Formulation of oleozon with *Phaleria macrocarpa* and *Cinnamomum burmanii* extract for diabetic wound treatment

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Abstract. In this work, the effect of ozonation on coconut oil and mixture of coconut oil and olive oil was studied. The properties of ozonated oils (oleozon) were analytically tested by the method of iodine number, acid number, peroxide number, and FT-IR as general chemical substances. Ozonation may increase the peroxide and acid number for both oils but decrease the iodine number. The best ozonation condition has been seen from an increase of 277.52% acid number, peroxide number about 114.77 meq O₂²⁻/kg oil, and decrease of iodine number up to 22%. Furthermore, ozonated oils were mixed with herbal extract and be tested the diabetic wound healing ability through antibacterial activity test. A mixture of 160 mL coconut oil that ozonated for 72 hours and 0.18 gram herbal extracts with n-hexane solvent showed the highest inhibition zone of 18.3 mm in *Staphylococcus aureus* bacteria.

1. Introduction

One of the most potentially dangerous complications in people with diabetes is diabetic wounds. High sugar levels in the blood induce to the growth of anaerobic bacteria, resulting an infection in the diabetic wound so that it becomes difficult to recover and get worse called gangrene or diabetic ulcers.

The ozonation of vegetable oils may produce a substance having the function of antiseptic against bacteria which cause skin diseases [4]. Olive oil is the most widely used oil as an ozonated vegetable oil, this is due to the high content of unsaturated fatty acids. In addition, studies on olive oil show the ability to heal skin wounds [8]. However, the price of olive oil is expensive and olive oil does not come from Indonesia, so research on the ability of other ozonated vegetable oil derived from Indonesia was carried out. Coconut oil is chosen because of its relatively cheap price and its huge availability in Indonesia. In addition, the content of lauric acid and capric acid in coconut oil is able to accelerate wound healing and reduce infection [7].

The reaction of ozone with vegetable oils produces Criegee ozonide, hydroperoxide, and aldehyde products [3]. In addition, mixing vegetable oil with natural ingredients can also increase the effectiveness of the oil. Saponins in *Phaleria macrocarpa* and sinamaldehid in *Cinnamomum burmanii* are also known to have antibacterial effects [2,5].

There are three goals to be achieved from this research. The first goal is to get the best ozonation condition on vegetable oil based on its physical and chemical properties. Second, to get the oleozon composition with *Phaleria macrocarpa* and *Cinnamomum burmanii* extract which is effective in healing diabetic wound based on their antibacterial effect. Third, to give better ozonation condition on vegetable oil and herbal extract for diabetic wound treatment.

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compare the ability of coconut oil and olive oil in healing diabetic wound based on their bacterial disinfection capabilities.

2. Experimental section

2.1. Materials
Materials used in this research are coconut oil (Barco), olive oil (D’VIRGIN), *Phaleria macrocarpa*, *Cinnamomum burmannii*, isopropyl alcohol and *n*-hexane solvents, material for the determination of iodine number, material for the determination of acid number, material for the determination of peroxide number, and material for the determination of antibacterial activity.

2.2. Instrumentation
Equipment used in this research are ozonator, reactor, stirrer, chiller, laboratory glassware for the extraction and determination of iodine, acid, peroxide number, analytical balance, FT-IR spectrometer, LC-MS spectrometer, and equipment for the determination of antibacterial activity.

2.3. Procedure

2.3.1. Oil ozonation and herbal extraction. This study uses the main component of ozonator as an ozone-producing tool to apply in the ozonation process of vegetable oil mixture. The ozonation reaction of the oil will occur in a batch reactor equipped with a 4 L/min air flow rate and a coolant to keep the reaction temperature at 25 °C. At this stage, the ozonation of vegetable oil has been done continuously for 72 hours to see the relationship between the length of time ozonation with the resulting antibacterial effects. The vegetable oil mixture will be ozonated with variation of oil concentrations.

After oil ozonation, herbal extraction then performed. This stage aims to produce herbal plant extracts from *Phaleria macrocarpa* and *Cinnamomum burmannii*. These two herbs will be macerated using isopropyl alcohol (IPA) and *n*-hexane solvents for 5 days and stirring every 4 hours. After obtaining the extract by evaporating, the extract will be mixed with the ozonated vegetable oil to add antibacterial properties.

2.3.2. Characterization of oleozon. The test method used iodine number, acid number, peroxide number, and FT-IR spectrum. Iodine number is used to know the amount of double bond in oil. Acid number to indicate the amount of free fatty acids present in the oil. Peroxide number to show the level of oxidation of ozone to fatty acids. Then, FT-IR spectrum is used to find out the differences in functional groups that increase or decrease before and after the ozonation.

2.3.3. Characterization of compound in herbal extract. The test method used Liquid Chromatography–Mass Spectrometry (LC-MS). This test was performed to identify the compounds contained in the *Phaleria macrocarpa* and *Cinnamomum burmannii* extract.

2.3.4. Antibacterial activity determination. In this stage, ozonated vegetable oil with herbal extracts is put into the culture of *Staphylococcus aureus* bacteria that have been prepared before. The ability of bacterial disinfection qualitatively seen in the form of visual observations by looking at the diameter of the inhibit zone formed. The test was conducted by using the paper disc method.

3. Result and Discussion

3.1. Iodine number
Iodine number can be used to determine the content of double bonds in vegetable oils. The success of ozonation is marked by a decrease of the iodine number obtained. Based on Table 1 it can be seen that
on all variations of oil mixture the oil ozonation for 72 hours has decreased the iodine number. It can be inferred that the ozonator succeeds in attacking and lowering the double bond on pure coconut oil and also a mixture of coconut and olive oil.

The decrease of iodine number occurs because ozone reacts with double bonds in unsaturated fatty acids in vegetable oils. This reaction is called the Criegee reaction that produces ozonide, hydroperoxide, aldehydes, peroxides, dioxide, and polyperoxide [3]. The highest decrease in iodine number is in the ozonation process of pure coconut oil, which changes up to 22%. This is due to coconut oil is MCFA (Medium Chain Fatty Acid) which more responsive or faster to receive ozone than olive oil. Thus, coconut oil requires a faster ozonation time than olive oil.

Table 1. Iodine numbers for pure and oils ozonated after 72 h.

| Oil                                | Iodine number (g iod/100 g oil) | % Change |
|------------------------------------|---------------------------------|----------|
| Untreated oil                      | 2.06                            |          |
| 72 h ozonated oil                  | 1.61                            | 21.84    |
| Coconut oil                        | 3.45                            | 1.45     |
| Mix of coconut oil and olive oil (85:15) | 4.85                            | 8.86     |
| Mix of coconut oil and olive oil (75:25) | 4.42                            |          |

3.2. Acid number

The acid number indicates the degree to which the triglycerides in the oil have broken down to release free fatty acids. The success of ozonation is marked by an increase of the acid number obtained. Based on the available data in Table 2, it can be seen that the acid numbers of oil after the ozonation have increased. It means that the ozonation in this research was successful.

The increase in acid number is triggered by increased peroxide decomposition and oxidation of aldehydes to carboxylic acids during the ozonation reaction. The highest increase in acid number is in the ozonation process of pure coconut oil, which changes up to 277.52%. This is proportional to the results obtained in the iodine number, where the highest iodine number decline belongs to pure coconut oil. The content of polyphenols and tocopherols in olive oil that serves as an antioxidant slows or prevents other molecular oxidation processes so that the ozonation process in pure coconut oil runs better than a mixture of coconut oil and olive oil.

Table 2. Acid numbers for pure and oils ozonated after 72 h.

| Oil                                | Acid number (mg KOH/g oil) | % Change |
|------------------------------------|-----------------------------|----------|
| Untreated oil                      | 0.2154                      | 277.52   |
| 72 h ozonated oil                  | 0.8131                      |          |
| Coconut oil                        | 0.8968                      | 225.15   |
| Mix of coconut oil and olive oil (85:15) | 2.9160                     |          |
| Mix of coconut oil and olive oil (75:25) | 1.4203                     | 98.94    |

3.3. Peroxide number

Peroxide number shows the level of oxidation of ozone to fatty acids, how much ozone binds with fatty acids to produce peroxide. The success of ozonation is marked by an increase of peroxide number obtained. The higher peroxide number, the more ozone that react with the carbon-carbon double bond and indicates the more ozones content in the oil. The following Table 3 presents the results of peroxide numbers. The highest numbers were obtained for a mix of ozonated coconut oil and olive oil with a concentration ratio of 85:15.
High peroxide number is connected with the reaction of ozone with carbon-carbon double bonds in unsaturated fatty acids. It produces ozonides, hydroperoxides, aldehydes, peroxides, diperoxides and polyperoxides according to well-known mechanism [3]. The resulting peroxide acts as an oxidizing agent in lowering the growth factor of *Staphylococcus aureus* bacteria.

| Table 3. Peroxide numbers for pure and oils ozonated after 72 h. |
|---------------------------------------------------------------|
| <table>
| Oil | Peroxide number (meq O₃²/kg oil) |
|-----|----------------------------------|
|     | Untreated oil | 72 h ozonated oil |
| Coconut oil | 0 | 102.91 |
| Mix of coconut oil and olive oil (85:15) | 0 | 114.77 |
| Mix of coconut oil and olive oil (75:25) | 0 | 110.64 |
| </table> |

3.4. **FT-IR spectroscopy**

FT-IR spectroscopy is used to highlight differences in the functional groups during the oil ozonation, in particular, the decrease of the bands corresponding to both C=C and =C–H stretches, and the increase of the band corresponding to ozonide CO stretch. Based on the spectrum obtained, the percent value of transmittance can be seen in Table 4.

The result shows that the concentration decreases in the bonds, C=C, and =C-H when an increase in transmittance occurs. Increased transmittance percent indicates a reduced number of moles of some group or bond in oleozon (percent transmittance is inversely proportional to the absorbance value). It shows also that the concentration increases in the C=O and C-O bonds when a decrease in transmittance occurs. It is because the reaction of ozone with carbon-carbon double bonds in unsaturated fatty acids produces some compounds include ozonides and aldehydes [3]. From the result of FT-IR spectra obtained for each bond, there are some percentage of transmittance which are not constant, therefore it can be said that FT-IR analysis is not as sensitive as other oleozon properties test.

| Table 4. FT-IR Analysis results. |
|----------------------------------|
| <table>
| Oil | Aromatic bond (C=C) (1400-1600 cm⁻¹) | Alkene bond (=C-H) (675-1000 cm⁻¹) | Carbonyl bond (C=O) (1650-1820 cm⁻¹) | Ozonide bond (C-O) (1050-1260 cm⁻¹) |
|-----|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|     | Wave length (cm⁻¹) | %T | Wave length (cm⁻¹) | %T | Wave length (cm⁻¹) | %T | Wave length (cm⁻¹) | %T |
| Pure coconut oil | 1462.94 | 86.29 | 721.62 | 88.79 | 1742.85 | 57.24 | 1110.07 | 76.16 |
| Ozonated coconut oil | 1463.08 | 89.36 | 721.59 | 90.79 | 1743.02 | 66.96 | 1109.36 | 81.85 |
| Pure coconut oil and olive oil (85:15) | 1462.65 | 86.14 | 721.61 | 88.03 | 1743.01 | 57.97 | 1110.58 | 77.18 |
| Ozonated coconut oil and olive oil (85:15) | 1462.42 | 85.99 | 721.65 | 88.09 | 1742.93 | 58.61 | 1110.07 | 76.58 |
| Pure coconut oil and olive oil (75:25) | 1462.36 | 86.04 | 721.65 | 87.58 | 1743.15 | 58.46 | 1110.98 | 77.91 |
| Ozonated coconut oil and olive oil (75:25) | 1462.43 | 85.99 | 721.03 | 86.86 | 1743.00 | 58.82 | 1110.19 | 77.03 |
| </table> |
3.5. LC-MS spectroscopy

The compounds contained in herbal extract were analyzed by LC-MS. The observed results are mass arrays which containing information about the mass to charge ratio (m/z) of the detected peak and peak intensity (% area). The alleged composition contained in the extract was carried out by matching the m/z value of the spectra mass array to the accurate mass of the literature compound using a database in ChemSpider or PubChem applications. The analysis results of the compound based on the LC-MS method for each extract sample were summarized in Table 5.

**Table 5. LC-MS Analysis results.**

| Compound                  | m/z (theoretical) | 100 g herbal with n-hexane solvent | 200 g herbal with n-hexane solvent | 100 g herbal with IPA solvent | 200 g herbal with IPA solvent |
|---------------------------|-------------------|-------------------------------------|------------------------------------|-------------------------------|-------------------------------|
| Cinnamaldehyde           | 132               | -                                   | ✓                                  | ✓                             | ✓                             |
| Cinnamyl acetate         | 176.215           | -                                   | -                                  | -                             | -                             |
| Linalool                 | 154.253           | -                                   | ✓                                  | ✓                             | -                             |
| Eugenol                  | 164.204           | -                                   | -                                  | -                             | -                             |
| Caryophyllene            | 204.357           | ✓                                   | ✓                                  | ✓                             | ✓                             |
| Diosgenin (Saponin)      | 204.357           | ✓                                   | ✓                                  | ✓                             | ✓                             |
| Diosgenin (Saponin)      | 414.63            | -                                   | -                                  | ✓                             | -                             |
| Bacoside A3 (Saponin)    | 929.107           | -                                   | -                                  | -                             | ✓                             |
| Asparaguside A (Saponin) | 578.787           | ✓                                   | ✓                                  | ✓                             | ✓                             |
| Asparaguside B (Saponin) | 596.802           | -                                   | ✓                                  | -                             | -                             |
| Elatin (Flavonoid)       | 594.522           | ✓                                   | -                                  | -                             | -                             |
| Eupatin (Flavonoid)      | 360.318           | ✓                                   | ✓                                  | -                             | -                             |
| Hispidone (Flavonoid)    | 346.335           | -                                   | ✓                                  | -                             | -                             |
| Laurifolin (Flavonoid)   | 356.374           | ✓                                   | -                                  | -                             | -                             |

The type of extraction performed in this study is liquid-solid extraction which there are several factors that must be considered for the extraction to run properly, such as particle size, solvent type, operating temperature, and stirring. It can be seen in table above that the compounds detected on extraction using n-hexane solvent are more than using isopropyl alcohol solvent. In the extraction process, the compound will be more easily attracted or dissolved with the solvent having the same polarity level. In this case, herbal extract is an organic compound that tends to have nonpolar properties, making it more soluble in nonpolar solvents (n-hexane) as well.

When analyzed from the differences in feed mass used, the extraction results of 100 g feed mass should be better than 200 g feed mass. Although the operating temperature used was same, the 200 g feed mass has a longer time to reach the operating temperature than the 100 g feed mass. Thus, the diffusion process at 100 g feed mass runs faster than the 200 g feed mass. In addition, stirring can make the diffusion rate increase and the transfer of material from the surface of the particles into the solution increases rapidly. Stirring also prevents precipitation. Stirring on 100 g feed mass is easier than on 200 g feed mass. As a result, the diffusion process at 100 g feed mass runs faster than the 200 g feed mass.

3.6. Antibacterial activity

The antibacterial activity test is necessary to know how effective the mixed sample of the ozonated vegetable oil with herbal extract to inhibit the growth of *Staphylococcus aureus* bacteria. As we know, *Staphylococcus* is a group of bacteria that can cause various diseases from the infection of some tissues in the body. Diseases caused by *Staphylococcus* are varying from no treatment to severe (such as diabetic ulcer) and potentially cause death. Table 6 and Figure 1 present the results of antibacterial activity test.
Based on the results, it was found that ozonated oil (oleozon) is a good oil in disinfecting bacteria, seen from the inhibition zone that formed. Moreover, after adding *Phaleria macrocarpa* and *Cinnamomum burmannii* extract the antibacterial effect will increase. A mixture of 160 mL coconut oil that ozonated for 72 hours and 0.18 gram herbal extracts with *n*-hexane solvent showed the highest inhibition zone of 18.3 mm in *Staphylococcus aureus* bacteria. This result is much better when compared with a study conducted by Nisa (2014) i.e. the 42-hour ozonated olive oil sample showed a 2 mm inhibition zone in *Staphylococcus aureus* bacteria. The highest inhibition zone produced by a mixture of coconut oil and herbal extracts with *n*-hexane solvent was also comparable with the test results of LC-MS extracts, where the compounds on the extraction using the *n*-hexane solvent were more detected than using the isopropyl alcohol (IPA) solvent. 72 hours of ozonation time is a good time in the ozonolysis reaction of oleozon and the addition of herbal extracts proven to increase disinfection properties of oleozon.

**Table 6. Antibacterial activity results.**

| Sample | Symbol | Diameter of inhibition (mm) |
|--------|--------|----------------------------|
| 160 mL Ozonated coconut oil (CO) | Blank | 12.2 |
| 160 mL Ozonated CO+0.16 g extract, IPA solvent | N | 13.7 |
| 160 mL Ozonated CO+0.17 g extract, IPA solvent | M | 10.1 |
| 160 mL Ozonated CO+0.18 g extract, *n*-hexane solvent | J | 18.3 |
| 160 mL Ozonated CO+0.2 g extract, *n*-hexane solvent | K | 12.7 |
| 160 mL Ozonated coconut oil (CO) & olive oil (OO) (85:15) | L | 15.1 |
| 160 mL Ozonated CO&OO (85:15)+0.16 g extract, IPA solvent | B | 12.6 |
| 160 mL Ozonated CO&OO (85:15)+0.17 g extract, IPA solvent | I | 16.1 |
| 160 mL Ozonated CO&OO (85:15)+0.18 g extract, *n*-hexane solvent | D | 14.0 |
| 160 mL Ozonated CO&OO (85:15)+0.2 g extract, *n*-hexane solvent | C | 12.6 |
| 160 mL Ozonated coconut oil (CO) & olive oil (OO) (75:25) | E | 11.3 |
| 160 mL Ozonated CO&OO (75:25)+0.16 g extract, IPA solvent | H | 12.5 |
| 160 mL Ozonated CO&OO (75:25)+0.17 g extract, IPA solvent | G | 11.7 |
| 160 mL Ozonated CO&OO (75:25)+0.18 g extract, *n*-hexane solvent | F | 12.6 |
| 160 mL Ozonated CO&OO (75:25)+0.2 g extract, *n*-hexane solvent | A | 14.6 |

**Figure 1.** Inhibition of bacteria *Staphylococcus aureus* on medium with ozonated oil.
4. Conclusion

The results presented here show that oil ozonation was successful, either for pure coconut oil or a mixture of coconut oil and olive oil. Ozonation may increase the peroxide and acid numbers for both oils and also decrease the iodine numbers. Evaluating from the analysis of chemical and physical properties results, the best ozonation condition is picked up from an increase of 277.52% acid number, peroxide number about 114.77 meq O₂⁻/kg oil, and decrease of iodine number up to 22%.

The addition of herbal extracts proven to increase disinfection properties of oleozon. A mixture of 160 mL coconut oil that ozonated for 72 hours and 0.18 gram herbal extracts with n-hexane solvent showed the highest inhibition zone of 18.3 mm in Staphylococcus aureus bacteria.

The highest increase in acid number and highest decrease in iodine number is owned by coconut oil, which means the acceptance of ozonation in coconut oil is better than mixture of coconut oil and olive oil. These results are also proportional to the antibacterial activity test result where the highest inhibitory zone is generated by coconut oil. It means that the acid number and iodine number determining for antibacterial test, but not with peroxide number. Meanwhile, high peroxide number is also effective to inhibit bacteria. From the properties of ozonated oils and antibacterial activity test results, it can be concluded that coconut oil is potential to become Olozon® as an alternative to olive oil which is more expensive and less availability in Indonesia.

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