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Research Article

# Evaluation of Wound Healing Activity of Ozonated Linseed and Sunflower Oils

Aljanzeer Roshan[1], Nayal Ream[1*], Abajy Mohammad Yaser[2], Alhaj Sakur Amir[3]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, University of Aleppo, Syria.*
[2] *Department of Biochemistry and Microbiology, Faculty of Pharmacy, University of Aleppo, Syria.*
[3]*Department of Food and Analytical chemistry, Faculty of Pharmacy, University of Aleppo, Syria.*

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**ABSTRACT**
This study evaluated the effect of ozonation on chemical properties of linseed and sunflower oils as well as the therapeutic effects of topical ozonated oils on cutaneous wound healing in rats. The ozone gas was pumped into both oils till the peroxide value reached 600 mmol/kg. The acidity value and the IR spectroscopy were determined for both oils before and after ozonation. Longitudinal wounds were made in the skin of rats and treatment with oils was applied once a day till cure for each group, the length of the wound was measured daily, and healing time was determined. Results showed an increase in acidity value and peroxide value, as well as a decrease of the bands corresponding to both C=C and =C–H stretching and increase in the band corresponding to ozonide C-O stretching. The two ozonated oils promoted wound healing and their therapeutic efficacy was better than the positive control. Further study is needed to evaluate the safety of long using of ozonated oils.

**Keywords**: ozone, wounds, linseed oil, sunflower oil, rats.

## INTRODUCTION

Ozone is normally present as a gas; it's made up of three atoms of oxygen with a cyclic structure[1]. Ozone was used to treat diseases since 1914; it was used in World War I for the treatment of gaseous gangrene[2]. Wounds are considered as one of the most inevitable accidents in our life, they occur as a result of physical or chemical factors. The natural response to heal the wound starts immediately after tissue injury.This process include many complex phases, such as hemostasis, inflammation, re-epithelization, granulation tissue formation and the late remodeling phase of repair[3]. These events include coordination of a many types of cells and proteins in addition to the presence of endogenous growth factors that play an important regulatory role to coordinate healing process[4]. Ozone is one of the best antimicrobial agents[5,6] and has been used clinically as a therapeutic agent for many chronic wounds such as diabetic wounds[5,7,8]. Ozone, as a gas, is unstable and cannot be stored. Its half-life is 40 minutes at 20℃[1], and ozonated water has a half-life of about 10 hours at room temperature and five days in the fridge[2], but ozonated oils has a longer half-life because ozone gas remains as ozonid between the double bound in the unsaturated fatty acids of these oils[2,6,9]. Since ozone does not penetrate cell but react with unsaturated fatty acids to form reactive oxygen species (ROS), therefore, it is possible to consider the ozonated oils as an effective way to deliver ozone messengers to the skin[6]. Linseed oil is considered as one of the richest plant oil of fatty acids, which could play an important role in wound healing as they interfere in the composition of the cell membrane[10]. This study aimed to determine the quality of ozonized linseed oil and sunflower oil through analytical methods such as peroxide and acidity values and *to* confirm the structural changes undergone by oils during the ozonation using IR techniques. As well as to evaluate the topical therapeuticeffect of linseed oil, and sunflower oil, before and after ozonationon wound healing in rats.

## MATERIALS AND METHODS

*Solvents and Reagents*

glacial acetic acid (Panreac®), Chloroform (SCP®), Potassium iodide (SRL®), Sodium thiosulfate (BDH®), Starch (HIMEDIA®), Ether (SCP®), Potassium hydroxide (PROLABO®). Phenolphthalein, Ethanol, Sunflower oil and Linseed oil were purchased from local market. all other chemical were of analytical grad. Mebo® ointment (beta-sitosterol 0.2% and plant extracts), UNIPHARMA pharma.Ind.,Syria, Syria under license from BGCMI

*Animals*

36wister rats (18 male and 18 female), aged 4 months and weight (200-250 g) were used in this study, experiments were performed under the standard conditions of temperature (22 ± 3)℃ and 12h light/dark cycle. Animals were fed with aspecial rat's diet with *ad libitum* access to water.

*Oil ozonation*

$O_3$/oxygen mixture was bubbled by using a generator medical ozone (BOZON SPE ECONIKA®), within a glass

**Author for Correspondence: reamnayal@gmx.net*

Table 1: peroxide value and acidity value before and after ozonationprocess of linseed and sunflower oils.

| The oil | Peroxide value mmol/Kg | Acidity value mg KOH/g |
|---|---|---|
| Sunflower oil | 23 ± 2.4 | 1.962 ± 0.28 |
| Ozonatedsunflower oil | 590 ± 12 | 7.293 ± 0.56 |
| Linseed oil | 63 ± 3 | 1.793 ± 0.11 |
| Ozonatedlinseed oil | 624 ± 21 | 16.265 ± 0.561 |

Values are expressed as mean ± SD, n = 6

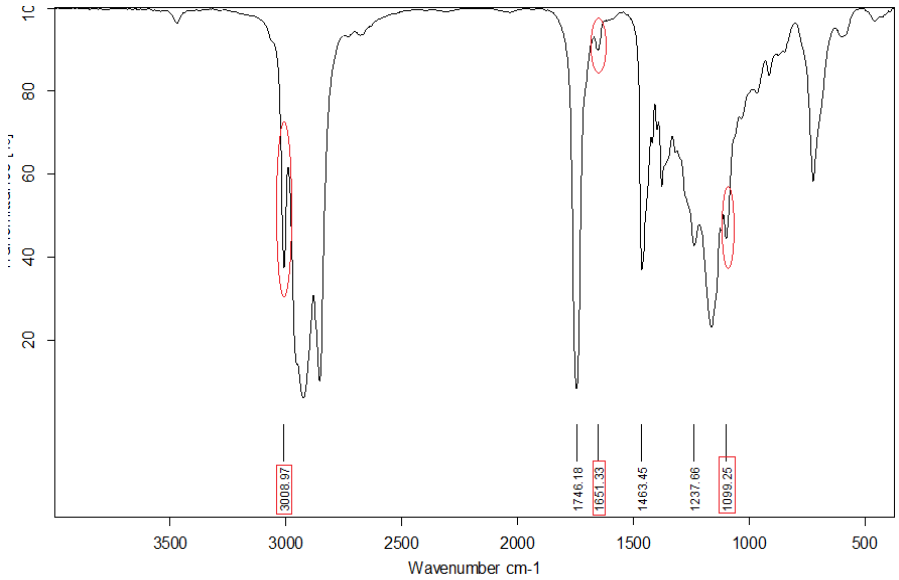



Figure 1: Infrared (IR) spectrum for sunflower oil.

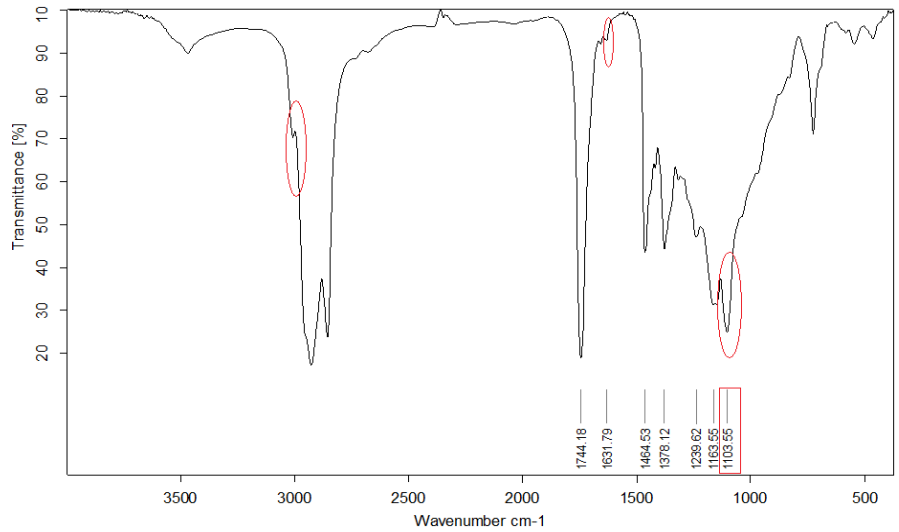



Figure 2: Infrared (IR) spectrum forozonated sunflower oil.

tube containing 90 ml of either linseed oil or sunflower oil, $O_3$ flow-rate was kept constant at 200 ml /min and $O_3$ concentration was 60 mg/l.

Pumping process was stopped when the peroxide value reached 600 meq/kg sample for both oils.

*Analytical study of the oil*

*Peroxide Value*[11]

Peroxide value represents the quantity of peroxide expressing in milliequivalentsof active $O_2$ contained in 1000 g of the sample. To determine this value, 0.1 g of the sample was dissolved in amixture of glacial acetic acid and chloroform (3: 2). After the iodometric titration, the peroxide value (PV) was calculated from the equation:

PV = 10 (V1-V2) / W

Where V1: volume (ml) for thiosulfate titrant used for the sample

V2: volume (ml) for thiosulfate titrant used for the blank

W (g): sample weight.

*Acidity value*[12]

This index expresses, in mg, the quantity of potassium hydroxide required to neutralize the free acids presents in 1 g of the substance.

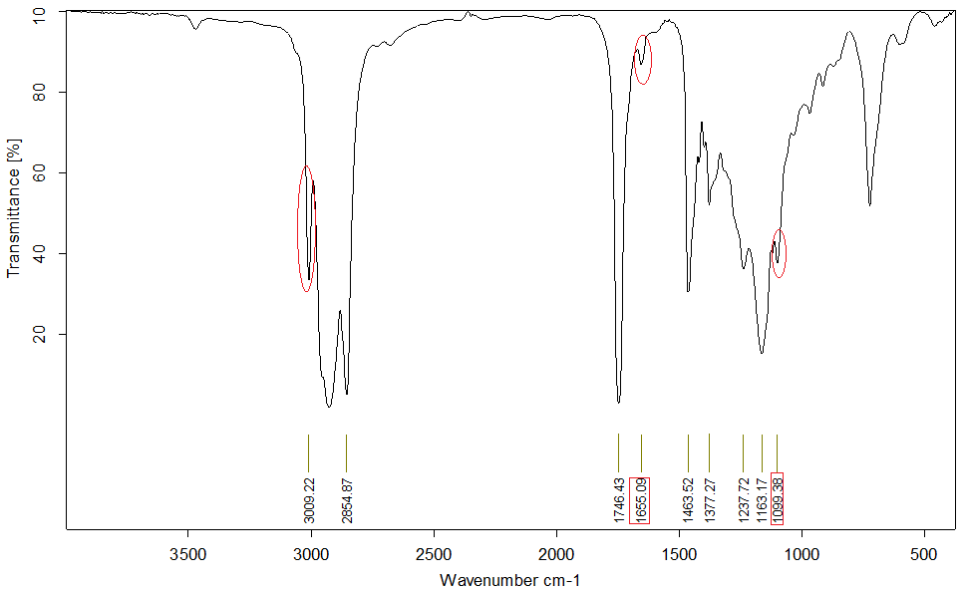



Figure 3: Infrared(IR) spectrum of linseed oil.

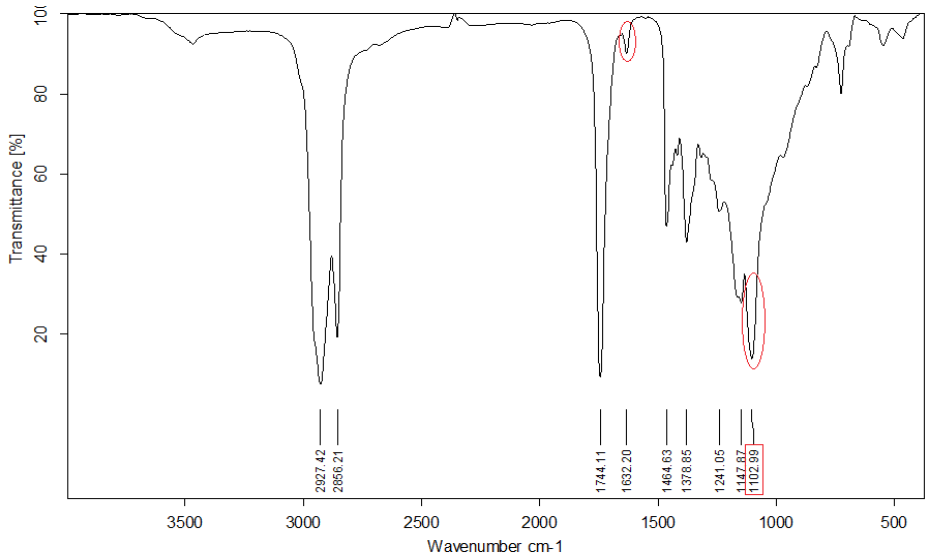



Figure 4: Infrared(IR) spectrum ofozonated linseed oil.

To determine this value, 1 g of oil was dissolved in a mixture containing 50% Ether + 50% ethanol. Titration was done using 0.1 M potassium hydroxide in the presence of phenolphthalein until the pink color appeared, this value (AV) was calculated from the following equation:

AV = 5.610 n / m

Where: n (ml): Volumeof potassium hydroxide used during titration

m (g): Sample weight.

*IR Spectroscopy*[9]

2 µl of sample was sandwiched between two KBr disks particular for IR spectrum with avoiding of air bubbles formation. The percent transmittance (T %) was measured in the range (800 – 4000) $cm^{-1}$.

*in vivo study*

*Incision wound model*

Hair of the lumbar regionof each rat was shaved, thenrats were anesthetized using intramuscular ketamine at a dose of 5mg /$kg^2$. After sterilizing the area with alcohol, a longitudinal incision of 1cm length and 0.2 mm depth was made, the wound was left undressed.

*Topical application of oils*

Treatment has begun immediately after making the incision. Animals were divided into six groups of six members (3males +3 females) in each, to distinguish these groups, rat tail were colored using non-toxic dyes:

Group 1: positive control group (treated with Mebo® ointment)

Group 2: negative control group (without treatment)

Group 3: was treated with sunflower oil.

Group 4: was treated with ozonated sunflower oil.

Group 5: was treated with linseed oil.

Group 6: was treated with ozonated linseed oil.

Oils were applied topically once a day.

*Assessment of wound healing activity*

The length of wound was measured starting from the fifth day, and the time needed for wound healing was recorded.

*Statistical study*

Table 2: length of the wound (cm) for each group from the fifth day until complete healing.

| Groupe | Day 5 | Day 6 | Day 7 | Day 8 | Day9 | Day10 | Day 11 | Day 12 | Day 13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.9± 0.122 | 0.720± 0.075 | 0.560± 0.114 | 0.470± 0.148 | 0.370± 0.148 | 0.220± 0.228 | 0.100± 0.141 | 0.000 | 0.000 | 0.0000 |
| 2 | 1.00± 0.000 | 0.816± 0.076 | 0.760± 0.052 | 0.716± 0.076 | 0.633± 0.028 | 0.566± 0.057 | 0.516± 0.076 | 0.366± 0.057 | 0.233± 0.057 | 0.133± 0.115 |
| 3 | 0.93± 0.075 | 0.837± 0.047 | 0.712± 0.118 | 0.687± 0.103 | 0.600± 0.115 | 0.475± 0.095 | 0.400± 0.081 | 0.337± 0.094 | 0.100± 0.115 | 0.0000 |
| 4 | 0.72± 0.061 | 0.458± 0.091 | 0.200± 0.228 | 0.116± 0.183 | 0.083± 0.132 | 0.000 | 0.0000 | 0.000 | 0.0000 | 0.0000 |
| 5 | 0.95± 0.057 | 0.812± 0.025 | 0.695± 0.052 | 0.537± 0.062 | 0.412± 0.094 | 0.275± 0.095 | 0.100± 0.115 | ±0.050 0.100 | 0.0000 | 0.0000 |
| 6 | 0.68± 0.054 | 0.391± 0.049 | 0.158± 0.174 | 0.033± 0.081 | 0.000 | 0.000 | 0.0000 | 0.000 | 0.0000 | 0.0000 |

n =6, values are expressed as mean ± SD, 1: positive control group, 2:negative control group , 3:sunflower oilgroup, 4:ozonated sunflower oil group, 5: linseed oil group, 6:ozonated linseed oil group.

Table 3: healing time for each group.

| Group | Healing period (days) |
|---|---|
| 1 | 11 ± 1.00 * |
| 2 | 14.67 ± 0.577 # |
| 3 | 13.5 ± 0.577 # |
| 4 | 8.17 ± 1.47 * # |
| 5 | 11.5 ± 0.577 * |
| 6 | 7.67 ± 0.816 * # |

Results have been expressed as mean ± SD. One way ANOVA was used to analyze the results, where the value of $P \leq 0.05$ was considered an indication of a statistical difference.

## RESULTS

*Analytical results of the oils*

Results of determination of peroxide value (PV) and the acidity value (AV) showed an increase in bothvalues after the ozonation process. As shown in table1, the peroxide valuewas increased from 23 mmol / Kg to 590 mmol/ Kg for sunflower oil and from 63 to 624 mmol / Kg for linseed oil. The acidity value was increased from 1.962mgKOH / g to 7.293 mg KOH / g and from 1.793 to 16.265 mg KOH / g for sunflower oil and linseed oil, respectively.

Result of Infra red (IR) spectroscopy Measurement of IR spectrum showed that the band corresponding to the double bound carbon-carbon stretching for sunflower oil (which appeared at 1651$cm^{-1}$) Figure (1) and linseed oil (which appeared at 1655$cm^{-1}$) Figure (3) was decreased after ozonation process (Fig.2 and 4). Also, the band corresponding to =C–H stretching for sunflower oil and linseed oil which appeared at 3009 $cm^{-1}$ (Fig.1& 2) was decreased after oil ozonation.

On the other hand, C-O stretching region for both oils which appeared at 1099 $cm^{-1}$ (Fig. 1 and 2) was increased in ozonized oils and appeared at 1102$cm^{-1}$ (Fig. 3 and 4)

*Results of In vivo study*

*Wound length*

No significant difference was observed in wound length among the groups before the fifth day of incision. Inthe sixth day a decrease of 40% was noticed in wound length in the groups treated with ozonated linseed and ozonated sunflower oils. This decrease in length continued so quickly until healing, table 2. However, the decrease in wound length was less than in the rest groups.

*Healing period*

Results in table 3 showed that theshortest healing time was for the ozonated linseed oil group (7.67 days), followed by the ozonated sunflower oil group, (8.17) days.

When comparing the results of all groups with negative group, we noticed that sunflower oil has no sufficient effectiveness. Also, when comparing the results with the positive control it could be noticed that the effectiveness of linseed oil was equal to mebo ointment (positive control).

Values are mean±SD, #P ≤0.05 comparing with Positive control, *P ≤ 0.05 comparing with Negative group. (1) Positive group, (2) Negative group, (3) Sunflower oil, (4) Sunflower ozonated oil, (5) Linseed oil, (6) Linseed ozonated oil.

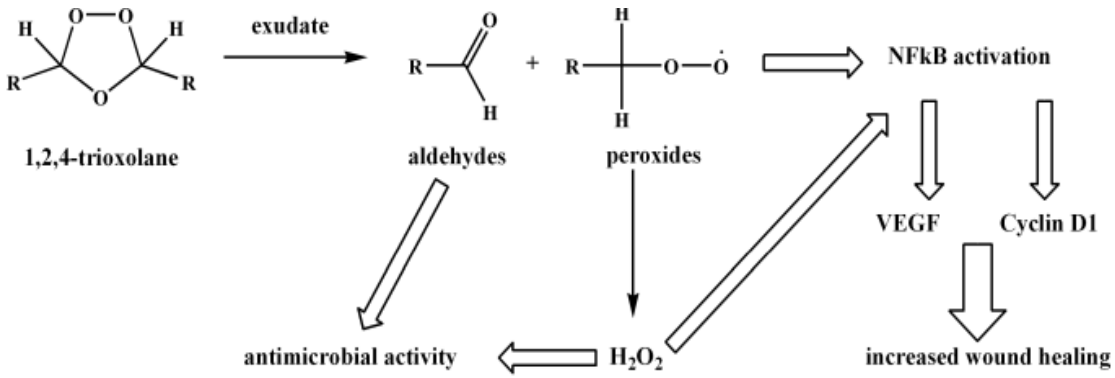
## DISCUSSION

The reaction of ozone with plant oils often occurs with the carbon-carbon double bound present in unsaturated fatty acids [9,13,14,16]. This interaction leads to formation of many oxygenated compounds, such as hydroperoxid, ozonide, aldehydes, peroxides, diperoxides and polyperoxide[6,14,15].

In this study Peroxide value was used as control of the ozonation process because of its simplicity and rapidity. Whereas, the acidity value which is representative of the acidity level of the oil was used as an index of the degradation by-products that could be formed during the ozonation process.

The peroxide value after oil ozonation was increased due to the formation of peroxide compounds, but someof these compounds undergone decomposition and yield other compounds such as carboxylic acids which resulting in increasing the acidity value[14].

The increase in peroxide and acidity values was higher in ozonized linseed oil in compare to sunflower oil because linseed oil has a higher proportion of unsaturated fatty acids. The acidityvalue was in linseed oil 2 fold more than that of sunflower oil, this indicates that the decomposition of peroxidic compounds to acid is very high in the former oil.




IR spectroscopy was used to highlight differences in the functional groups during the oil ozonation (9). Our results showed that the band corresponding to the double bound carbon-carbon C=C and=C–H stretching for both oils was decreased after oil ozonation, while the band corresponding to ozonide CO stretching for both oils was increased.

The band corresponding to the double bound carbon-carbonof linseed oil was more clear in the IR spectrum than Sunflower oil because the later. haslower proportion of unsaturated fatty acids[17].

As known ozonized oils represent an interesting pharmaceutical approach to the management of a variety of dermatological disorders such as wounds. Wound healing is a critical process in the skin and it has known to be affected by oxidative stress. (9)therefore, In this study the effectiveness of ozonated oils was more than the positive control.

Wound healing process has three basic phases[18]

Phase I: indicates the inflammation stage (2–3 days).

PhaseII: corresponds to the intermediate phase and normally lasts about 2 weeks represented by the synthesis of extracellular matrix and an active proliferation of fibroblasts and keratinocytes.

Phase III: also called remodelingphase, needs month or years to occur. in this phase the scar is transformed to the final mature healed wound.

Previous studied have showed that the beneficial effects of ozoanted oils on wound healingin the first stage might be assumed to be due to decreased bacterial infection. Also, exposing these wounds to ozonated oil leads to activation of NFkB transcription factor, which in turn controls the growth factors genes such as vascular endothelial growth factor (VEGF) and CyclinD1 which play a fundamental role in wounds healing in the second stage[6].

This study showed that the linseed oil possessed wound healing activity that is becauseit is one of the richest sources of fatty acids such as α-Linoleic acid which constitutes (44-57%) of the total quantity of fatty acids, and linolenicacid(15-29%)[10]. These two fatty acids are considered as important constituents of cell membrane and responsible for its protection. So they can help in the wounds healing process[10,17]. Linseed oil also contains antioxidants such as Tocopherol, carotenoids, and phenolic compounds which stimulate the natural process for wounds healing[10].

## CONCLUSION

Ozonation of linseed and sunflower oils occured on carbon-carbon double bound. The peroxide and acid values were increased in both oils but they were higher in ozonized linseed oil. Linseed oil showed wound healing activity whereas sunflower oil did not. Both ozonated oils have similar wound healing activity, which is better than the positive control.

Further studies on the development of pharmaceutical formula for topical application for these oils is needed.

## ACKNOWLEDGMENT

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