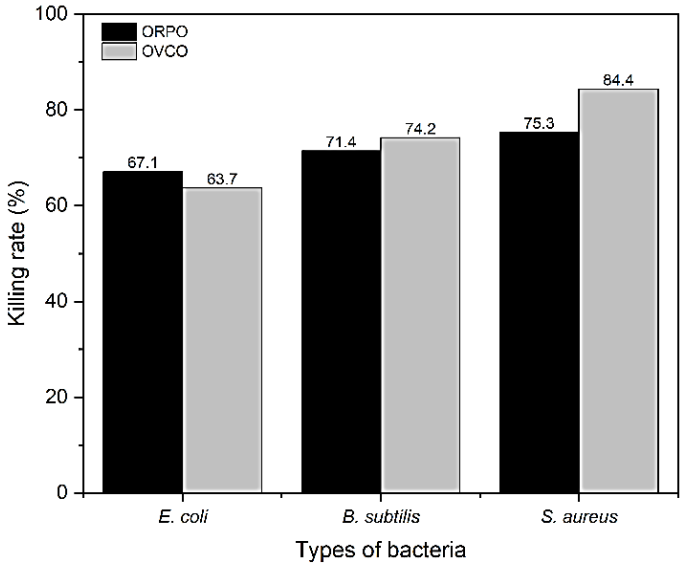

Figure 2. Antibacterial activities of OVO using a plating method.

Figure 2 shows that *B. subtilis* and *S. aureus* are the most sensitive towards OVCO and ORPO. The results indicate that OVCO can kill up to 74.2% of *B. subtilis* and up to 84.35% of *S. aureus* within a 30-minute incubation period compared to ORPO. Hence, OVCO has been identified as having the highest antibacterial activity against the gram-positive bacteria *S. aureus* compared to *B. subtilis* and *E. coli*. This high level of effectiveness is likely due to the presence of lauric acid in VCO (40-60%) compared to RPO (0.2%). Lauric acid is known to have antibacterial, antiviral, antifungal, and antiprotozoal properties and can also enhance the immune system [18]. Studies have shown that ozonation of oils by reacting with saturated fatty acids like lauric acid can enhance their antibacterial activity [17]. Thus, the high lauric acid content of VCO and the potential benefits of ozonation enhanced the synergistic effect of OVCO for antibacterial activity. Therefore, OVCO was chosen to be further optimized for its antibacterial properties against *S. aureus* by varying the ozone treatment time and flow rates.

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3.5. Optimization of the OVO conditions

The effects of two parameters (A: ozonation exposure time and B: ozone flow rate) for treating OVCO were analyzed using a percentage of killing rate towards *S. aureus* as the response test to determine their bactericidal activities. The ozonation conditions (120–360 mins and 1–3 l/min) used in this study are consistent with previous research on vegetable oil ozonation. These parameters balance effective ozone interaction with the oil's unsaturated compounds while minimizing oxidative degradation and unwanted by-products. In this design, a total of 13 runs were carried out in triplicate, and the experimental results are shown in Table 4.

Table 4. Response Surface Method with Face-Centered Central Composite Design and Responses

Run	Factor 1: Ozone Exposure Time (mins)	Factor 2: Ozone Flow Rate (l/min)	Response: Killing Rate (%)
1	120	3	78.3
2	360	2	100
3	360	1	100
4	240	3	87.01
5	240	1	99.78
6	240	2	91
7	120	1	90.33
8	240	2	92.7
9	240	2	98
10	120	2	90.3
11	240	2	93.4
12	360	3	97.8
13	240	2	93

Table 4 suggests that runs 1 through 13 of an experiment show significant antibacterial activity against *S. aureus*. The highest percentage of killing rate (100%) was achieved in runs 2 and 3 with 360 mins of ozone exposure time and 2 l/min and 1 l/min flow rate, respectively. Run 1, with 120 mins of ozone exposure time and 3 l/min ozone flow rate, had the lowest percentage of killing rate at 78.3%. The findings indicate that longer ozone exposure times with lower flow rates improve the bactericidal activity of OVCO. Longer reaction times allow for more complete interactions between ozone and the double bonds in the oil. In contrast, higher ozone flow rates may decrease the contact time and reduce the effective ozone concentration available for reaction [19].

Table 5. Analysis of Variance (ANOVA) for killing rate (R1)

Source	Sum of Squares	Degree of Freedom, df	Mean Square	F-value	p-value	Impact
Model	373.31	2	186.66	21.02	0.0003	Significant
A: Ozone Exposure Time	251.81	1	251.81	28.36	0.0003	
B: Ozone Flow Rate	121.50	1	121.50	13.68	0.0041	
Residual	88.81	10	8.88			
Lack of Fit	61.48	6	10.25	1.50	0.3620	Not significant
Pure Error	27.33	4	6.83			
Cor Total	462.12	12				

The ANOVA analysis in Table 5 suggests that the model and selected parameters used in the Design Expert software are linear and significant, with a p-value less than 0.05. The optimal

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ozone conditions for the antibacterial study were 357.52 mins of ozonation with a 1.39 l/min ozone flow rate.

The ANOVA analysis in Table 5 tests the significance of the ratio of mean square variation due to the regression coefficient and residual error. The ANOVA result for R1 shows an F-value of 21.02, implying that the terms in the model significantly affect the responses. A large value of F indicates that most of the variation in the response can be explained by the regression models. The associated p-value estimates whether F is large enough to indicate statistical significance. A p-value of 0.0003, lower than 0.05, indicates that the model is statistically significant. Results show that both selected parameters in the optimization study are significant for antibacterial properties, which is crucial for the potential use of OVCO in skincare and medical treatments.

3.6. Kinetic study of OVCO against *S. aureus*

Throughout the study, OVCO inhibits a significant antibacterial effect against *S. aureus*, and the killing rate increases as the ozonation time increases and the flow rate decreases. The growth pattern for the killing rate of *S. aureus* when exposed to OVCO ozonated for 120 mins and 360 mins with a flow rate of 1 l/min was examined.

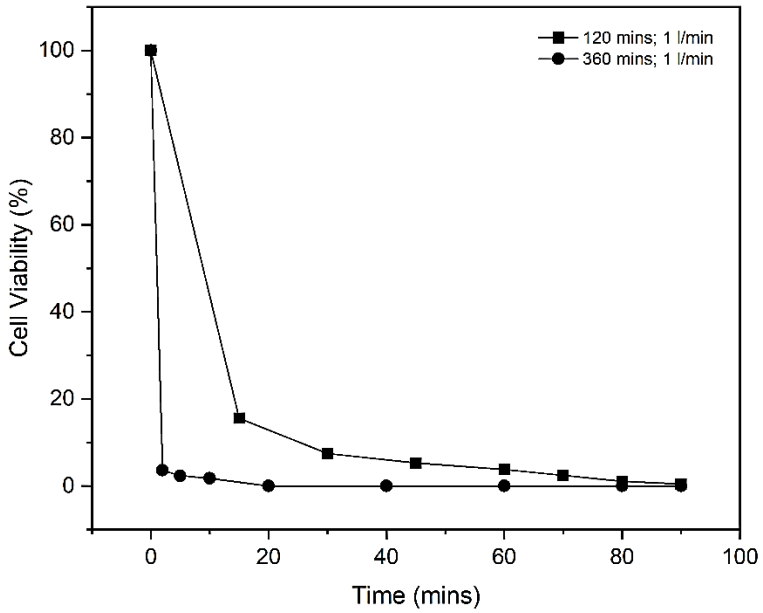

Figure 3. Kinetic study of OVCO against *S. aureus* at different times

Figure 3 depicts the quantitative evaluation of antibacterial properties for OVCO using a biocidal kinetic test. At the start of the incubation phase, the cell survival rate remained constant at 100%. After 60 mins of incubation, the residual live *S. aureus* was detected at 3.78% and 0.01% for 120 mins and 360 mins of OVCO ozonation, respectively. The killing rate for OVCO ozonated at 360 mins was very fast, which killed 96.4% of the bacterial cells within 2 2-minute incubation period. The graph shows a sharp decline in cell viability to 3.6%. The difference in the kinetic pattern of the killing rate proves that the ozone exposure time significantly affects the bactericidal properties. However, if a shorter ozonation period of vegetable oil can still result in strong antibacterial properties, it would be more efficient and cost-effective for manufacturers to produce the OVO along with environmental benefits, since 120 mins of OVCO can kill almost 100% of bacteria within 60 mins.

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3.7. Cytotoxicity Test

The cytotoxicity of OVCO was further evaluated using HFF1 fibroblasts, a model for normal skin cells. Based on Figure 4, it was observed that OVCO demonstrated minimal toxicity and can be safely administered, as 100% cell viability was achieved at a concentration of 1 mg/ml for samples exposed to ozone for 120 mins. This suggests that OVCO treated with 120 mins of ozone exposure is the safest formulation among the tested samples. However, extended ozone exposure times resulted in reduced cell viability. Specifically, samples exposed to ozone for 240 mins displayed slightly higher than 80% cell viability at 1 mg/ml, while samples exposed for 360 mins showed less than 80% cell viability.

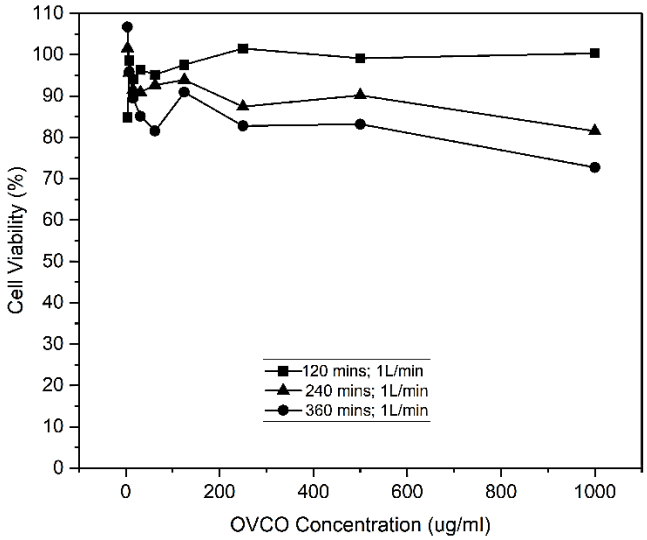

Figure 4. Dose-dependent cytotoxic test of the OVCO at different treatment times

Despite the observed decline in cell viability at higher ozone exposure times, OVCO consistently maintained over 80% cell viability at concentrations up to 500 mg/ml, indicating low cytotoxicity. These findings align with previous studies on ozonated oils, such as ozonated olive oil [3] and sunflower oil [2], demonstrating low toxicity to normal skin cells while retaining therapeutic benefits, including antimicrobial and wound-healing properties. The findings demonstrate the potential of OVCO as a therapeutic agent, particularly for dermatological and medical applications. The combination of low toxicity at therapeutic concentrations and strong antibacterial properties makes it a promising option for skincare and medical treatments. The peroxide value of OVCO is significantly lower than that of other OVOs, which enhances its safety profile. Maintaining a low peroxide value is essential to minimize oxidative damage to healthy cells, making this formulation highly suitable for managing chronic wounds, burns, and various dermatological conditions where protecting healthy tissue is a priority. These characteristics support its role as a safe and practical choice for skin tissue care [20].

4. CONCLUSION

Ozonation of vegetable oils (RPO, RBO, PO, and VCO) resulted in increased antibacterial properties against *E. coli, B. subtilis*, and *S. aureus* compared to untreated samples. From the screening processes, OVCO was found to have the highest bactericidal activity among the studied OVOs, with notable potency against gram-positive bacteria. The high bactericidal activity of OVCO is believed to result from the combined effects of lauric acid and ozonation by-products such as peroxides and aldehydes. Furthermore, optimization of ozonation

conditions through DOE achieved a 100% bacterial killing rate within 2 minutes, using an ozone exposure time of 360 minutes and a flow rate of 1 l/min. The kinetic study confirmed that longer ozone exposure times reduce the time needed to reach a 100% bacterial killing rate. The cytotoxic test also demonstrated that OVCO is non-toxic to normal skin cells. However, despite its strong antibacterial properties, OVO requires the addition of a surfactant as a solubilizing agent to improve its bioavailability. Future studies could focus on enhancing the properties of OVO formulations without chemical surfactants to increase their efficacy further. These findings provide a strong foundation for exploring the pharmaceutical potential of OVO.

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