In-Vitro Antimicrobial, Antibiofilm, Antihemolytic, and Anti-Inflammatory Activities of Ozonated *Ziziphus spina-christi* Oil Supported by GC–MS Analysis

Marwa Yousry A. Mohamed *

Ozonation has emerged as an effective approach to enhance the biological efficacy of plant-derived oils through chemical modification. In this study, crude and ozonated *Ziziphus spina-christi* oils were chemically characterized by gas chromatography–mass spectrometric (GC-MS) analysis and evaluated *in vitro* for their biological activities. GC–MS profiling revealed that the crude oil consisted predominantly of oxygenated monoterpenes and aromatic esters, particularly eucalyptol and methyl salicylate, along with lower proportions of fatty acids and their derivatives. Following ozonation, a marked increase in oxygenated compounds was observed, including epoxidized fatty alcohol esters and alkyl ether alcohols. Ozonated oil showed pointedly enhanced antibacterial activity, minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC), with inhibition zones of 37.0 ± 0.6, 31.0 ± 0.1, 25.0 ± 0.8, and 29.0 ± 0.8 mm against *Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi,* respectively. Ozonated oil inhibited biofilm formation by more than 80% at sub-MBC levels and reduced bacteria-induced hemolysis by up to 98.1% at 75% MIC. Time-kill kinetics confirmed rapid bactericidal effects within 180 min. Moreover, ozonated oil exhibited superior anti-inflammatory activity with an IC_{50} of 2.40 ± 0.09 µg/mL. These findings highlight ozonated *Z. spina-christi* oil as a promising multifunctional bioactive agent.

DOI: 10.15376/biores.21.2.4701-4719

Keywords: Ziziphus spina-christi oils; Ozonization; Antibiofilm activity; Anti-inflammatory impact

Contact information: Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University (IMSIU), P.O. Box 90950 Riyadh 11623, Saudi Arabia;
** Corresponding author: Mymohamed@imamu.edu.sa*

INTRODUCTION

Medicinal plants are an important source of bioactive chemicals with substantial therapeutic potential, especially for the advancement of nontraditional and adjunct biomedical techniques (Alawlaqi *et al*. 2023; Alsalamah *et al*. 2025a). *Ziziphus spina-christi* (L.) Desf. (Rhamnaceae), often known as Christ's thorn jujube or Sidr, is a perennial tree that grows in dry and semi-arid areas of the Middle East, North Africa, and Asia. Botanically, the plant has thorny branches, oval leathery leaves, and drupe-like fruits (Dafni *et al*. 2005; Abdulrahman *et al*. 2022). Its lengthy history of usage in conventional therapies for the management of skin problems, wounds, gastrointestinal issues, and inflammatory conditions demonstrates its pharmacologic significance (Almasri *et al*. 2025; Bolatkyzy *et al*. 2025).

The oil derived from *Z. spina-christi* has sparked significant attention due to its high concentration of fatty acids, terpenoids, sterols, and phenolic molecules (Alla *et al*. 2025). For centuries, this oil has been exploited to treat wounds, protect the skin, and relieve inflammatory symptoms, demonstrating its promise as a multipurpose natural medicinal agent (Kadioglu *et al*. 2016). Plant-derived oils have gained popularity in recent years as part of novel biomedical techniques due to their biocompatibility and extensive biological activity (Qanash *et al*. 2023). *In vitro* studies are critical in this procedure since they are the first stage of assessing bioactivity, health risks, and mechanistic insights before moving on to *in vivo* investigations (Al-Rajhi and Abddelghany 2023).

Ozonation has evolved as a new and successful approach for increasing the biological effectiveness of natural oils (Al-Rajhi *et al*. 2025b). Ozonation produces oxygenated derivatives such as ozonides and peroxides when it reacts with unsaturated fatty acids, which have been linked to better antibacterial and anti-inflammatory properties (Alsalamah *et al*. 2025b). This chemical change provides a promising route for increasing the medicinal properties of natural oils without using synthetic chemicals (de Sousa *et al*. 2023). Gas chromatography-mass spectrometry (GC-MS) is an important analytical technique for assessing these alterations, since it allows for a complete comparison of the chemical profiling of crude and ozonized oils while also correlating compositional variations to biological function (Al-Rajhi *et al*. 2025c).

Microbial infections remain to be a serious health concern, particularly because biofilm formation develops resistance to antimicrobial drugs and leads to chronic and ongoing infections (Sharma *et al*. 2023). Furthermore, many pathogenic bacteria have hemolytic function, which worsens disease severity (Sahu *et al*. 2023). Natural oils have shown promise antibacterial, antibiofilm, and antihemolytic capabilities, suggesting alternatives to traditional treatments (Alsolami *et al*. 2023). In parallel, inflammation underpins a wide range of clinical disorders, and natural oils high in bioactive lipids and antioxidants have been found to be highly effective in moderating inflammatory responses and preventing damage to tissues (Rabbaa *et al*. 2025).

Recent studies, including the work of Alsalamah *et al*. (2026) on ozonated camphor oil, have demonstrated that ozonation can enhance the biological activity of essential oils, particularly in terms of antifungal and anticancer properties. However, important gaps remain in understanding the broader biological implications of this modification, especially regarding antibacterial efficacy against clinically relevant pathogens, inhibition of biofilm formation, and mitigation of bacterial virulence factors such as hemolysis. Furthermore, limited attention has been given to correlating ozonation-induced chemical transformations with multifunctional biological activities or to evaluating the kinetics of antimicrobial action. The current work is among the most recent to correlate ozonation-induced chemical transformations with the inhibition of key bacterial virulence factors such as biofilm formation and hemolysis. These combined methodological and conceptual advances distinguish the present study from previous reports and provide new insights into the multifunctional therapeutic potential of ozonated plant oils. Therefore, the present study aims to address these gaps by integrating GC–MS-based chemical characterization with a comprehensive set of *in vitro* biological assays, including antibacterial, antibiofilm, antihemolytic, and anti-inflammatory evaluations. In addition, time-kill kinetic analysis was employed to provide dynamic insight into bactericidal activity. This combined approach suggests a more holistic understanding of how ozonation improves the

therapeutic potential of plant-derived oils and supports their development as multifunctional bioactive agents.

EXPERIMENTAL

Chemicals and Test Microorganisms

The oil of *Ziziphus spina-christi* utilized in this study was produced using a cold-pressing extraction method and procured from a regional supplier in Saudi Arabia (product code: 004562). All experimental chemicals and reagents were of analytical grade and obtained from Sigma-Aldrich (Saudi Arabia). Bacterial strains used for antibacterial evaluation were kindly supplied by Prof. Tarek Mohamed (Cairo, Egypt). The microbial panel consisted of *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella typhi* (ATCC 6539).

Ozonation of *Z. spina-christi* Oil

Ozone was produced using an electrically driven ozonation generator. A 1.5 L Drechsel flask containing 6.0 mL of *Z. spina-christi* oil was placed in a cooling bath maintained at −7 °C and positioned near the plasma reactor outlet. Ozone gas was introduced into the oil at a controlled flow rate of 0 to 8 L/min for four hours, leading to the formation of a partially solidified material. Upon completion of the ozonation process, the treated oil was removed from the flask, transferred to an airtight container, and stored at 4 °C until further use (Al-Rajhi *et al*. 2024).

Phytochemical Characterization of *Z. spina-christi* Oil and Ozonized Oil by GC–MS

Chemical composition analysis was carried out using a GC-MS system equipped with an Rt-562 capillary column (96.0 m × 0.26 mm × 0.20 µm; PerkinElmer, Germany), an automated sample injector (2410-PerkinElmer, Germany), and a flame ionization detector (FID). Chromatographic signals recorded by the FID were processed using Compass CDS software (PerkinElmer, Germany). Helium was employed as the carrier gas with a split ratio of 97:1. The instrument was operated with a nine-minute pre-run period, achieving equilibrium within 0.6 min. The oven temperature program consisted of two heating stages: an initial increase at 6 °C/min to 215 °C, followed by a second ramp at the same rate up to 292 °C, starting from an initial temperature of 43 °C. Analyses were conducted under programmed heating conditions between 102 and 286 °C, with thermal stabilization reached after 20 min. The carrier gas flow was fixed at 1.1 mL/min (Abdelghany *et al*. 2020).

Assessment of Antimicrobial Activity of Tested Oil Preparations

The antibacterial activity of native *Z. spina-christi* oil and its ozonized derivative was examined *in vitro* using the agar well diffusion assay against selected bacterial strains. Mueller–Hinton agar was used as the growth medium. Bacterial suspensions (100 µL; 1.8 × 10^6 CFU/mL) were uniformly spread over the agar surface. Wells were aseptically prepared using a sterile cork borer, and predetermined volumes of the oil samples were introduced into each well. Gentamicin at a concentration of 0.07 mg/mL was used as the reference antibiotic, while 5% dimethyl sulfoxide (DMSO) served as the negative control.

Bacterial cultures were incubated at 37 °C for 48 h. Antimicrobial effectiveness was quantified by measuring the diameter of the clear inhibition zones formed around the wells (Al-Rajhi *et al*. 2025b).

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

The minimum inhibitory concentration (MIC) values of the investigated samples were established using the broth microdilution method in nutrient-supplemented media. Serial two-fold dilutions were prepared to obtain concentrations ranging from 0.98 to 1000 µg/mL. Aliquots of 100 µL from each dilution were dispensed into 96-well microplates. Fresh microbial suspensions were prepared and standardized to the turbidity of a 1.0 McFarland reference. Each well was inoculated with 2.5 µL of sterile 0.9% NaCl, resulting in a final microbial density of 2.0×10^6 CFU/mL. The microplates were incubated at 38 °C for 72 h for bacterial strains. The MIC values were defined as the lowest concentration that completely suppressed visible microbial growth, as determined by optical assessment. Positive controls (inoculated medium without test samples) and negative controls (test samples without inoculum) were included. For minimum bactericidal concentration (MBC) determination, 100 µL from wells showing total growth inhibition were subcultured onto fresh agar plates. The MBC corresponded to the lowest concentration at which no microbial growth was detected after incubation (Bazaid *et al*. 2025).

Antibiofilm Activity of *Z. spina-christi* Oil Samples

The influence of the tested oil samples on bacterial biofilm formation was investigated using a 96-well microplate assay. Briefly, each well received 300 µL of freshly prepared trypticase soy yeast (TSY) broth inoculated with bacterial cells at sub-lethal concentrations corresponding to 75%, 50%, and 25% of the previously determined MBC values. The final bacterial density was adjusted to 1×10^6 CFU/mL. Control wells contained TSY medium with methanol but lacked plant extracts. The microplates were incubated at 38 °C for 48 h to allow biofilm development. After incubation, bacterial cells were removed by discarding the supernatant, and each well was gently rinsed with sterile distilled water to eliminate non-adherent bacteria. The plates were air-dried for 35 min and subsequently stained with 0.1% crystal violet solution for 15 min at room temperature to visualize biofilm formation. Excess stain was removed by washing with sterile distilled water. Thereafter, 250 µL of 95% ethanol was added to solubilize the dye bound to biofilm cells. Following a 16-min incubation, absorbance was measured at 580 nm using an ELISA microplate reader. Antibiofilm activity was calculated according to the following formula,

$$\text{Antibiofilm activity (\%)} = [(OD_{\text{control}} - OD_{\text{sample}}) / OD_{\text{control}}] \times 100 \quad (1)$$

where OD_{control} represents the optical density of untreated bacterial biofilm and OD_{sample} corresponds to treated wells (Selim *et al*. 2025).

Hemolysis Inhibition Assay

The hemolytic activity of *Z. spina-christi* oil samples at sub-inhibitory concentrations (25.0%, 50.0%, and 75.0% of MIC) was evaluated following the method described (Yahya *et al*. 2022). Bacterial cultures were standardized to an optical density of 0.4 at 595 nm and subsequently centrifuged at 20,000 × g for 24 min in the presence of the designated sub-MIC concentrations or without treatment. Aliquots (500 µL) of the resulting

supernatants were mixed with freshly prepared erythrocyte suspensions (2.0%) in 0.80 mL of saline solution and incubated at 36 °C for 120 min. The mixtures were then centrifuged at 16,000 × g for 10 min at 4 °C.

Complete hemolysis was achieved by treating erythrocytes with 0.1% sodium dodecyl sulfate (SDS) and used as a positive control, while non-hemolyzed erythrocytes incubated in LB broth served as the negative control. All experiments were conducted in triplicate. Hemoglobin release was quantified by measuring absorbance at 545 nm. Hemolytic activity of treated samples was expressed as mean ± standard error relative to untreated controls. The level of hemolysis was calculated using the formula,

$$\text{Hemolysis (\%)} = [(a - b) / (t - b)] \times 100 \tag{2}$$

where a denotes absorbance of the test sample, b represents baseline absorbance of erythrocytes in sterile medium, and t corresponds to total hemolysis induced by 0.10% SDS.

Time-Kill Kinetic Assay

Time-dependent bactericidal activity of the tested oil specimens against examined bacteria was assessed using a kill-kinetics assay. Fresh bacterial cultures were adjusted to a 0.7 McFarland standard and inoculated into broth media to obtain a final concentration of 1.20×10^6 CFU/mL. The cultures were then exposed to MIC levels of the tested oil formulations.

At predetermined time intervals (0, 30, 60, 90, 120, 150, and 180 min), 0.5 mL aliquots were withdrawn, plated onto agar media, and incubated overnight at 38 °C. Control samples consisted of bacterial cultures grown without test compounds. Colony-forming units (CFU) were enumerated at each time point, and reductions in CFU/mL were calculated relative to the initial inoculum. Time-dependent antimicrobial effects were evaluated by monitoring changes in bacterial population dynamics throughout the experiment.

Data interpretation was performed in accordance with standardized protocols to assess the killing efficiency of the tested samples compared with the reference antimicrobial agent (To *et al*. 2026).

Evaluation of Anti-Inflammatory Activity of Tested Oil Formulations

Anti-inflammatory potential was determined using the protein denaturation assay. A total volume of 450.0 µL of a 1.0% aqueous bovine serum albumin (BSA) solution was combined with 50 µL of the test samples at concentrations ranging from 5.0 to 50.0 µg/mL. The pH of the reaction mixture was adjusted to 6.4 using 1.0 N hydrochloric acid. The mixtures were incubated at ambient temperature for 25.0 min, followed by heating at 55 °C for 35 min in a water bath. After cooling to room temperature, absorbance was recorded at 652 nm using a Hitachi 312 Plus spectrophotometer (Japan). Diclofenac sodium was employed as the standard anti-inflammatory drug, while dimethyl sulfoxide (DMSO) was used as the control.

Statistical Analysis

All experimental data are expressed as mean ± standard deviation (SD) from three independent experiments. Statistical comparisons among groups were performed using Student's t-test and one-way analysis of variance (ANOVA). Data analysis was carried out

using GraphPad Prism software version 8 (San Diego, CA, USA). Statistical significance was established at a probability level of $P < 0.05$.

RESULTS AND DISCUSSION

Transformation of Chemical Composition in *Z. spina-christi* Oil after Ozonization

GC–MS analysis of crude *Ziziphus spina-christi* oil identified 14 compounds spanning multiple chemical classes. The dominant constituents were oxygenated monoterpenes and aromatic esters, with eucalyptol (31.5%) and methyl salicylate (22.4%) representing the most abundant compounds. In addition, the oil contained moderate to low levels of fatty acids, fatty acid esters, and long-chain alcohols (Table 1, Fig. 1A).

Table 1. Chemical Composition of Crude *Z. spina-christi* Oil (GC–MS Analysis)

RT (min)	Compound Name	Molecular Weight	Molecular Formula	Area (%)	Class
5.23	Eucalyptol	154	$C_{10}H_{18}O$	31.5	Oxygenated monoterpene
7.41	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)	152	$C_{10}H_{16}O$	6.7	Monoterpene ketone
8.89	Methyl salicylate	152	$C_8H_8O_3$	22.4	Aromatic ester
29.86	17-Octadecynoic acid	280	$C_{18}H_{32}O_2$	13.4	Alkynoic fatty acid
34.12	2-Methyl-Z,Z-3,13-octadecadienol	280	$C_{19}H_{36}O$	2.2	Branched unsaturated fatty alcohol
34.24	12-Methyl-E,E-2,13-octadecadien-1 ol	280	$C_{19}H_{36}O$	2.7	Branched unsaturated fatty alcohol
34.88	1,2-15,16-Diepoxyhexadecane	254	$C_{16}H_{30}O_2$	1.8	Diepoxy alkane
34.98	11,14-Eicosadienoic acid, methyl ester	322	$C_{21}H_{38}O_2$	2.3	Polyunsaturated fatty acid methyl ester
40.23	Undec-10-ynoic acid, hexadecyl ester	406	$C_{27}H_{50}O_2$	1.7	Fatty acid alkynyl ester
40.51	9-Hexadecenoic acid	254	$C_{16}H_{30}O_2$	2.2	Monounsaturated fatty acid
40.58	Z-8-Methyl-9-tetradecenoic acid	240	$C_{15}H_{28}O_2$	1.8	Branched monounsaturated fatty acid
41.08	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	312	$C_{20}H_{40}O_2$	3.7	Alkyl ether alcohol
41.48	12-Methyl-E,E-2,13-octadecadien-1 ol	280	$C_{19}H_{36}O$	4.8	Branched unsaturated fatty alcohol
41.77	9-Octadecadienoic acid (Z)-	282	$C_{18}H_{34}O_2$	2.8	Polyunsaturated fatty acid

Although present at lower relative abundances, unsaturated fatty acids and their derivatives remain functionally important due to the presence of reactive carbon–carbon double bonds. Following ozonation, the number of detected compounds increased to 16, accompanied by a pronounced shift toward oxygenated species, including epoxidized fatty alcohol esters and alkyl ether alcohols (Table 2, Fig. 1B). Concurrently, the relative abundance of several native unsaturated fatty acids decreased. These changes are attributed to the preferential reaction of ozone with unsaturated bonds, leading to oxidative cleavage and formation of oxygen-rich derivatives such as epoxides, peroxides, and esterified products. As a result, the ozonated oil exhibits increased chemical diversity and oxygen content, transforming it from a mixed-composition oil into a system enriched with functionally active oxygenated compounds. This ozonation-induced transformation converts the oil from a lipid-rich, highly unsaturated matrix into a chemically modified oil enriched with oxygenated and potentially bioactive compounds in the same line with other reports (Radzimierska-Kaźmierczak *et al*. 2021; Vieira *et al*. 2025). Furthermore, the present findings are consistent with published work on examined ozonated oils, which report reduced unsaturation and increased formation of oxygen-rich compounds associated with enhanced biological activities (Dominguez Lacueva *et al*. 2025; Sathwik *et al*. 2025).

Table 2. Chemical Composition of Ozonated *Z. spina-christi* Oil (GC–MS Analysis)

RT (min)	Compound Name	Molecular Weight	Molecular Formula	Area (%)	Class
26.83	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	4.8	Fatty acid
28.96	Oleic Acid	282	$C_{18}H_{34}O_2$	2.2	Fatty acid
29.86	9,12-Octadecadienoic acid (Z,Z)-	280	$C_{18}H_{32}O_2$	16.1	Fatty acid ester
34.13	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	354	$C_{21}H_{38}O_4$	2.2	Fatty acid ester
34.25	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyc lopropyl]methyl]-, methyl ester	334	$C_{22}H_{38}O_2$	2.3	Fatty acid ester
34.87	17-Octadecynoic acid	280	$C_{18}H_{32}O_2$	2.8	Fatty acid
40.23	Cholestan-3-ol, 2-methylene-, (3α,5α)	400	$C_{28}H_{48}O$	2.7	Sterol
40.50	9-Octadecadienoic acid (Z)-	436	$C_{25}H_{40}O_6$	9.4	Fatty acid
41.14	12-Methyl-E,E-2,13-octadecadien-1 ol	280	$C_{19}H_{36}O$	4.6	Unsaturated fatty alcohol
41.71	9,12,15-Octadecatrienoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl]et hyl ester, (Z,Z,Z)	436	$C_{25}H_{40}O_6$	6.3	Polyunsaturated fatty acid ester
41.87	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	268	$C_{16}H_{28}O_3$	7.6	Epoxidized unsaturated fatty alcohol ester
43.39	1-Heptatriacotanol	536	$C_{37}H_{76}O$	8.3	Long-chain fatty alcohol
43.95	Z,Z-3,15-Octadecadien-1-ol acetate	308	$C_{20}H_{36}O_2$	3.4	Unsaturated fatty alcohol ester
44.18	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy	444	$C_{30}H_{52}O_2$	.25	Epoxidized polycyclic hydrocarbon
44.92	13-Heptadecyn-1-ol	252	$C_{17}H_{32}O$	4.7	Alkynol
45.13	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	312	$C_{20}H_{40}O_2$	19.5	Long-chain alkyl ether alcohol

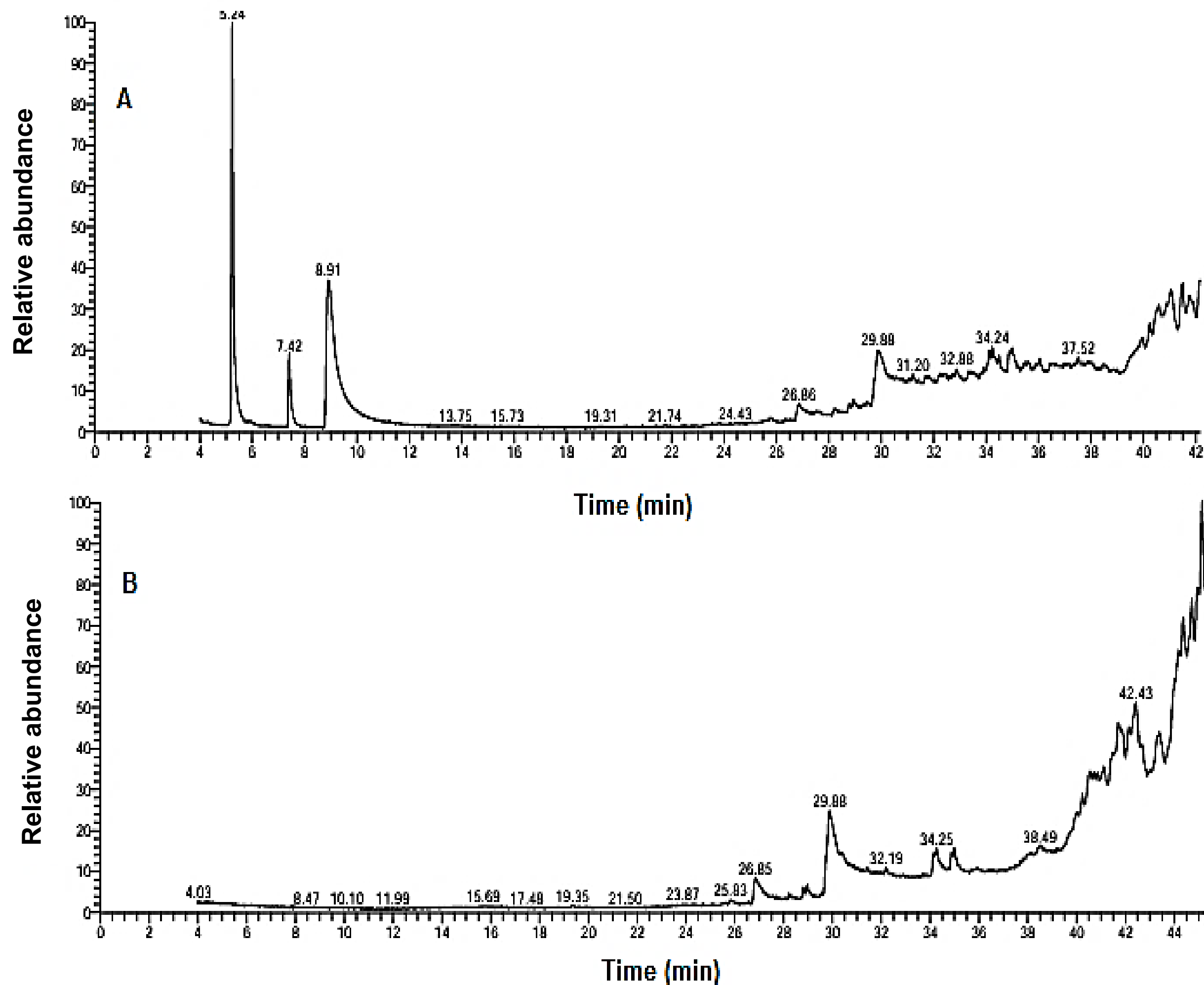

Fig. 1. Distribution of various molecules with different classes: (A) Crude *Z. spina-christi* oil; and (B) After ozonation

Although GC–MS analysis confirmed significant compositional changes following ozonation, the present study did not include standard physicochemical parameters such as peroxide value, FT-IR spectroscopy, or viscosity measurements. These analyses are commonly used to quantify the degree of ozonation and assess process reproducibility. Therefore, the extent of oxidation in this study is inferred primarily from compositional shifts rather than direct oxidation indices. Future studies should incorporate these measurements to provide a more comprehensive evaluation of ozonated oil properties and ensure process standardization.

Antibacterial Potential and MIC, and MBC of Crude and Ozonized Oil

The antibacterial assay demonstrated that ozonized *Ziziphus spina-christi* oil exhibited enhanced activity compared to the crude oil against both Gram-positive and Gram-negative bacteria. Among Gram-positive strains (*Bacillus subtilis* and *Staphylococcus aureus*), the inhibition zones increased from 33 ± 1 and 27 ± 0.2 mm to 37 ± 0.6 and 31 ± 0.1 mm, respectively. Similarly, for Gram-negative bacteria (*Pseudomonas*

aeruginosa and *Salmonella typhi*), the inhibition diameters rose from 18 ± 0.5 and 21 ± 0.8 mm to 25 ± 0.8 and 29 ± 0.8 mm, respectively, following ozonation (Fig. 2 and Table 3).

The crude *Z. spina-christi* oil showed moderate inhibitory effects, which can be attributed to its native lipid constituents, particularly unsaturated fatty acids and fatty acid esters identified by GC–MS analysis. These molecules exert antibacterial effects primarily through partial disruption of bacterial cell membranes; however, their efficacy is limited, especially against Gram-negative bacteria with more complex cell envelope structures. In contrast, the enhanced antibacterial impact of the ozonized oil correlates strongly with the presence of oxygenated and bioactive compounds such as epoxidized fatty alcohol esters, alkyl ether alcohols, eucalyptol, and methyl salicylate. These oxygen-rich derivatives are reported to increase membrane permeability, induce oxidative stress, and interfere with essential cellular processes, resulting in broader and more effective antibacterial activity (Khwaza *et al*. 2025). Additionally, the observed outcomes are in the same line with previous investigations on ozonated oils, which report improved antimicrobial efficacy following ozonation (Slavinskienė *et al*. 2024).

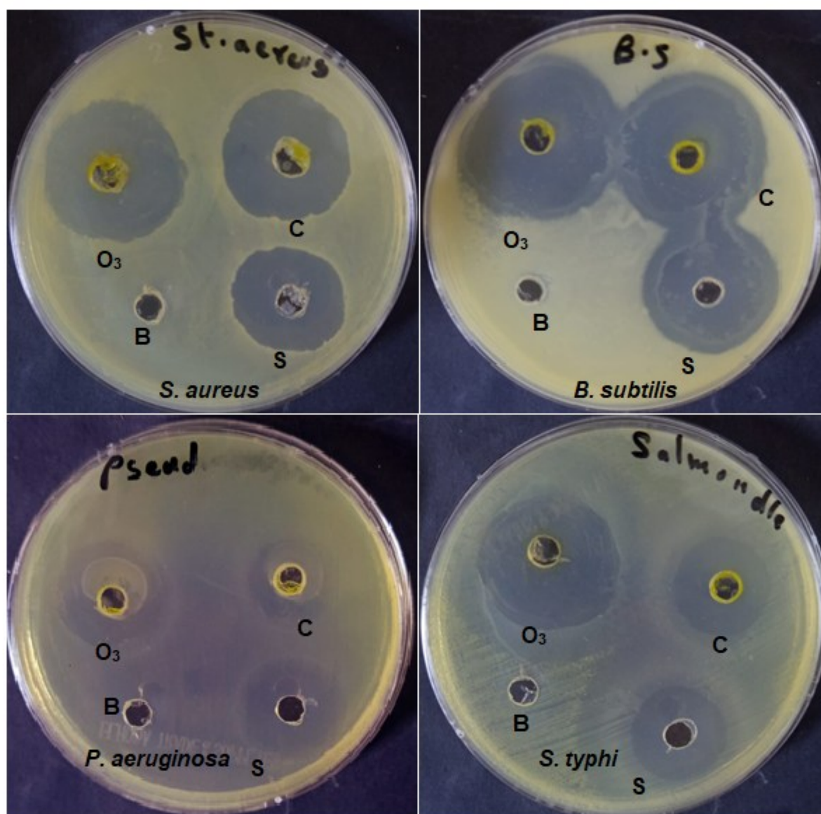

Fig. 2. Antibacterial action of *Z. spina-christi* oil using agar diffusion technique *versus S. aureus, B. subtilis*, *P. aeruginosa* and *S. typhi*). [C: crude oil; O_3: Ozonized oil; B: Blank, S: Standard drug]

The MIC and MBC results revealed that ozonized *Z. spina-christi* oil had a significantly higher antibacterial potency ($P \leq 0.05$) than crude oil. The crude oil had a higher MIC and MBC value, indicating that greater quantities were required to suppress bacterial growth and achieve bactericidal activity (Table 4). These data demonstrated modest antibacterial activity of crude oil and bacteriostatic characteristics at lower levels. In contrast, ozonized oil had significantly decreased MIC and MBC values over both

Gram-positive and Gram-negative bacteria, indicating increased bactericidal activity. These findings are consistent with the GC-MS profile of the ozonized oil, which showed higher quantities of oxygenated chemicals capable of damaging bacterial membranes and increasing cell death (Augello *et al*. 2024). Similar reductions in MIC and MBC levels after ozonation have been documented in the literature, which are attributed to improved oxygen functionality and the generation of reactive lipid derivatives (Qanash *et al.* 2025). The current results were supported by similar studies but on other oils. Al-Rajhi *et al.* (2025c) reported that the tested bacteria were more effectively inhibited by ozonized mustard oil (OMO), which exhibited MIC values ranging from 15.6 to 62.5 µg/mL and MBC values from 15.6 to 125 µg/mL. In comparison, non-ozonized mustard oil (NOMO) showed higher MIC (31.25 to 125 µg/mL) and MBC (62.5 to 500 µg/mL) values against *E. faecalis, S. aureus*, *K. pneumoniae*, and *S. typhi*, indicating lower antibacterial potency. As another example, Al-Rajhi *et al.* (2024) reported that ozonated black seed oil exhibited superior MIC (7.8 to 15.62 µg/mL) and MBC (7.8 to 31.25 µg/mL) values compared with non-ozonated oil against multiple pathogenic strains, confirming that ozonation consistently enhances the antimicrobial activity of plant-derived oils.

Table 3. Antibacterial Action (mm) of *Z. spina-christi* Oil Types *versus* Tested Bacteria

Tested Bacteria	Crude Oil	Crude Oil + O_3	Standard Drug (Gentamicin)
B. subtilis	33±1	37±0.6	30±0.8
S. aureus	27±0.2	31±0.1	24±1.0
P. aeruginosa	18±0.5	25±0.8	23±0.5
S. typhi	21±0.8	29±0.8	20±0.6

The outcomes are tabulated as means ± SD

Table 4. MIC and MBC Levels of *Z. spina-christi* Oil Types for Examined Bacteria

Tested Bacteria	MIC Levels		MBC Levels	
	Crude Oil	Crude Oil +O_3	Crude Oil	Crude Oil +O_3
B. subtilis	31.25 ±0.4^{a}	15.62±0.2^{b}	62.5±0.2^{a}	31.25±0.2^{b}
S. aureus	31.25 ±0.2^{a}	15.62±0.3^{b}	31.25±0.3^{a}	15.62±0.1^{b}
P. aeruginosa	62.5 ±0.3^{a}	15.62±0.4^{b}	125±0.2^{a}	31.25±0.3^{b}
S. typhi	31.25±0.2^{a}	15.62±0.2^{b}	62.5±0.4^{a}	31.25±0.4^{b}

The results are reported as means ± SD; various letters above numbers refer to significant difference where $P \leq 0.05$

The Action of Ozonation on Antibiofilm Activity of the Tested Oils

The antibiofilm results demonstrated that ozonized *Z. spina-christi* oil exhibited higher biofilm inhibition compared to the crude oil against both Gram-positive and Gram-negative bacteria (Figs. 3 and 4). The crude *Z. spina-christi* oil showed moderate antibiofilm activity, which can be attributed to its native lipid constituents, particularly unsaturated fatty acids and fatty acid esters identified by GC–MS analysis. These compounds may partially interfere with bacterial adhesion and early biofilm formation; however, their effectiveness against established biofilms is limited. In contrast, ozonized oil demonstrated higher antibiofilm efficacy, which is directly related to the compositional alterations reported in the GC-MS results.

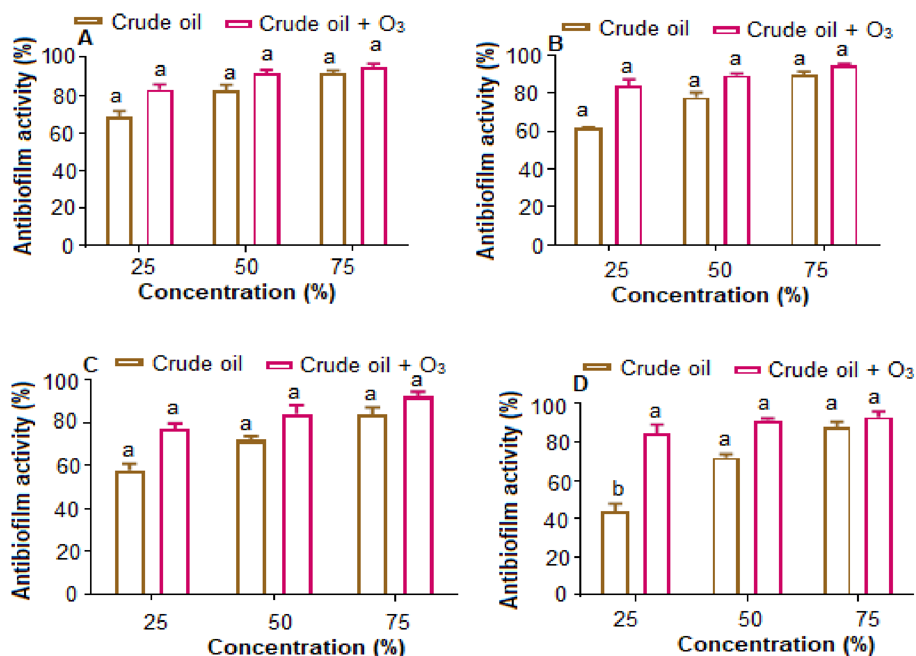

Fig. 3. Antibiofilm activity of crude and ozonized *Z. spina-christi* oil towards Gram-positive and Gram-negative bacteria: (A) B. *subtilis* (B) *S. aureus*, (C) *P. aeruginosa*, and (D) *S. typhi*. The outcomes are shown as means ± SD; various letters indicate significant difference where P ≤ 0.05

Ozonation resulted in the production of oxygenated and bioactive molecules such as epoxidized fatty alcohol esters, alkyl ether alcohols, eucalyptol, and methyl salicylate, which have been shown to disrupt biofilm architecture via a variety of mechanisms. Regarding the effect on Gram-positive bacteria, ozonized oil most likely disrupts cell wall integrity and reduces extracellular polymeric substance formation, thereby weakening biofilm structure (Silva *et al*. 2020**)**. Regarding the impact on Gram-negative bacteria, the oxygenated compounds have been shown to promote membrane permeability, disrupt quorum sensing, and collapse the extracellular biofilm matrix, lowering biofilm formation and permanence. The results obtained are consistent with previously published research on ozonated oils, which found that increased oxygen functionality improves antibiofilm action towards both Gram-positive and Gram-negative bacteria by triggering oxidative stress, influencing communication between cells, and influencing biofilm stability (Pinto *et al*. 2025). According to Al-Rajhi *et al.* (2025c), at 25% of the MIC, NOMO caused lower biofilm inhibition (44.4%, 64.4%, 14.3%, and 16.4%) compared to OMO, which achieved higher inhibition rates of 79.7%, 91.2%, 93.7%, and 19.3% against *faecalis, S. aureus, K. pneumoniae, and S. typhi*, respectively

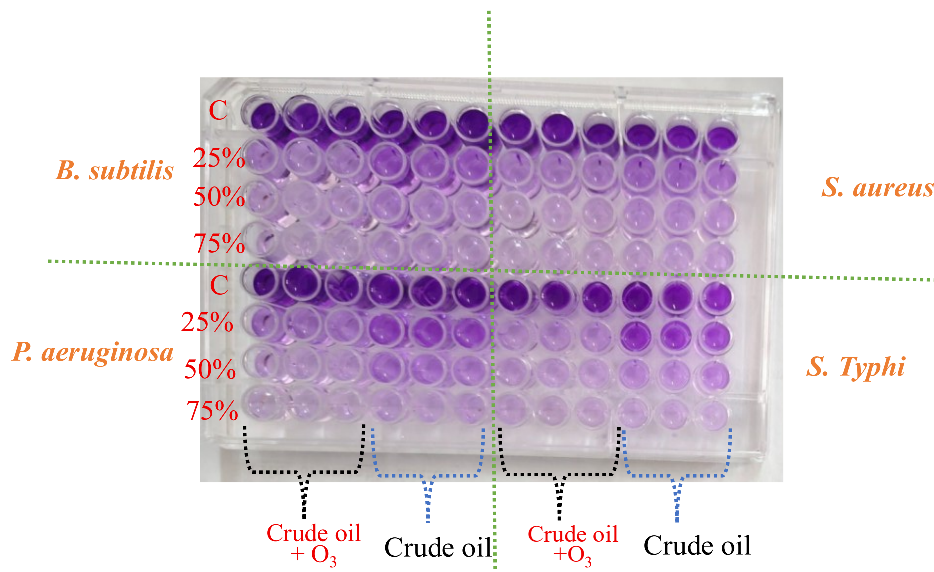

Fig. 4. Antibiofilm action of Z. *spina-christi* cured oil, and ozonized form towards *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *S. typhi* at different sub-inhibitory MBC levels (25%, 50%, and 75%) in 96 well plats.

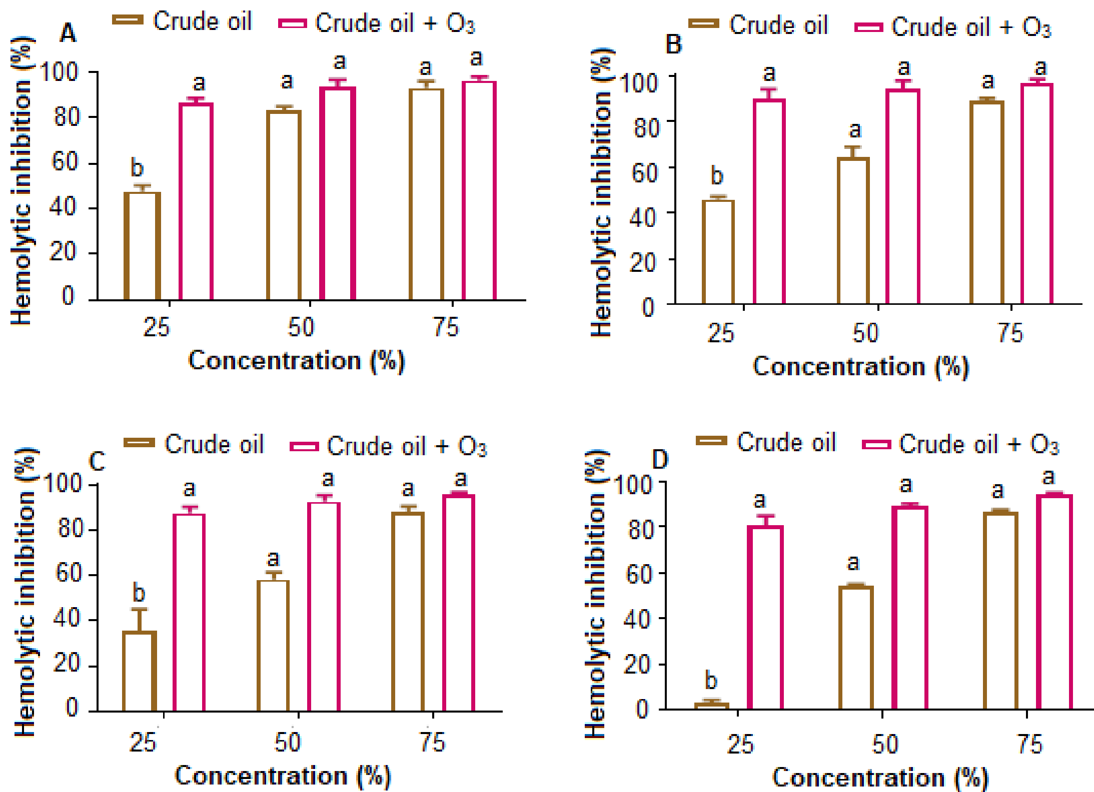

Fig. 5. Anti-hemolytic impact of crude and ozonized *Z. spina-christi* oil at different concentrations % of oil MIC in presence of various bacteria: (A) B. *subtilis* (B) *S. aureus*, (C) *P. aeruginosa*, and (D) *S. typhi* (Data are drawn as means ± SD; different small letter refers to significant difference where P ≤ 0.05)

The Role of Ozonation on Anti-Hemolytic Action of the Oil Forms

The antihemolytic testing revealed that ozonized *Z. spina-christi* oil slightly outperformed crude oil in protecting erythrocytes toward bacteria-induced hemolysis across all tested bacterial strains (Figs. 5 and 6).

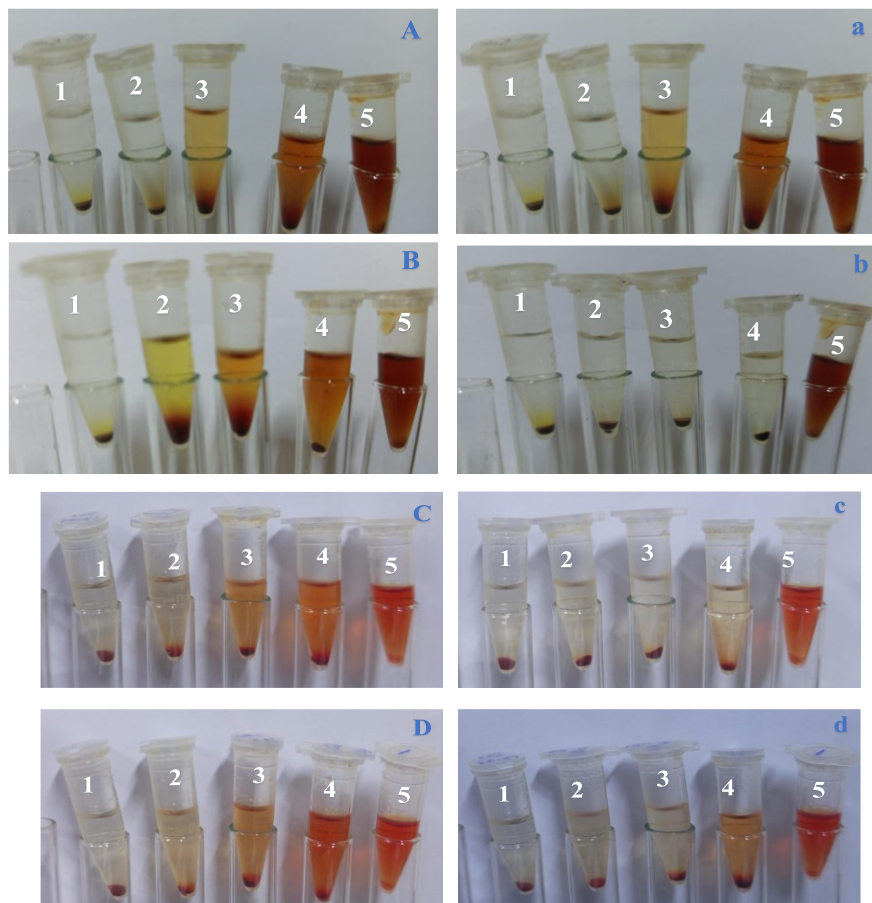

Fig. 6. Antihemolytic activity of Z. *spina-christi* oil (Cure oil – Capital letters), ozonized oil (Small-case letters) in presence of various bacteria: (A, a) *B. subtilis* (B) *S. aureus*, (C) *P. aeruginosa*, and (D) *S. Typhi* at different sub-inhibitory levels where 1: 1/4 MIC, 2: 1/2 MIC, 3: 3/4 MIC, 4: Positive Control, and 5: Negative Control)

The crude oil has moderate preventive effects, owing to its high amount of unsaturated fatty acids detected by GC-MS, which can partially maintain erythrocyte membranes but are still susceptible to bacterial toxins and oxidative damage, as illustrated in previous work (Rahim *et al*. 2025). In the presence of Gram-positive bacteria such as *S. aureus* and *B. subtilis*, ozonized *Z. spina-christi* oil had more antihemolytic effect than crude oil. This improvement can be attributed to oxygenated chemicals determined by GC-MS, including eucalyptol, methyl salicylate, and epoxidized fatty derivatives, which are known to break bacterial cell walls and limit hemolysin synthesis. According to published studies, ozonated oils are more efficient versus Gram-positive bacteria owing to their simplest cell wall structure, which reduces toxin-mediated erythrocyte degradation (Silva *et al*. 2020; Bazaid *et al*. 2025). Regarding Gram-negative bacteria, both oils provided less protection than Gram-positive strains as *P. aeruginosa* and S. *typhi*; however, the ozonized oil revealed significantly increased ($P \leq 0.05$) antihemolytic activity especially upon using

25% of MIC of the oil. The occurrence of alkyl ether alcohols and epoxidized esters in ozonized oil is thought to raise membrane permeability and interfere with bacterial outer membranes, lowering lipopolysaccharide-induced oxidative stress. This finding is consistent with previous research demonstrating that ozonated oils may partially counteract the innate resistance of Gram-negative bacteria *via* oxidative processes (Grandi *et al*. 2022).

Time–Kill Kinetic Activity Against Gram-Positive and Gram-Negative Bacteria

The time-kill kinetic analysis found that ozonized *Z. spina-christi* oil had a faster and more pronounced bactericidal effect than crude oil toward both Gram-positive and Gram-negative bacteria. The crude *Z. spina-christi* oil demonstrated a steady decline in the number of bacteria over time, indicating a slow bactericidal effect. This moderate action is due to its native lipid contents, specifically unsaturated fatty acids detected by GC-MS analysis, which have been shown to damage bacterial membranes to a small amount but require sustained contact to accomplish considerable death, as reported by Sharmin *et al*. (2025). In contrast, ozonized sider oil showed a rapid, time-dependent drop in relevant bacterial counts, indicating increased bactericidal activity.

Table 5. Time–Kill Kinetic of Crude *Z. spina-christi* Oil against Tested Gram-Positive and Gram-Negative Bacteria at Different Time Points

Time (min)	***B. subtilis*** (CFU)	***S. aureus*** (CFU)	***P. aeruginosa*** (CFU)	***S. typhi*** (CFU)
0	52×10^5 ±1.4	33×10^5±0.2	57×10^5±0.6	45×10^5±0.8
30	49×10^4±1.0	55×10^4±0.5	37×10^4±2.0	94×10^4±1.2
60	66×10^3± 0.7	34×10^3±0.4	212×10^3±1.4	153×10^3±1.0
120	39×10^2 ±0.1	42×10^2 ±1.5	117×10^2±0.2	93×10^2 ±2.0
150	102×10 ±1.0	94×10^2 ±0.9	113×10±0.1	53×10 ±0.6
180	25±0.4	112	128±1	145
360	0	0	0	5

Results are recoded as means ± SD.

Table 6. Time–Kill Kinetic of Ozonized *Z. spina-christi* Oil towards Examined Gram-Positive and Gram-Negative Bacteria at Various Time Intervals

Time (min)	***B. subtilis*** (CFU)	***S. aureus*** (CFU)	***P. aeruginosa*** (CFU)	***S. typhi*** (CFU)
0	52×10^5 ±1.4	33×10^5±0.2	57×10^5±0.4	45×10^5±0.8
30	114×10^3±1.0	38×10^3±1	28×10^4±1.0	11×10^4±0.7
60	31×10^2± 0.8	27×10^2±1.0	125×10^2±1	23×10^3±1
120	127 ±1.0	134±0.6	232±1	32×10±1
150	0	35	23	151
180	0	0	0	0
360	0	0	0	0

Results are recoded as means ± SD.

This increased killing efficiency is directly related to the GC-MS profile of the ozonized oil, which indicated higher quantities of oxygenated and bioactive chemicals such

as epoxidized fatty alcohol esters, alkyl ether alcohols, eucalyptol, and methyl salicylate. These chemicals are known to cause oxidative stress, enhance membrane permeability, and disrupt critical cellular processes, resulting in bacterial cell death. Against Gram-positive bacteria, ozonized oil is anticipated to damage the peptidoglycan layer more efficiently, resulting in a rapid loss of cell viability. Against Gram-negative bacteria, oxygenated substances can penetrate or disrupt the outer membrane, bypassing inherent resistance and increasing bactericidal activity (Tables 5 and 6).

The determined time-kill kinetics are comparable with previous research on ozonated oils, which resulted in faster bacterial killing rates and better efficacy due to higher oxygen functionality and the generation of reactive lipid derivatives. These investigations show that ozonated oils act on several targets, lowering the potential of bacterial persistence over time (Ugazio *et al*. 2020; Gulcin 2025).

The Impact of Ozonation on Anti-Inflammatory Activity of the Examined Oil Types

According to the anti-inflammatory assay, the ozonized *Z. spina-christi* oil exhibited slightly higher activity with IC_{50}= 2.40±0.09 µg/mL than the crude oil and showed comparable efficacy relative to the standard drug which had an IC_{50}= 2.09±0.02 µg/mL. The moderate activity (IC_{50}= 3.34±0.07 µg/mL) of the crude oil is mainly attributed to its high content of unsaturated fatty acids, such as oleic and linoleic acid derivatives (Fig. 7). In contrast, the enhanced anti-inflammatory effect of the ozonized oil correlates well with the GC–MS results, which revealed increased levels of oxygenated and bioactive compounds, including eucalyptol, methyl salicylate, epoxidized alcohol esters, and alkyl ether alcohols.

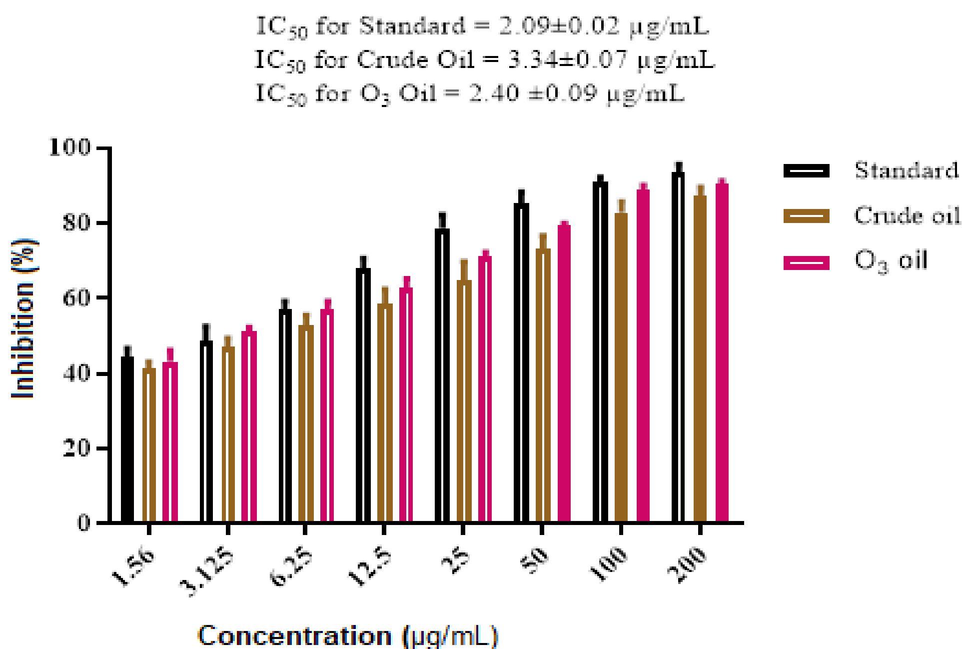

Fig. 7. Anti-inflammatory efficacy of crude and ozonized *Z. spina-christi* oil in comparison with a standard anti-inflammatory drug (Data are drawn as means ± SD.)

These findings are consistent with published reports on prepared ozonated oils, where increased oxygen functionality and formation of reactive derivatives were associated

with improved anti-inflammatory properties, confirming that ozonation enhances the therapeutic potential of *Z. spina-christi* oil (Cho *et al*. 2023).

CONCLUSIONS

1. This study has provided new insight into how ozonation alters the functional properties of *Ziziphus spina-christi* oil by inducing targeted chemical transformations that directly influence its biological performance. GC–MS analysis demonstrated that ozonation reduced the degree of unsaturation in the native oil and promoted the formation of oxygenated derivatives, including epoxides, esters, and ether-linked alcohols.
2. The findings revealed that the increased abundance of oxygenated compounds played a central role in improving antibacterial efficacy, accelerating bactericidal kinetics, and inhibiting biofilm formation. In addition, the enhanced anti-inflammatory activity suggested a dual functional role in both infection control and modulation of host inflammatory responses.

ACKNOWLEDGEMENTS

This work was supported and funded by the Deanship of Scientific Research at Imam Mohammad Ibn Saud Islamic University (IMSIU) (grant number IMSIU-DDRSP2601)

REFERENCES CITED

Abdelghany, T. M., Hassan, M. M., El-Naggar, M. A. (2020). "GC/MS analysis of *Juniperus procera* extract and its activity with silver nanoparticles against *Aspergillus flavus* growth and aflatoxins production," *Biotechnol Rep (Amst).* 27, article e00496. https://doi.org/10.1016/j.btre.2020.e00496

Abdulrahman, M. D., Zakariya, A. M., Hama, H. A., Hamad, S. W., Al-Rawi, S. S., Bradosty, S. W., and Ibrahim, A. H. (2022). "Ethnopharmacology, biological evaluation, and chemical composition of *Ziziphus spina-christi* (L.) Desf.: A review," *Adv Pharmacol Pharm Sci.* 2022, article 4495688. https://doi.org/10.1155/2022/4495688

Alawlaqi, M. M., Al-Rajhi, A. M. H., Abdelghany, T. M., Ganash, M., Moawad, H. (2023). "Evaluation of biomedical applications for linseed extract: Antimicrobial, antioxidant, anti-diabetic, and anti-inflammatory activities *in vitro*," *J. Funct. Biomater.*14(6), article 300. https://doi.org/10.3390/jfb14060300

Alla, C., Ali, A., Mehiou, A., Salhi, Y., Bouanani, N., Legssyer, A., and Ziyyat, A. (2025). "Phytochemical composition of *Ziziphus lotus* (L.) Lam and its impact on the metabolic syndrome: A review," *Adv. Pharmacol. Pharm. Sci.* 2025, article 8276090. https://doi.org/10.1155/adpp/8276090

Almasri, R. S., Bedir, A. S., and Al Raish, S. M. (2025). "Comprehensive ethnopharmacological analysis of medicinal plants in the UAE," *Nutrients* 17(3), article 411. https://doi.org/10.3390/nu17030411

Al-Rajhi, A. M. H., Abdelghany, T. M., Almuhayawi, M. S., Alruhaili, M. H., Saddiq, A. A., Baghdadi, A. M., Al Jaouni, S. K., Albasri, H. M., Waznah, M. S., Alraddadi, F. A., and Selim, S. (2024). "Effect of ozonation on the phytochemicals of black seed oil and its biological activities *in vitro*," *Sci. Rep.* 14(1), article 30445. https://doi.org/10.1038/s41598-024-81157-9

Al-Rajhi, A. M. H., Abdelghany, T. M., Selim, S., Almuhayawi, M. S., Alruhaili, M. H., Alharbi, M. T., and Al Jaouni, S. K. (2025a). "Phytochemical characterization of peanut oil and its ozonized form," *AMB Express* 15(1), article 76. https://doi.org/10.1186/s13568-025-01849-x

Al-Rajhi, A. M. H., Alsalamah, S. A., Qanash, H., Bazaid, A. S., Aldarhami, A., Felemban, H. R., Gattan, H. S., AlZamil, N. M., Daws, D., and Abdelghany, T. M. (2025b). "Biochemical description of ozonized propolis and its biological activities," *PLoS One* 20(9), article e0332224. https://doi.org/10.1371/journal.pone.0332224

Al-Rajhi, A. M., and Ghany, T. A. (2023). "Nanoemulsions of some edible oils and their antimicrobial, antioxidant, and anti-hemolytic activities," *BioResources* 18(1), 1465-1481. https://doi.org/10.15376/biores.18.1.1465-1481

Al-Rajhi, A. M. H., Alsalamah, S. A., and Mashlawi, A. M. (2025c). "Assessment of mustard oil and its ozonated form for antimicrobial and antioxidant potential," *Appl. Biol. Chem.* 68, article 84. https://doi.org/10.1186/s13765-025-01058-x

Alsalamah, S. A., Alghonaim, M. I., Ismail, Mashraqi, A., and Abdelghany, T. M. (2025b). "Innovative strategy for extraction of green cardamom *via* supercritical fluid extraction," *Bioresour*. *Bioprocess*. 12, article 146. https://doi.org/10.1186/s40643-025-00951-z

Alsalamah, S. A., Alghonaim, M. I., Mohammad, A. M., Khalel, A. F., Alqahtani, F. S., and Abdelghany, T. M. (2025a). "Ozone-modified properties of pumpkin seed oil as a therapeutic agent," *Sci. Rep.* 15(1), article 25959. https://doi.org/10.1038/s41598-025-11123-6

Alsalamah, S. A., Almotayri, A. M., Alghonaim, M. I., Dawoud, R. A., Fageehi, A., Kashmiry, A. A., Kafy, S. M. and Selim, S. (2026). "Ozone driven modulation of camphor oil–chemical composition, anti-yeast potential, and anti-ovarian cancer mechanistic exploration," *BioResources* 21(2), 4246-4262. https://doi.org/10.15376/biores.21.2.4246-4262

Alsolami, A., Bazaid, A. S., Alshammari, M. A., Qanash, H., Amin, B. H., Bakri, M. M., and Abdelghany, T. M. (2023). "Ecofriendly fabrication of jojoba nanoemulsion and its biological activities," *Biomass Conv. Bioref*. https://doi.org/10.1007/s13399-023-05162-0

Augello, S., Cameli, V., Montanari, A., Tacconi, S., Uccelletti, D., Dini, L., and Schifano, E. (2024). "The antifungal potential of ozonated extra-virgin olive oil against *Candida albicans*," *Biomolecules* 14(11), article 1472. https://doi.org/10.3390/biom14111472

Bazaid, A. S., Binsaleh, N. K., Barnawi, H., Alharbi, B., Alsolami, A., Selim, S., Al Jaouni, S. K., Saddiq, A. A., Ganash, M., Abdelghany, T. M., and Qanash, H. (2025). "In vitro activity of *Euphorbia trigona* extracted *via* supercritical fluid extraction," *Bioresour. Bioprocess.* 12(1), article 28. https://doi.org/10.1186/s40643-025-00855-y

Bolatkyzy, N., Shepilov, D., Turmanov, R., Berillo, D., Vassilina, T., Ibragimova, N., Berganayeva, G., and Dyusebaeva, M. (2025). "Medicinal plants for skin disorders:

Phytochemistry and pharmacology," *Molecules* 30(15), article 3281. https://doi.org/10.3390/molecules30153281

Cho, K. H., Kim, J. E., Bahuguna, A., and Kang, D. J. (2023). "Ozonated sunflower oil exerted potent anti-inflammatory activities," *Antioxidants* 12(8), article 1625. https://doi.org/10.3390/antiox12081625

Dafni, A., Levy, S., and Lev, E. (2005). "The ethnobotany of *Ziziphus spina-christi* in Israel," *J. Ethnobiol. Ethnomed*. 1, 8. https://doi.org/10.1186/1746-4269-1-8

de Sousa, D. P., Damasceno, R. O. S., Amorati, R., Elshabrawy, H. A., de Castro, R. D., Bezerra, D. P., Nunes, V. R. V., Gomes, R. C., and Lima, T. C. (2023). "Essential oils: Chemistry and pharmacological activities," *Biomolecules* 13(7), article 1144.

Dominguez Lacueva, P., Corella Guillamón, P., and Cantalejo Díez, M. J. (2025). "Changes in quality parameters and antimicrobial activity of ozonated olive oils," *Foods* 14(6), article 999. https://doi.org/10.3390/foods14060999

Grandi, G., Cavallo, R., Zanotto, E., Cipriani, R., Panico, C., Protti, R., Scapagnini, G., Davinelli, S., and Costagliola, C. (2022). "*In vitro* antimicrobial activity of ozonated oil in liposome eyedrop," *Open Med*. 17(1), 1057-1063. https://doi.org/10.1515/med-2022-0495

Gulcin, İ. (2025). "Antioxidants: A comprehensive review," *Arch. Toxicol.* 99, 1893-1997. https://doi.org/10.1007/s00204-025-03997-2

Kadioglu, O., Jacob, S., Bohnert, S., Naß, J., Saeed, M. E., Khalid, H., Merfort, I., Thines, E., Pommerening, T., and Efferth, T. (2016). "Anti-inflammatory activity of *Ziziphus spina-christi*," *Phytomedicine* 23(3), 293-306. https://doi.org/10.1016/j.phymed.2016.01.004

Khwaza, V., and Aderibigbe, B. A. (2025). "Antibacterial activity of selected essential oil components," *Antibiotics* 14(1), article 68. https://doi.org/10.3390/antibiotics14010068

Pinto, N. V., Viana, M. C., Fernandes, F. A. S., Fletes-Vargas, G., Rodríguez-Rodríguez, R., Borges, G. S. M., and Carneiro, G. (2025). "Ozonized vegetable oils as bioactive compounds," *Ozone: Sci. Eng*. 1-15. https://doi.org/10.1080/01919512.2025.2552726

Qanash, H., Alsalamah, S. A., Bazaid, A. S., Alghonaim, M. I., Duhduh, A., and Hudani, I. (2025). "Therapeutic potential of ozonated *Ocimum basilicum*," *Pharmaceuticals* 18(8), article 1223. https://doi.org/10.3390/ph18081223

Qanash, H., Alotaibi, K., Aldarhami, A., Bazaid, A. S., Ganash, M., Saeedi, N. H., and Ghany, T. A. (2023). "Effectiveness of oil-based nanoemulsions with molecular docking," *BioResources* 18(1), 1554-1576.

Rabbaa, S., Bouchab, H., Tahri-Joutey, M., Laaziouez, Y., Limami, Y., Pires, V., Nasser, B., Andreoletti, P., Cherkaoui-Malki, M., and El Kebbaj, R. (2025). "Protective antioxidant potential of argan oil," *Int. J. Mol. Sci.* 26(17), article 8300. https://doi.org/10.3390/ijms26178300

Radzimierska-Kaźmierczak, M., Śmigielski, K., Sikora, M., Nowak, A., Plucińska, A., Kunicka-Styczyńska, A., and Czarnecka-Chrebelska, K. H. (2021). "Olive oil with ozone-modified properties," *Molecules* 26(11), article 3074. https://doi.org/10.3390/molecules26113074

Rahim, M. A., Uzma, M., Al-Asmari, F., Koraqi, H., Ramadan, M. F., Castro-Muñoz, R., and Zongo, E. (2025). "Omega fatty acids: Extraction, stability, and health benefits," *Food Sci. Nutr.* 13(10), article e71008. https://doi.org/10.1002/fsn3.71008

Slavinskienė, G., Grigonis, A., Ivaškienė, M., Sinkevičienė, I., Andrulevičiūtė, V., Ivanauskas, L., Juodžentė, D., Ramanauskienė, K., and Daunoras, G. (2024). "Chemical properties and antibacterial activity of ozonated oils," *Vet. Sci.* 11(4), article 161. https://doi.org/10.3390/vetsci11040161

Sathwik, M., Komarraju, S., D. S., and Muralidharan, S. (2025). "Technical considerations of ozonated oils in medical applications," *Cureus* 17(4), article e83185. https://doi.org/10.7759/cureus.83185

Sahu, T., Pande, B., Verma, H. K., Bhaskar, L. V. K. S., Sinha, M., Sinha, R., and Rao, P. V. (2023). "Infection and childhood mortality in sickle cell disease," *Thalassemia Rep.* 13(3), 206-229. https://doi.org/10.3390/thalassrep13030019

Selim, S., Abdelghany, T. M., Almuhayawi, M. S., Nagshabandi, M. K., Tarabulsi, M. K., Elamir, M. Y. M., Alharbi, A. A., and Al Jaouni, S. K. (2025). "Biosynthesis and activity of Zn-MnO nanocomposite," *Sci. Rep.* 15(1), article 2032. https://doi.org/10.1038/s41598-024-85005-8

Sharma, S., Mohler, J., Mahajan, S. D., Schwartz, S. A., Bruggemann, L., and Aalinkeel, R. (2023). "Microbial biofilm: Formation, resistance, and control," *Microorganisms* 11(6), article 1614. https://doi.org/10.3390/microorganisms11061614

Sharmin, S., Muzahid, A. A., Islam, M. M., Yeasmin, M. S., Dey, A. K., Uddin, M. J., Rana, G. M. M., Barmon, J., Alam, S., Bhuiyan, M. N. H., and Ahmed, N. U. (2025). "GC-MS profiling and antibacterial activity of *Litchi chinensis* seed," *Scientifica* 2025, article 7644558. https://doi.org/10.1155/sci5/7644558

Silva, V., Peirone, C., Amaral, J. S., Capita, R., Alonso-Calleja, C., Marques-Magallanes, J. A., Martins, Â., Carvalho, Á., Maltez, L., Pereira, J. E., Capelo, J. L., Igrejas, G., and Poeta, P. (2020). "Efficacy of ozonated oils on MRSA biofilms," *Molecules* 25(16), article 3601. https://doi.org/10.3390/molecules25163601

To, S. W., Sani, M. H., Malek, N. A. N. N., Susanto, H., Endharti, A. T., Permatasari, H. K., and Zhang, T. (2026). "Phytochemical analysis of *Bauhinia kockiana* and antibacterial synergy," *Appl. Biochem. Biotechnol*. 198(3), 1869-1898. https://doi.org/10.1007/s12010-025-05497-6

Ugazio, E., Tullio, V., Binello, A., Tagliapietra, S., and Dosio, F. (2020). "Ozonated oils as antimicrobial systems in topical applications," *Molecules* 25(2), article 334. https://doi.org/10.3390/molecules25020334

Vieira, M. H., Boreski, D., Franzen Matte, B., Arias, J. L. d. O., Júnior, C. M., Bauab, T. M., Gabriel, S. A., and Chin, C. M. (2025). "Standardization and antimicrobial mechanisms of ozonized sunflower oil," *Int. J. Mol. Sci*. 26(18), article 9156. https://doi.org/10.3390/ijms26189156

Yahya, R., Al-Rajhi, A. M. H., Alzaid, S. Z., Al Abboud, M. A., Almuhayawi, M. S., Al Jaouni, S. K., Selim, S., Ismail, K. S., and Abdelghany, T. M. (2022). "Aloe vera gel based on chitosan nanoparticles: Molecular docking and efficacy," *Polymers* 14(15), article 2994. https://doi.org/10.3390/polym14152994

Article submitted: January 13, 2026; Peer review completed: March 21, 2026; Revised version received: April 1, 2026; Accepted: April 2, 2026; Published: April 13, 2026.
DOI: 10.15376/biores.21.2.4701-4719