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BIOLOGICAL AND PHYSICOCHEMICAL STUDIES OF OLEYL OLEATE AS AN ACTIVE AGENT TO BE APPLIED IN COSMETIC FORMULATION.

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Abstract

This study aimed to synthesize oleyl oleate via esterification, confirm its structure using spectroscopic methods, and evaluate its antimicrobial properties and physicochemical characteristics for cosmetic use. Oleyl oleate synthesis was confirmed with FTIR and NMR spectroscopy. Oleic acid and oleyl alcohol were reacted in a 1:2 molar ratio at 50°C for 5 hours in hexane. The product yield was approximately 85%. Antimicrobial activity was tested against Gram-positive and Gram-negative bacteria using agar diffusion method, Minimum Inhibition Concentration (MIC), and Minimum Bactericidal Concentration (MBC) assays against Gram-positive and Gram-negative bacteria. Dilutions of 1:1 to 1:4 (v/v) were tested, and the bacterial inoculum was standardized to 10^7 cells/mL. The compound showed higher activity against Gram-positive bacteria, with MBC of 1:2 (v/v) for Bacillus subtilis. Physicochemical properties such as Sun Protection Factor (SPF), peroxide value, saponification value, and iodine value were also measured. Physicochemical assessments yielded an SPF value of 8.0, peroxide value of 17.98 meq/kg, saponification value of 82.48 mg KOH/g, and iodine value of 82.37 g/100g. These properties meet cosmetic ingredient standards. Unlike traditional extraction methods that rely on limited and costly natural wax sources like jojoba oil, this study demonstrates a simple, catalyst-free synthetic route using readily available materials, offering a scalable and economical alternative. The study demonstrates that oleyl oleate has notable antimicrobial activity, particularly against Gram-positive bacteria, and physicochemical properties suitable for cosmetic formulations, indicating its potential for wide spread use in the cosmetic industry.

Keywords: oleyl oleate, wax ester, antimicrobial, physicochemical, cosmetics.

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Introduction

The cosmetic industry is rapidly evolving, incorporating innovative ingredients for advanced formulations. Wax esters, commonly found in the natural lipids of plants, animals, and microbes, and they have a wide range of applications in various sectors, such as cosmetics (Al-Arafi et al., 2021). One of the examples of synthetically produced wax ester is oleyl oleate. Due to the presence of both ester functional group and long chain carbon in oleyl oleate structure, it is plausible to be used as industrial bio lubricant and emollient (Al-Arafi et al., 2022). Natural sources such as jojoba oil and carnauba wax are used to obtain wax esters, but these are limited by high production costs and seasonal availability (Lopes et al., 2011).

Oleyl oleate, a synthetic wax ester, offers a viable alternative with similar chemical properties. Despite advancements in synthesis methods, there is limited data on the antimicrobial and physicochemical properties of synthetic wax esters (Azmi et al., 2022). Recent study by Aguilar et al. (2023) has focused on the incorporation of bio waxes derived from hydro processed crude palm oil into cosmetic creams,

demonstrating favorable sensory and stability properties, but have not extensively evaluated the antimicrobial potential of synthetic wax esters. This study aims to fill that gap by evaluating the biological efficacy and physical characteristics of oleyl oleate synthesized via direct esterification. The objective is to validate its suitability as a cost-effective active agent in cosmetic formulations. By addressing limitations in natural wax esters and optimizing synthetic production, this research aims to provide cost-effective and high-quality solutions for the cosmetic industry.

Methods

Materials

The study employed analytical-grade reagents, including oleic acid, oleyl alcohol, and hexane as the solvent. The microorganisms utilized in the research, which included Gram-positive bacteria *(Bacillus subtilis* and *Staphylococcus aureus)* and Gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli*), were obtained from the Microbiology Laboratory at Universiti Sains Islam Malaysia.

Synthesis of Oleyl Oleate

The synthesis of oleyl oleate was carried out through a direct esterification reaction. Oleic acid and oleyl alcohol were mixed in a molar ratio of 1:2, dissolved in hexane, and subjected to a reaction in a water bath shaker at 50°C with continuous agitation at 150 rpm for 5 hours. The product was purified using rotary evaporation.

Product Verification

To verify the identity and chemical structure of the synthesized oleyl oleate, FTIR (Nicolet IS 50) and NMR (DMSO solvent, ^{1}H and ^{13}C) were used to confirm structure. FTIR analysis identified the characteristic functional groups, while ^{1}H-NMR and ^{13}C-NMR spectra offered detailed information on the molecular structure, validating the formation of the ester bond.

Antimicrobial Activity

Antimicrobial activity was tested using agar diffusion, MIC, and MBC assays against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Salmonella typhimurium.* Dilutions of 1:1 to 1:4 (v/v) oleyl oleate in Mueller-Hinton broth were used (Table 1). Inoculum was standardized to 10^{7} cells/mL using McFarland standard. Replicates (n = 2) were conducted for each assay. For the agar diffusion assay, the inhibitory effects of oleyl oleate at the specified concentrations were assessed by measuring the diameter of inhibition zones after incubating inoculated agar plates at 37°C for 24 hours. Streptomycin sulphate was used as a positive control, and a negative control was included for reference. To determine the MIC, serial dilutions of oleyl oleate were prepared in a 96-well microtiter plate and inoculated with the test bacteria. After overnight incubation at 37°C, bacterial viability was assessed by adding 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution. The MIC was defined as the lowest concentration at which no formazan blue coloration, indicating bacterial metabolic activity, was observed. For the minimum bactericidal concentration (MBC), samples from wells showing no visible growth in the MIC assay were subcultured onto Mueller-Hinton agar plates and then incubated overnight at 37°C. The lowest concentration that completely inhibited bacterial growth was recorded as the MBC.

Table 1. Ratio Concentration of Oleyl Oleate to Mueller-Hinton Broth.

Concentration (v/v) (Ester: Broth)	Oleyl Oleate (mL)	Mueller-Hinton Broth (mL)
1:1	2	2
1:2	2	4
1:3	2	6
1:4	2	8

Physicochemical Studies

The physicochemical properties of the synthesized oleyl oleate were analyzed to assess its suitability for cosmetic formulations. The sun protection factor (SPF) was determined using UV-visible

spectrophotometry by measuring the absorbance of a 1% (w/v) oleyl oleate solution in ethanol across the wavelength range of 290–320 nm at 5 nm intervals. Specifically, 1 g of oleyl oleate was dissolved in 10 mL of absolute ethanol, and the absorbance was recorded in a 1 cm path length quartz cuvette. The SPF value was calculated using the Mansur equation:

$$\text{Mansur eq} = CF \times \sum_{290}^{320} EE \times I \times Abs \quad (1)$$

where CF is the correction factor, EE(λ) is the erythemal effect spectrum, I(λ) is the solar intensity spectrum, and Abs(λ) is the absorbance at each wavelength.

Peroxide, saponification, and iodine values were determined via titration methods in accordance with AOCS standards. The peroxide value was determined by reacting 5.0 g of sample with 30 mL of a 3:2 (v/v) mixture of acetic acid and chloroform, followed by the addition of 1 mL of saturated potassium iodide (KI). After 5 minutes of reaction in the dark, 30 mL of distilled water was added, and the liberated iodine was titrated with 0.05 M sodium thiosulphate with 1 ml starch as an indicator. The peroxide value (meq/kg) was calculated using the formula:

$$\text{Peroxide value} = [(B - S) \times N \times 1000]/W \quad (2)$$

where S is the sample titration volume (mL), B is the blank titration volume (mL), N is the normality of sodium thiosulphate, and W is the sample weight (g).

For saponification value determination, 1 g of sample was refluxed with 25 mL of ethanolic KOH for 1 hour. The excess KOH was titrated with 0.5 N hydrochloric acid (HCl) using phenolphthalein as an indicator. The saponification value (mg KOH/g) was calculated as:

$$\text{Saponification value} = [(B - S) \times N \times 56.1] \,/\, W \quad (3)$$

where B is the volume of HCl used for the blank, S is the volume used for the sample, N is the normality of HCl, and W is the weight of the sample.

The iodine value was measured using the Wijs method following AOCS procedures. The sample (0.3 g) was reacted with 25 mL of Wijs solution (iodine monochloride in acetic acid) in the dark for 30 minutes. Then, 20 mL of 10% potassium iodide solution and 150 mL of distilled water were added. The excess iodine was titrated with 0.1 N sodium thiosulphate using starch as an indicator. The iodine value (g I_2/100g) was calculated by:

$$\text{Iodine Value} = [(B - S) \times N \times 126.9] \,/\, (W) \quad (4)$$

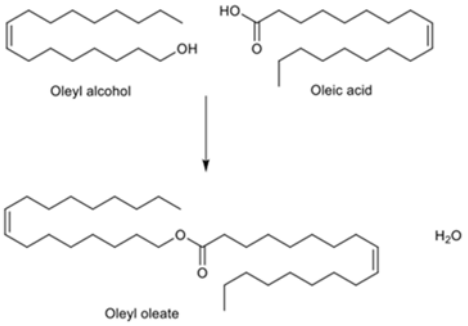
where B is the volume of thiosulphate for the blank, S is the volume for the sample, N is the normality of thiosulphate, and W is the weight of the sample.

Result and Discussion

Synthesis and Characterization

Oleyl oleate was successfully synthesized via a direct esterification reaction using oleic acid and oleyl alcohol in a molar ratio of 1:2 without a catalyst. The reaction conditions, including a temperature of 50°C, 150 rpm agitation, and a reaction duration of 5 hours, facilitated the formation of oleyl oleate with high efficiency. The use of hexane as a solvent provided an optimal environment for the reaction. This method aligns with previous studies that suggest high alcohol-to-acid ratios improve ester yield by shifting the equilibrium towards product formation, as stated in Le Chatelier's principle (Trivedi et al.,

2015). Scheme 1 depicts the chemical reaction to yield oleyl oleate. In the esterification process, the oleyl alcohol and oleic acid molecules react, causing the elimination of a water molecule. The final product was obtained with a reaction yield of approximately 85%, indicating efficient conversion under the given conditions.



Scheme 1. Synthesis of Oleyl Oleate.

The purity of the product was confirmed through FTIR and NMR analyses, which showed characteristic peaks corresponding to ester functional groups with minimal presence of unreacted starting materials. The FTIR spectrum confirmed the successful synthesis of oleyl oleate. A strong absorption peak at 1710.77 cm^{-1} was observed, corresponding to the ester C=O stretching vibration (Figure 1).

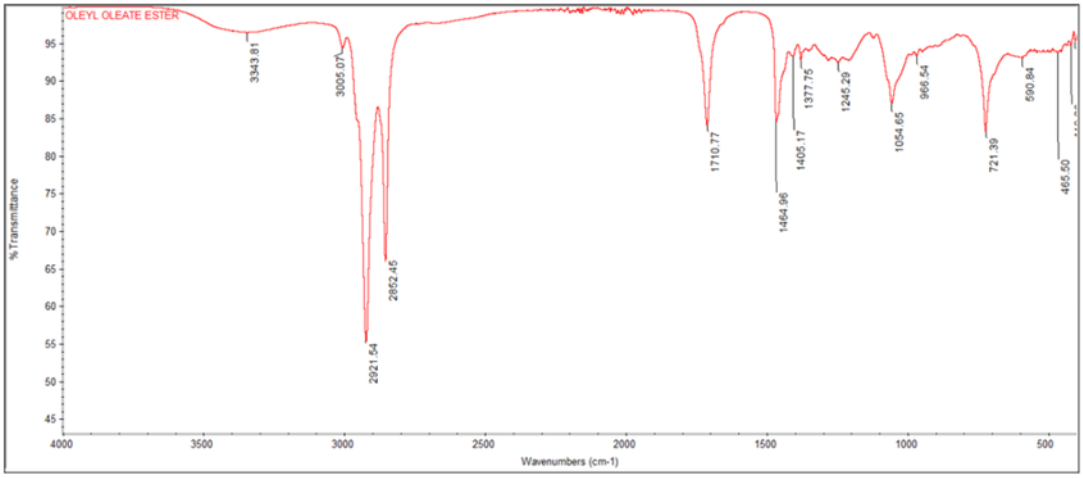


Figure 1. FTIR spectra of oleyl oleate.

Additional peaks at 2921.54 and 2852.45 cm^{-1} indicated the presence of aliphatic C−H stretching, while a weaker signal at 3005.07 cm^{-1} represented the unsaturated double bonds in the oleyl oleate structure. These results are consistent with earlier findings on wax esters (Radzi et al., 2010). Table 2 summarizes the FTIR absorption peaks observed for oleyl oleate.

Table 2. FTIR Absorption Peaks of Oleyl Oleate

Type of Vibration	Frequency (cm^{-1})	Intensity	Compound Class
C=O stretching	1710.77	Strong	Unsaturated ester
C-H stretching	2921.54- 252.45	Medium	Alkane
=C-H stretching	3005.07	Weak	Alkene

NMR Analysis provided further confirmation of the product's structure (Figure 2). The ^{1}H-NMR spectrum displayed signals for methyl (-CH_3) at 0.9 ppm, methylene (-CH_2) at 1.2–1.4 ppm, and methine (-CH_4) at 1.6 ppm. The signal at 2.26 ppm for CH_2-COO-R confirms esterification (Table 2). In the ^{13}C-NMR spectrum, a peak at 173.2 ppm corresponds to the carbonyl carbon (C=O) of the ester group, while signals at 130.6 ppm and 64.24 ppm are attributed to the olefinic (C=C) carbons and the methylene

carbon adjacent to the ester oxygen (CH_2–O–C=O), respectively. The presence of the 64.24 ppm peak is characteristic of ester formation and is not typically observed in the ^{13}C-NMR spectra of unreacted oleic acid or oleyl alcohol, based on literature reports, thereby confirming the successful synthesis of oleyl oleate.

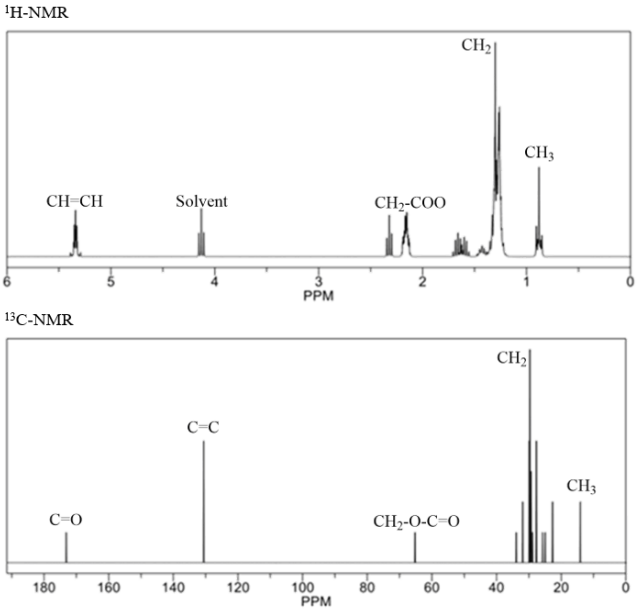


Figure 2. NMR Spectrum for ^{1}H-NMR and ^{13}C-NMR of oleyl oleate.

Table 3 presents the ^{1}H-NMR signals. A small peak at ~4.2 ppm may correspond to residual oleyl alcohol or residual solvent.

Table 3. ^{1}H-NMR Functional Groups of Oleyl Oleate.

Assignment	Chemical Shifts (^{1}H, ppm)
CH=CH	5.32
CH_2-COO-	2.26
CH_4	1.6
CH_2	1.2-1.4
CH_3	0.9

Table 4 lists the ^{13}C-NMR signals observed for oleyl oleate. There are other peaks due to the presence of multiple chemically non-equivalent carbons in the long hydrocarbon chains of oleyl oleate. However, it highlights only representative peaks corresponding to key functional groups.

Table 4. ^{13}C-NMR of Oleyl Oleate.

Assignment	Chemical Shifts (^{13}C, ppm)
C=O (Ester)	173.2
C=C (Alkene)	130.6
CH_2–O–C=O (Methylene adjacent to ester)	64.59
CH_2 (Methylene)	32.82
CH_3 (Terminal methyl)	14.12

Antimicrobial Properties

Agar Diffusion Test

The antimicrobial activity of oleyl oleate was evaluated using the agar diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. Agar diffusion

tests demonstrated that oleyl oleate exhibited significant inhibitory effects against Gram-positive bacteria, particularly *Bacillus subtilis* and *Staphylococcus aureus*, with inhibition zones ranging from 20-30 mm depending on the concentration. In contrast, the inhibition zones for Gram-negative bacteria were smaller, indicating lower susceptibility. This is due to the presence of the outer membrane in Gram-negative organisms, specifically the lipopolysaccharide layer, restricts the diffusion of hydrophobic substances, contributing to their resilience compared to Gram-positive bacteria (Dangol et al., 2020). Table 5 presents the diameter of inhibition zones observed for each bacterium at different concentrations of oleyl oleate while Figure 3 provides a visual representation of these data.

Table 5. Diameter of Inhibition zone for antimicrobial test of oleyl oleate via agar diffusion method.

Microorganism	Diameter of Inhibition Zone (mm)				
	Oleyl Oleate (sample)				Streptomycin Sulfate
	1:1	1:2	1:3	1:4	10 mg/mL
Bacillus subtilis	30 ± 0.5	27 ± 0.5	26 ± 0.5	20 ± 0.5	45 ± 0.5
Staphylococcus aureus	29 ± 0.5	25 ± 0.5	28 ± 0.5	24 ± 0.5	75 ± 0.5
Salmonella typhimurium	21 ± 0.5	22 ± 0.5	20 ± 0.5	10 ± 0.5	20 ± 0.5
Escherichia coli	24 ± 0.5	10 ± 0.5	11 ± 0.5	14 ± 0.5	30 ± 0.5

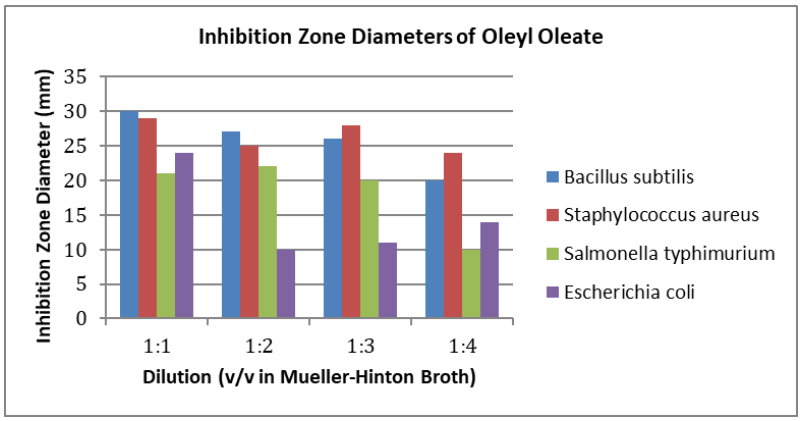


Figure 3. Bar graph showing the inhibition zone diameters (mean ± SD) *for Bacillus subtilis, Staphylococcus aureus, Salmonella typhimurium,* and *Escherichia coli* across four oleyl oleate dilutions (1:1 to 1:4 v/v in Mueller-Hinton broth). Gram-positive bacteria exhibited significantly larger zones of inhibition compared to Gram-negative strains ($p < 0.05$), indicating greater susceptibility to oleyl oleate.

MIC and MBC

Based on Table 6, the MIC values *for Bacillus subtilis* and *Staphylococcus aureus* were determined to be 1:3 and 1:1 (v/v), respectively, with oleyl oleate showing stronger inhibition against *Bacillus subtilis*. No MIC was observed for Gram-negative bacteria within the tested concentration range. MBC analysis confirmed the bactericidal effect of oleyl oleate at concentrations of 1:1 and 1:2 for *Bacillus subtilis*, with a reduced efficacy observed at 1:3. These results highlight the superior activity of oleyl oleate against Gram-positive bacteria, attributed to their lack of an outer membrane, which makes them more susceptible to antimicrobial agents.

Table 6. MIC and MBC values for antimicrobial activity.

Sample	MIC (v/v)	MBC (v/v)
Bacillus subtilis	1:3	1:2
Staphylococcus aureus	1:1	1:1

Physicochemical Properties

Table 7 summarizes four parameters in the physicochemical analysis which demonstrated the suitability of oleyl oleate for cosmetic applications. The SPF value of oleyl oleate was found to be 8.0, indicating

low UV protection. This aligns with studies showing wax esters' potential as emollients with SPF-enhancing properties (Perera et al., 2020). The peroxide value was recorded at 17.98 meq/kg, which suggests good oxidative stability suitable for cosmetic formulations, as values below 20 are generally considered acceptable. This is supported by Yildirim et. al. (2009), peroxide values exceeding 20 indicate notably low product quality. The saponification value of 82.48 mg KOH/g and iodine value of 82.37 g/100 g reflect the compatibility of oleyl oleate with cosmetic formulations, consistent with quality standards.

Table 7. Summarized Physicochemical Properties of Oleyl Oleate.

Physicochemical Properties	Oleyl Oleate Wax Ester
SPF Value	8.0
Peroxide Value	7.98
Saponification Value	82.48
Iodine Value	82.37

The findings from this study confirm the successful synthesis of oleyl oleate and its potential application in the cosmetic industry. Its antimicrobial properties against Gram-positive bacteria and favourable physicochemical attributes position it as a promising alternative to natural wax esters like jojoba oil. Furthermore, the simplicity of the synthetic process offers economic and environmental advantages, making oleyl oleate a viable option for large-scale production.

The structural features of oleyl oleate, specifically its long-chain unsaturated ester configuration, are central to its physicochemical and biological performance. The presence of the double bond in both oleic acid and oleyl alcohol contributes to its fluidity and lipophilicity, enhancing its ability to integrate into and disrupt lipid membranes of microbial cells, particularly Gram-positive species lacking an outer membrane (Yang et al., 2017). This structural feature not only influences antimicrobial efficacy but also affects its emollient texture in formulations. The ester linkage in oleyl oleate reduces the surface tension and improves spread ability on the skin, enhancing sensory characteristics such as smoothness and hydration retention. Its amphiphilic nature allows it to function as a stabilizer in oil-in-water emulsions, further supporting its multifunctionality in cosmetic systems.

The selective antimicrobial activity of oleyl oleate, particularly its higher efficacy against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, presents promising implications for its integration into cosmetic formulations. This selectivity can be beneficial in skin-care products where targeted antimicrobial action is desirable without disrupting the skin's microbiome. Gram-positive bacteria are commonly involved in skin disorders such as acne and dermatitis; thus, an agent that effectively inhibits their growth while preserving Gram-negative bacteria may contribute to both therapeutic and microbiome-friendly formulations (Polak-Szczybyło et al., 2020).

In addition to its antimicrobial properties, oleyl oleate exhibits physicochemical characteristics that further support its application in cosmetics. The calculated SPF value of 8.0 places it in the category of low sun protection. While this level is insufficient for use as a primary sunscreen agent, it suggests that oleyl oleate could function as a supporting ingredient in daytime moisturizers or as a component in night creams, where high SPF is not required. Its moisturizing effect enhances skin hydration, contributing to the overall protective function of skincare products. The iodine value of 82.37 g I_2/100g reflects a high degree of unsaturation, consistent with the presence of two cis-double bonds in the molecule. This level of unsaturation enhances its emollient properties and skin absorption but also makes it susceptible to oxidative degradation. These findings imply that antioxidants may be required in formulations to improve the oxidative stability and shelf life of products containing oleyl oleate. Furthermore, the saponification value of 82.48 mg KOH/g indicates a low level of ester hydrolysis potential, which aligns with the relatively high molecular weight of oleyl oleate (532.9 g/mol). This low saponification value suggests a higher proportion of long-chain fatty acids, which are preferred in cosmetics due to their mildness, low irritancy, and ability to improve skin feel and provide lasting hydration (Perera et al., 2020). In summary, the combination of antimicrobial selectivity, moisturizing

properties, and structural stability supports the use of oleyl oleate as a multifunctional ingredient in skin care formulations.

However, several limitations should be acknowledged. The study did not include a control formulation or base formulation without oleyl oleate, which would help isolate and compare the compound's specific effects. Additionally, the synthesis process was performed at laboratory scale, and potential challenges related to scaling up the esterification reaction, such as maintaining purity and yield were not addressed. Future experiments could include long-term stability studies under various storage conditions, compatibility assessments of oleyl oleate in complex cosmetic formulations, and expanded antimicrobial testing involving a broader range of microbial species.

Conclusion

This study successfully synthesized oleyl oleate through a straightforward esterification reaction between oleic acid and oleyl alcohol, with structural confirmation provided by FTIR and NMR analyses. The compound demonstrated favourable physicochemical properties, including a low saponification value (82.48 mg KOH/g), a high iodine value (82.37 g/100 g), and an SPF value of 8.0. These characteristics suggest its suitability as a multifunctional cosmetic ingredient, particularly for formulations emphasizing moisturization and mild sun protection. Furthermore, antimicrobial testing indicated that oleyl oleate exhibits selective activity against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, while showing limited efficacy against Gram-negative strains.

The combination of its structural stability, emollient properties, and targeted antimicrobial effects supports oleyl oleate's potential as an alternative to natural wax esters in cosmetic products. However, while these findings are promising, broader claims regarding its industrial applicability should be made with caution. Additional studies including scale-up synthesis, formulation stability assessments, and in vivo evaluations are necessary to fully validate its performance and safety in commercial applications. Furthermore, replacement of hexane with greener alternatives like supercritical carbon dioxide could improve the environmental safety and sustainability of the synthesis process. In conclusion, oleyl oleate presents as a cost-effective and versatile candidate for future cosmetic formulations. With further research and development, it may contribute to more accessible and sustainable options within the personal care industry.

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Author Contribution

Fadhlin Hasya Shaiful Bahri: Conducted experiments, synthesized oleyl oleate, performed antimicrobial assays, analyzed physicochemical properties, interpreted data, and prepared initial manuscript draft.
Salina Mat Radzi (corresponding author): Conceptualized the research, designed methodology, supervised overall research process, critically reviewed data and analysis, managed manuscript revisions, and finalized the manuscript for submission.
Nur Amalina Mohd Amin: Assisted in experimental design, provided technical expertise in spectroscopic analysis (FTIR and NMR), interpreted structural data, and contributed to manuscript writing and revision.
Maryam Mohd Rehan: Supported physicochemical characterization experiments, data analysis, and contributed significantly to interpretation of results, particularly in the physicochemical properties evaluation section.
Nurul Jannah Abdul Rahman: Assisted with microbial assays including MIC and MBC experiments, interpreted antimicrobial data, and participated in drafting and critically revising relevant sections of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Declaration on the Use of Generative AI

Generative AI tool was used to assist in generating text summaries and improving manuscript structure. All outputs

were critically reviewed and edited by the authors.

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