Emulsifier and antimicrobial activity against 
Propionibacterium acnes and Staphylococcus epidermidis of oxidized fatty acid esters from hydrolyzed castor oil

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Abstract. This study aimed to synthesize esters from oxidized fatty acids produced by castor oil hydrolysis as emulsifiers and antimicrobial compounds. Castor oil was hydrolyzed using KOH and the fatty acids were then oxidized using KMnO4. The success of oxidation proven by determined the iodine number. Esterification was conducted with varied alcohols, namely methanol, ethanol, isopropanol, and 1-butanol using ZnCl2 as catalyst and mole ratio was 1:2. The conversion percentage of esterification was determined using titrimetric method and products were characterized using FTIR. From the hydrolysis of castor oil, 84% of fatty acids were produced. Decreasing iodine number from 43.38 mg/g to 13.11 mg/g and increasing intensity of the -OH group absorption in the FTIR spectrum showed the success of fatty acids oxidation. Emulsifier test showed all products have emulsifier ability and emulsions were stable up to 24 hours with a water-in-oil (w/o) emulsion type. The best ability as an emulsifier demonstrated by methyl ester. Antimicrobial assay against Propionibacterium acnes and Staphylococcus epidermidis showed all ester products could inhibit the growth of both bacteria. Largest inhibition zone obtained from isopropyl ester for P. acnes by 16 mm and butyl ester for S. epidermidis by 17 mm.

1. Introduction
Castor oil is a vegetable oil extracted from the seeds of the castor plant (Ricinus communis L.) and has been used since ancient times as an important base material in the oleochemical industry [1]. The world market demand for castor oil and its products continue to increase due to several advantages, among them are the renewable sources, not competing with food, biodegradable, low production costs, and environmentally friendly [2].

In Indonesia, the production of Ricinus communis L. in 2015 reached 1.5 thousand tons [3]. However, its processing into a product with a high economic value has not been maximized, because there is no refined castor oil processing industry and modified castor oil processing industry [4].

Castor oil is more unique than other vegetable oils because of its fatty acid content. The highest content of fatty acids in castor oil is ricinoleic acid, about 87-90%. Ricinoleic acid is an unsaturated fatty acid consisted of 18 atom carbon with one double bond at carbon 9 and one hydroxyl group at carbon 12. The hydroxyl group makes a ricinoleic acid difference than other fatty acids because it has three reaction sites, namely the hydroxyl group, the double bond, and the carboxylic group. So ricinoleic acids can undergo many more reactions than other fatty acids [1].
In the cosmetic industry, ricinoleic acid and its derivatives were widely used because of their properties as an emulsifier and antimicrobial agent.

A study was conducted in 2018 to identify the emulsifying and antimicrobial activity against Propionibacterium acnes and Staphylococcus epidermidis of oxidized fatty acids ethyl ester obtained from hydrolysis of castor oil. The results of the study concluded that oxidation of fatty acids before esterification with ethanol generated a better emulsifying and antimicrobial activity of the fatty acids ethyl ester [5]. However, it was not yet known whether the length of alkyl carbon affects its ability as an emulsifier and its antimicrobial activity. The hypothesis is the longer the carbon alcohol chain used in esterification can produce better emulsification and antimicrobial activity.

In this research, variations of alcohol were used in the esterification of oxidized fatty acids of hydrolyzed castor oil to determine their effect on emulsifier and antimicrobial activity against P. acnes and S. epidermidis. Alcohols used were methanol, ethanol, 2-propanol, and 1-butanol and the esterification was performed using ZnCl as a catalyst.

2. Materials and methods

2.1. Materials

The materials used were castor oil obtained from Heansa Kimia, potassium hydroxide, ethanol 96%, hydrochloric acid, potassium permanganate, sodium hydroxide, chloroform, Wijs solution, potassium iodide, aquadest, sodium thiosulfate, methanol p.a, ethanol p.a, 2-propanol. 1-butanol, anhydrous zinc chloride, n-hexane, phenolphthalein, starch, eosin, clindamycin, DMSO, beef extract, peptone, nutrient agar, Propionibacterium acnes and Staphylococcus epidermidis cultures obtained from Biochemistry Laboratory, Department of Chemistry, Universitas Indonesia.

2.2. Methods

2.2.1. Castor oil hydrolysis. 300 g castor oil and 192.6 mL of 5 M KOH solution in 96% alcohol were mixed until homogenous then heated in an oil bath at 70 °C for 1 hour accompanied by stirring. The mixture was then cooled at room temperature and then 250 mL of 5 M HCl was added to the mixture. Then the mixture was transferred to a separating funnel and allowed to stand for 24 hours to form two phases. The fatty acids from hydrolysis in the upper phase were then filtered [6].

2.2.2. Fatty acid oxidation. 120 mL of hydrolyzed fatty acids were cooled to 25-27 °C then added by the alkaline solution of KMnO₄ (60 mL of 0.5 M KMnO₄ with 360 mL of 2 M NaOH, pH 12) slowly accompanied by stirring for 30 minutes. The mixture was protected from temperature changes and direct light exposure. The mixture was then left for 24 hours until split into two phases. The organic phase contained MnO precipitate was separated and filtered [7].

2.2.3. Determination of iodine number. Iodine number was determined using a standard Wijs method [8].

2.2.4. Esterification and determination of conversion percentage. Oxidized fatty acid and alcohol (mole ratio of 1:2) were placed in a two neck flask equipped with a condenser reflux and a thermometer and then added by 0.3% ZnCl. The mixture was stirred until homogeneous and the reflux system was run for 6 hours at 60 °C. Ester product extraction was carried out using methanol and n-hexane. Methanol was removed by heating and oxidized fatty acids ester was obtained [9]. The titrimetric method was used to determine the conversion percentage of esterification. Unreacted fatty acid was moved into a 10 mL volumetric flask and the volume was adjusted using n-hexane. Thereupon 1 mL aliquot of the solution was titrated with 0.1 N NaOH.
2.2.5. **FTIR analysis.** Identification using FTIR was carried out for castor oil, fatty acids from hydrolysis, oxidized fatty acids, and ester products.

2.2.6. **Emulsifier ability test and emulsion type determination.** 0.1 g of sample was put into a mixture of oil and water in the proportions according to table 1 and then shaken to formed an emulsion. The emulsion formed was observed in its stability after 24 hours.

| Tube Number | Variation 1 | Variation 2 |
|-------------|-------------|-------------|
| Water (mL)  | 1 2 3 4 5   | 1 2 3 4 5   |
| Oil (drops) | 2 4 6 8 10  | 2 2 2 2 2   |

The type of emulsions was determined by observing the mixture of emulsion and 1 drop of eosin dye under a microscope.

2.2.7. **Antimicrobial assay using the disk diffusion method.** 200 μL suspensions of bacteria (*P. acnes* or *S. epidermidis*) with a cell density of 1 x 10^7 cells / mL were placed aseptically into a sterile petri dish. Then 20 mL of nutrient agar (± 45-50 °C) was poured in and was shaken until homogeneous, then allowed to harden. Sterile disc paper was placed on top of media and 4 μL of the sample was dropped onto disc paper. The dish containing each of the test bacteria then incubated at 37 °C for 24 hours. The transparent area surrounding the disc paper was measured. DMSO, n-hexane, and methanol were used as negative control and 0.5% clindamycin antibiotic was used as a positive control.

3. Results and discussion

3.1. **Hydrolysis**
Hydrolysis of castor oil was conducted in an alkaline solution to generate more yield of fatty acids because basic hydrolysis is irreversible, whereas acidic hydrolysis is reversible [10]. In basic hydrolysis, -OH as a nucleophile attacks carbon from the carbonyl group followed by the release of the leaving group. Deprotonation causes the formation of carboxylic anions and forms fatty acid salts (soap). This process occurs gradually from triglycerides to diglycerides by breaking the ester bond on carbon number 1, then becoming monoglyceride by breaking the ester bond on carbon number 3 and finally being released three fatty acids from breaking the ester bond number 2 and forming glycerol [11].

In this study, KOH was used as nucleophile and ethanol acts as a reaction medium. The addition of KOH was done slowly because of its exothermic characteristics. The reaction was carried out at temperature 70 °C based on a study [6] that it was the optimum temperature of the castor oil hydrolysis reaction. Free fatty acids obtained from the resulting soap by neutralization that was carried out using excessive HCl. The hydrogen cation of HCl will be captured by the alkanoic anion to form free fatty acids. The addition of HCl was done slowly accompanied by stirring without heating to prevent dehydration in OH groups adjacent to the double bond [12].

From the hydrolysis of 900 grams of oil, 723.64 grams of fatty acids were obtained with a yield of 84%. Fatty acids from the hydrolysis of castor oil are dark yellow and viscous.

3.2. **Oxidation of fatty acids**
Oxidation of fatty acids aims to formed two hydroxyl groups (diols) from carbon 9 double bonds which can increase the polarity of ricinoleic acid. The oxidizing agent used in this study was diluted alkaline KMnO₄. To prevent the fatty acids undergo further oxidation, the reaction took place at pH 12 and temperature 25°C due to the very strong oxidizing properties of KMnO₄ [10]. At the end of the reaction, a mixture of fatty acids and black MnO₂ precipitate was obtained and then separated by filtration.
The success of the oxidation reaction was determined by the iodine number test. A decrease in iodine number indicated that the number of double bonds was decreased and thus the reaction was successful. In this study, the iodine value of oxidation products decreased from 43.38 mg/g to 13.11 mg/g. Ricinoleic acid was oxidized to produce 9,10,12-trihydroxy stearic acid.

3.3. Esterification and Conversion Percentage

Esterification of oxidized fatty acids was carried out chemically using variations of alcohols namely methanol, ethanol, isopropanol, and 1-butanol and ZnCl₂ as a catalyst. The ratio between oxidized fatty acids to alcohol used in this study was 1: 2 based on the previous study that this ratio gave the most yield [5].

A Lewis acid, ZnCl₂, was used as a catalyst replacing the common catalyst used in general esterification, sulfuric acid. This replacement aims to avoid the dehydration of OH groups obtained from previous oxidation by strong acids which can cause the breakdown of fatty acid chains. In the process, the carboxylic acid was bound to the acidic site of the catalyst through its carbonyl carbon and formed an activated complex. Then ester was produced by nucleophilic attack from alcohol together with water release as a by-product, and ZnCl₂ is recovered at the end of the reaction [13]. After the reaction, two solution phases were formed, the upper was the ester and the lower was the water phase.

The conversion percentage was determined using equation (1):

\[
\text{% Conversion} = \frac{\text{reacted fatty acid (mmol)}}{\text{total fatty acid (mmol)}} \times 100\%
\]  

Mmol of unreacted fatty acid was obtained from equation (2):

\[
\text{Mmol} = \frac{V \times N \times V_{ufa} \times DF}{V_{tfa}}
\]

Where:
- V: Volume of NaOH (mL)
- N: Normality of NaOH
- V_{ufa}: Volume of unreacted fatty acid (mL)
- DF: Dilution Factor
- V_{tfa}: Volume of titrated fatty acid

The mmol of reacted fatty acid can be obtained from the subtraction of total fatty acid to unreacted fatty acid.

Based on table 2 which shows the conversion percentage of esterification, the highest conversion percentage was obtained from esterification between oxidized fatty acid and ethanol.

Table 2. Conversion percentage of esterification.

| Alcohol used in Esterification | Conversion Percentage (%) |
|-------------------------------|---------------------------|
| Methanol                     | 91                        |
| Ethanol                      | 98                        |
| Isopropanol                  | 92                        |
| 1-Butanol                    | 75                        |

3.4. Identification using FTIR

FTIR spectrum of castor oil and its fatty acids are showed in figure 1. The absorption band for -OH groups showed at the wavenumber 3600-3200 cm⁻¹ and the C = O carbonyl absorption band appears at the wavenumber 1725-1700 cm⁻¹ for the fatty acids spectrum, shifted from 1750-1717 cm⁻¹ in castor oil spectrum. Based on the FTIR spectrum of oxidized fatty acids in figure 2, the presence of -OH absorption was seen sharper and wider than its fatty acids spectrum, indicated that the double bond of the fatty acids has been oxidized to diols [10].
Figure 1. FTIR spectra of castor oil and its fatty acids.

Figure 2. FTIR spectra of oxidized fatty acids.

Based on figure 3 below, the spectrum of all oxidized fatty acid esters showed the presence of O-H absorption bands at wavenumbers 3600–3200 cm\(^{-1}\), and C = O ester at wavenumber 1750-1717 cm\(^{-1}\) shifted to a larger wavenumber than the oxidized form. The absorption for C-O-C at wavenumber 1300-1000 cm\(^{-1}\) also shows the typical ester absorption [10].
3.5. Simple Qualitative Test as Emulsifier

In this study, a simple emulsifier test was conducted using two types of water-oil mixtures which were mixtures with water’s volume greater than the oil’s volume and mixtures with oil’s volume greater than the water’s volume. The samples tested were fatty acids from castor oil hydrolysis, oxidized fatty acids, and ester products. A mixture of oil and water was used as control. After 24 hours, the emulsion with fatty acids as emulsifier was seen separated into the oil phase and the water phase. Meanwhile, the emulsion formed with the addition of the oxidized fatty acid showed a more stable emulsion. These results showed that the oxidation of fatty acids can generate a better emulsifying ability of the fatty acids.

![FTIR spectra](image)

**Figure 3.** FTIR spectra of oxidized ricinoleic acid esters.

![Emulsion types](image)

**Figure 4.** Results of determination of emulsion type.

Emulsions formed with the addition of oxidized fatty acids esters as its emulsifiers generally maintained its emulsified form. Methyl ester as emulsifier formed the most stable emulsion compared to other esters as an emulsifier, while isopropyl ester emulsion showed the most unstable emulsion. The results of observations of the type of emulsion produced can be seen in figure 4. Red droplets showed eosin dye dissolved in the water phase, surrounded by the clear color that was the oil phase. Thus it can be concluded that the type of emulsion produced from oxidized fatty acids esters was water-in-oil (w/o) emulsion.
3.6. Antimicrobial Assay

Antimicrobial assay against gram-positive bacteria, *Propionibacterium acnes* and *Staphylococcus epidermidis*, was carried out using disc diffusion method. The bacterial growth inhibition by antimicrobial substances was indicated by the diameter of the clear area around the disc paper. The strength of the antimicrobial activity of the sample was determined based on the classification of the effectiveness of antimicrobial compounds in table 3 [14]. The inhibition zone diameter data of the samples tested in this study shows in table 4.

| Table 3. Classification of effectivity of antimicrobial compounds. |
|----------------------------------------------------------|
| Inhibitory zone diameter | The response of growth barriers |
|--------------------------|--------------------------------|
| >20 mm                   | Strong                        |
| 16-20 mm                 | Medium                        |
| 10-15 mm                 | Weak                          |
| <10 mm                   | Not effective                 |

| Table 4. Results of antimicrobial assay. |
|-----------------------------------------|
| Sample                          | *Propionibacterium acnes* | *Staphylococcus epidermidis* |
|---------------------------------------|--------------------------|----------------------------|
| Inhibitory zone (mm) | Effectiveness | Inhibitory zone (mm) | Effectiveness |
| Castor Oil                      | -                       | Not effective          | -               | Not effective          |
| Fatty Acids                     | 10                      | Weak                   | 8               | Not effective          |
| Oxidized Fatty Acids            | 21                      | Strong                 | 22              | Strong                 |
| Methyl ester                    | 13                      | Weak                   | 14              | Weak                   |
| Ethyl ester                     | 12                      | Weak                   | 13              | Weak                   |
| Isopropyl ester                 | 16                      | Medium                 | 16              | Medium                 |
| Butyl ester                     | 12                      | Weak                   | 17              | Medium                 |
| Clindamycin 0,5%                | 10                      | Weak                   | 11              | Weak                   |
| DMSO                             | -                       | Not effective          | -               | Not effective          |
| Methanol                        | -                       | Not effective          | -               | Not effective          |
| Ethanol                         | 12                      | Weak                   | 12              | Weak                   |
| Isopropanol                     | 7                       | Not effective          | 8               | Not effective          |
| 1-butanol                       | 10                      | Weak                   | 10              | Weak                   |
| n-hexane                        | -                       | Not effective          | -               | Not effective          |

From the data above, it can be seen that oxidized fatty acids have the strongest antibacterial activity. The antimicrobial activity of oxidized fatty acids was higher than fatty acids from the hydrolysis of castor oil. This indicated that the addition of two hydroxyl groups increases the polarity of fatty acids and their ability to interact with bacterial cell membranes. Meanwhile based on the classification of the effectivity of antimicrobial compounds in table 3, among the esters produced the strongest antibacterial activity was shown by oxidized isopropyl ester with medium strength against *P. acnes* and oxidized butyl ester with medium strength against *S. epidermidis*. Other ester products were classified as weak antimicrobial agents against both bacteria. These results showed that the ester products could be a potential antimicrobial compound, although there was not any trend seen in the chain length of alcohols used in esterification towards antimicrobial properties. Generally, the mechanism of how the ester class of antimicrobial agents in bacterial membrane lysis is similar to how emulsifier works, which relies on hydrophobic and hydrophilic balance. But from these results, we could imply that besides the chain length from the hydrophilic group and the bacteria tested, several things such as the position of the hydrophobic part and the structure of the alkyl chain used maybe contributes to the antimicrobial activity of the substances. More study is needed to clarify this.
4. Conclusion
Esterification of oxidized fatty acids from hydrolyzed castor oil with various alcohols has been accomplished and proved by the presence of absorption of C = O ester groups at 1750-1735 cm$^{-1}$ and C-O-C at 1300-1000 cm$^{-1}$ in FTIR spectrum. All esterification products could act as emulsifiers with emulsion type water-in-oil emulsion (w/o). The emulsions formed were stable up to 24 hours and the best activity was shown by methyl esters. All esters also have antimicrobial property against both bacteria, *P. acnes* and *S. epidermidis* and the largest inhibitory zone obtained for isopropyl ester against *P. acnes* by 16 mm and butyl ester against *S. epidermidis* by 17 mm.

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