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# Nanoemulsion Formulation and Antibacterial Activity of Nagasari (Mesua ferrea L.) Leaf Extract

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**ABSTRACT:** Nagasari (*Mesua ferrea* L.) has antibacterial activity. Its active compounds are difficult to absorb. Nanoemulsions can enhance the absorption and solubility of these compounds. This study aims to determine the physical stability of a Nagasari extract nanoemulsion, as evaluated by its antibacterial activity. The nanoemulsion was prepared using VCO (1%) as the oil phase, Tween 80 (16%) as the surfactant, and PEG 400 as the cosurfactant, with variations of 1% (FI), 3% (FII), and 5% (FIII). The nanoemulsions were evaluated for their physical properties, solubility, organoleptic properties, pH, emulsion type, transmission percentage, particle size, polydispersity index, zeta potential, and stability. The most effective nanoemulsion was tested against B. subtilis. The results show that FI was stable during freeze-thaw testing. Its pH was 6.2 ± 0.15. FI was found to be an o/w emulsion-type with the following characteristics: transmission (94.23 ± 1.18%), polydispersity index (0.345 ± 0.051), particle size (103.07 nm), and zeta potential (-6.47 ± 2.20 mV). The stability and physical characteristics of the nanoemulsion are affected by variations in the concentration of PEG 400 as a co-surfactant. Antibacterial activity testing of the nanoemulsions resulted in an inhibition zone measurement of 19.15 mm against B. subtilis.

**Keywords**: **nanoemulsion; nagasari; co-surfactant; *Bacillus subtilis*; PEG 400.**

## Introduction

Illnesses caused by viruses, bacteria, and parasites are significant health problems in developing countries, including Indonesia. Many *B.subtilis* in the intestine can cause diarrhea and other infections [1]. One of the biodiversity in Indonesia that has the potential to be developed as an antibacterial is the Nagasari plant. Tannins, phenolics, flavonoids, and terpenoids were found in the phytochemical screening of the ethanolic extract of Nagasari leaves. Nagasari leaves have a flavonoid content of 69.31 GAE/g and a phenolic content of 65.82 to 98.15 mg GAE/g [2,3].

The active compounds, such as flavonoids, tannins, and terpenoids, have poor permeability in the body due to their large particle size. Thus, they cannot be absorbed by passive diffusion. Their solubility is not good, and they are difficult to dissolve in water due to their hydrophobic nature. However, even in glycosides, most active compounds are poorly bioavailable and water-insoluble, significantly reducing their therapeutic efficacy [4]. That compound causes many problems in formulating drugs derived from natural ingredients [5,6]. In addition, particle size reduction can increase the surface area, well drug delivery, bioavailability, and therapeutic effect.

Nanoemulsions are used in the drug and pharmaceutical industry because they have a high solubilizing capacity and are thermodynamically stable. Furthermore, oil-in-water nanoemulsions are attractive because hydrophobic drugs can be carried in oil and emulsified in water, which increases drug solubility. The small droplet size of the nanoemulsion allows it to remain dispersed; it can also prevent creaming, flocculation, or sedimentation during storage [7,8].

Nanoemulsions are thermodynamically stable, transparent dispersions of oil and water stabilized by interfacial films of surfactant and cosurfactant molecules. They have a droplet size of less than 100 nm. The stability of nanoemulsions can be influenced by selecting ingredients such as oils and surfactants for their preparation. Virgin coconut oil (VCO) was chosen as the oil phase, as it can produce smaller droplets than nanoemulsions with an oil phase using MCT (medium-chain triglyceride), resulting in enhanced physical stability [9].

Tween 80 is a non-ionic surfactant that reduces the surface tension between water and oil. Non-ionic surfactants are

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commonly used to prepare nanoemulsions due to their low irritation potential. Tween 80 is a non-ionic surfactant with an HLB value of 8–18, which is used to produce oil-in-water emulsions [10]. However, surfactants alone are insufficient to reduce surface tension between the oil and water phases, necessitating the addition of a cosurfactant. The polyethylene glycol (PEG) 400 cosurfactant is a mid-chain hydrocarbon that can form hydrogen chains by filling the gaps in the nanoemulsion system, thereby maximizing the emulsification process in nanoemulsion preparation [11]. PEG 400 occupies a large area in the ternary diagram when combined with Tween 80 and is also safe, biocompatible, and highly soluble. The ratio of surfactant to cosurfactant greatly affects the stability and bioavailability of the resulting nanoemulsion, so optimizing this ratio is necessary to produce a high-quality nanoemulsion.

# Methods

## Materials

Nagasari (*M. ferrea* L.) leaf obtained from Notog Banyumas, Indonesia, ethanol 96% (Bratachem, Indonesia), virgin coconut oil (Bratisia Herbal, Indonesia), Tween 80 (Bratachem), PEG 400, tetracycline (BPOM, Indonesia), *Bacillus subtilis* bacteria (ATCC 6633), and MHA (PT Smart Lab, Indonesia) as the media of antibacterial activity testing.

## Extraction of Nagasari leaves

Samples were determined before use in the study to determine the correct identity of the plant. The Nagasari leaves were sorted in a wet state by separating the dirt and foreign matter, washing them with clean water, and draining them. Further, Nagasari leaves were then dried in an oven (brand-type, manufacturer country) at 40°C. Then, they were crushed using a blender (brand-type, manufacturer country) to form simplicia. These Nagasari leaf simplicias were extracted using a maceration extraction method of 96% ethanol. It was done in 2 repetitions with solvent change every 24 hours. The ethanol to simplicia ratio is 1:5. The maceration results were filtered. Meanwhile, the solvent was evaporated using an evaporator and water bath to obtain a thick extract [12,13].

## Formulation of Nagasari Nanoemulsion

Nanoemulsions were prepared using the formulas listed in Table 1. Nagasari extract was mixed with VCO and homogenized with a magnetic stirrer (brand-type, manufacturer country) at 500 rpm for 10 minutes to produce mixture 1. Tween 80 and PEG 400 were mixed and stirred at 250 rpm for 10 minutes to produce Mixture 2. Mixture 1 was added to Mixture 2 while the stirring speed was increased to 1250 rpm over 30 minutes. Distilled water was added to the mixture until the volume reached 100 ml. The mixture was sonicated (brand-type, manufacturer country) for a further 10 minutes.

## Physical Characteristics Evaluation

The physical characteristics evaluated were organoleptic, emulsion type, pH (brand-type, manufacturer country), viscosity (brand-type, manufacturer country), particle size (brand-type, manufacturer country) and size distribution analysis, transmittance percent (brand-type, manufacturer country), polydispersity index (brand-type, manufacturer country), zeta potential (brand-type, manufacturer country), and freeze-thaw physical stability tests. The bacterial activity test was conducted using the selected formula based on the physical characteristics and stability test results.

# Result and Discussion

## Nagasari Leaves (*Mesua ferrea* L.) Extraction

The determination results show that the plant used in this study is the leaf of Nagasari, of the family

**Table 1.** Nagasari (*M. ferrea* L.) nanoemulsion formulation.

| No. | Substance | Unit | Function | Concentration (% m/v) | | |
|---|---|---|---|---|---|---|
| | | | | FI | FII | FIII |
| 1 | Ethanol extract from Nagasari leaves | gram | Active substance | 0.1 | 0.1 | 0.1 |
| 2 | Virgin coconut oil | mL | Oil phase | 1 | 1 | 1 |
| 3 | Tween 80 | mL | Surfactant | 16 | 16 | 16 |
| 4 | PEG 400 | mL | Co-surfactant | 1 | 3 | 5 |
| 5 | Aquadest ad | mL | Solvent | 100 | 100 | 100 |

**Table 2.** The organoleptic test of Nanoemulsion.

| Formula | Day | Odor | Colour | Consistency |
|---|---|---|---|---|
| I PEG 400 1% | 1 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 7 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 14 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 21 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 28 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| II PEG 400 3% | 1 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 7 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 14 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 21 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 28 | Typical Nagasari leaves an odor | Yellow | Liquid |
| III PEG 400 5% | 1 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 7 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 14 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 21 | Typical Nagasari leaves an odor | Bright yellow | Liquid |
| | 28 | Typical Nagasari leaves an odor | Yellow | Liquid |

*Calophyllaceae*, with the scientific name *Mesua ferrea* L. The certificate of determination with registration number 045/HP.LL/IV/2022. The extraction of the leaves of Nagasari obtained a yield value of 35.60%. Ethanol is a suitable solvent for extracting Nagasari leaves. It has a relatively high yield value compared to methanol [14,15].

Nanoemulsion formulation should meet the requirements of physical characteristics and physical stability tests for good nanoemulsion formulation. The results of evaluating the physical characteristics and testing the stability of the preparation are as follows:

## Formulation and Organoleptic Test

The organoleptic test of the nanoemulsion preparation of Nagasari leaf extract is conducted to determine its stability during a 28-day storage period. This test includes observing the preparation's shape, smell, and colour. The results of the organoleptic observations of the nanoemulsion preparation of the Nagasari leaf extract can be seen in Table 2.

The table shows that the nanoemulsion preparation has a distinctive scent of Nagasari leaves; it is also a bright yellow colour and is in a liquid form. Moreover, no nanoemulsion FI colour, odour, or shape change is stored at room temperature from day 1 to day 28. On the other hand, FII and FIII show a shift in colour to yellow on day 28. The change may be due to the effect of the pH of the preparation. The pH significantly affects the stability of

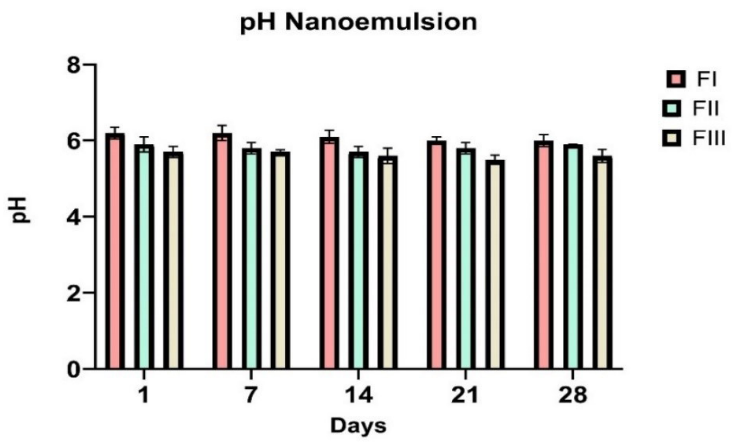



**Figure 1.** The pH test of the nanoemulsion.

Table 3. Transmittance percent test result of before and after freeze-thaw test.

| Day | Transmittance Percent (%) ± SD (n=3) | | |
|---|---|---|---|
| | F I | FII | FIII |
| 1 | 94.23 ± 1.18 | 98.00 ± 1.50 | 95.00 ± 0.47 |
| 24 | 90.17 ± 2.50 | 92.20 ± 3.30 | 92.57 ± 3.12 |

nanoemulsions. The optimal pH range generally results in greater stability, whereas extreme pH values can destabilise the emulsion [16].

## The Emulsion Type Test

The nanoemulsion type test is performed to determine the type of nanoemulsion formed. It is performed by dropping methylene blue on a preparation sample placed on a microscope slide. The nanoemulsion-type test results show that the methylene blue is uniformly distributed. Thus, in this study, the nanoemulsion preparation of ethanolic extract of Nagasari leaves is an oil-in-water nanoemulsion type, where the oil phase is dispersed. In contrast, the water phase is the dispersing medium. The concentration of the oil phase in the dosing formulation is lower than that of the water, so the emulsion type is an oil-in-water emulsion [17]. The type of emulsion has a significant effect on its stability. Water-in-oil (W/O) emulsions are generally more stable than oil-in-water (O/W) emulsions because the continuous oil phase has a lower dielectric constant, which reduces the effectiveness of electrostatic repulsion in preventing droplet coalescence. Other factors affecting emulsion stability include emulsifier type and concentration, droplet size, and the presence of stabilizers [18].

The nanoemulsion type depends on the surfactant's concentration and chemical properties, the oil phase, and the dissolved materials. In addition, surfactants with polar groups tend to be more likely to form the type of oil in water. In the formulation of nanoemulsion preparation, Tween 80 is used as a surfactant, where Tween 80 is hydrophilic; besides, the concentration of oil phase in the formulation is smaller than water, so the type of emulsion produced is oil-in-water [19].

## pH Test

The pH test uses a pH meter on days 1, 7, 14, 21, and 28. The pH level significantly impacts the stability of extract in nanoemulsions. Generally, higher pH values (alkaline conditions) can enhance flavonoid stability, whereas acidic conditions may lead to degradation. The results of monitoring the pH value are shown in Figure 1.

Flavonoids can also act as Pickering emulsion stabilizers, and their effectiveness is influenced by pH, particularly at pH 8, where they demonstrate improved dispersion and charge on stabilized droplets [20,21]. The result of the statistical analysis shows that the difference in the concentration of co-surfactant PEG 400 affects the pH of nanoemulsion preparation of ethanolic extract of Nagasari leaves, as the result of the One-Way ANOVA test indicates a significant difference between the formulations with a p-value of 0.000** (<0.05).

In addition, PEG 400 has a pH of 4.0-7.5. The higher the concentration of PEG 400 in the nanoemulsion formulation leads to the lower the pH of the preparation [16,22]. Furthermore, the pH values for each formulation are within the oral dosage form pH ranges, i.e. between 5.5 and 7.5 [23]. It shows that the nanoemulsion preparation of ethanolic extract of Nagasari leaves (*M. ferrea* L.) has pH stability and no significant change in pH based on 28 days of observation.

## Solubility Test

A solubility test was carried out to compare the

Table 4. Particle size, polydispersity index, and potential zeta of nanoemulsion.

| Formula | Particle size (nm) ± SD (n=3) | Polydispersity index±SD (n=3) | Zeta Potential (mV)±SD (n=3) |
|---|---|---|---|
| I | 103.07 | 0.345 ± 0.051 | -6.47 ± 2.20 |
| II | 72.20 | 0.286 ± 0,049 | -5.41 ± 1.38 |
| III | 63.53 | 0.317 ± 0.041 | -4.66 ± 1.25 |

**Table 5.** Result of organoleptic and percent transmittance of nanoemulsion before and after a freeze-thaw cycle.

| Parameter | Formula | | | | | |
|---|---|---|---|---|---|---|
| | FI | | FII | | FIII | |
| | Before | After | Before | After | Before | After |
| consistency | liquid | liquid | liquid | liquid | liquid | liquid |
| Colour | bright yellow | bright yellow | bright yellow | bright yellow | bright yellow | bright yellow |
| Odor | typical of extract odor | typical of extract odor | typical of extract odor | typical of extract odor | typical of extract odor | typical of extract odor |
| Transmittance percent (%) | 94.23±1.18 | 90.17±2.50 | 98.00±1.50 | 92.20±3.30 | 95.00±0.47 | 92.57±3.12 |

solubility of the nanoemulsion preparation and the ethanolic extract preparation of nagasari (*M. ferrea* L.). The results showed that the ethanolic extract of Nagasari leaves dissolved in 1.1 L of distilled water (very poor solubility), whereas the nanoemulsion dissolved in 1 mL of distilled water (excellent solubility) [24]. Thus, the solubility of Nagasari leaf extract increased before and after the preparation of the nano-emulsion. It shows that nanoemulsion preparation can be used to improve the solubility of drugs.

### Percent Transmittance Test

A percent transmittance test is performed on days 1 and 24 to measure the clarity of the formed nanoemulsion. The percentage transmission is measured with a UV-visible spectrophotometer at 650 nm. The clarity of nanoemulsions is typically assessed using spectrophotometry at 650 nm. This wavelength is often used because it enables the percentage transmittance to be accurately measured and indicates the nanoemulsion's clarity [24].

Table 3 shows that all the nanoemulsion preparations produced have clear characteristics, with a percent transmittance value close to 100%. In addition, the amount of surfactant and co-surfactant should be more than the oil phase to cover the oil droplets when emulsified in water and produce droplet sizes in the nanometer range [25,26]. The percent transmittance of the three nanoemulsion formulations decreased after the freeze-thaw test. Extreme temperature changes influence the decrease in percent transmittance during the freeze-thaw cycle. Nanoemulsion looks like a little cloudy solution. The rise in the transmittance percentage is attributed to alterations in the clarity's shape due to the particles in the preparation reassembling to form bigger globules [27,28].

### Particle Size Analysis and Polydispersity Index

Particle size analysis determines the particle size and distribution in the resulting nanoemulsion preparation. The particle size distribution shows the size uniformity of the resulting nanoemulsion. The particle size distribution index (PDI) values obtained using the three formulas

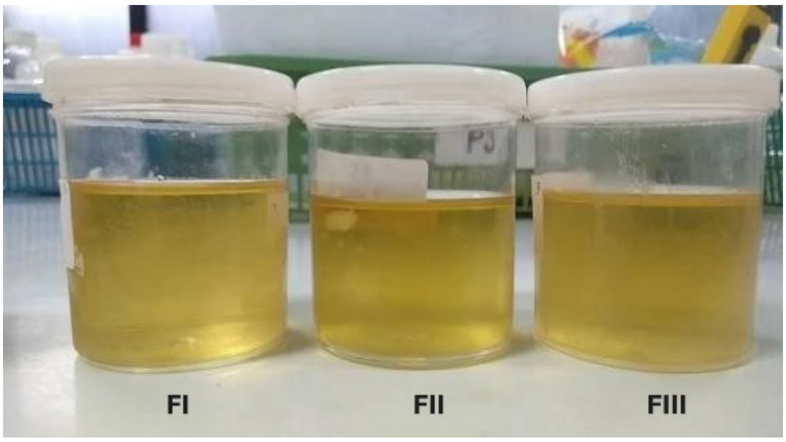



**Figure 2.** Nagasari extract nanoemulsion.

**Table 6.** Result of inhibition zone diameter of nanoemulsion.

| No. | Sample | Inhibition zone diameter (mm) | Criteria |
|---|---|---|---|
| 1. | Blank Formula | 6.00 ± 0.00 | Moderate |
| 2. | FI nanoemulsion | 19.15 ± 0.50 | Strong |
| 3. | Extract of Nagasari | 15.80 ± 0.28 | Strong |
| 4. | Tetracycline (control +) | 38.65 ± 0.21 | Very strong |
| 5. | Aquadest *(c*ontrol-) | 0 ± 0.00 | Negative |

ranged from 0.29 to 0.34, which indicates that the particle sizes in the resulting nanoemulsions produced using all three formulas are uniform [29]. In addition, this test is performed on all formulations using the Zetasizer series particle size analyzer (Malvern). The results are analyzed using the Zetasizer software. The results of the particle size analysis are shown in the Table 4.

The nanoemulsion of Nagasari leaf extract (*M. ferrea* L.) shows particle sizes of F1, F2, and F3 with average values of 103.07 nm, 72.2 nm, and 65.53 nm, based on the measurement results shown in Table 4. Thus, the particle size in each formula is within the required range of 20-200 nm. The results of the statistical analysis of the One-way ANOVA test show that there is an insignificant difference with a p-value of 0.523 (>0.05), so the difference in the concentration of the co-surfactant PEG 400 does not affect the particle size of the nanoemulsion preparation of the ethanolic extract of Nagasari leaves. The polydispersity index (PDI) is a value used to estimate the particle size distribution range in a nanoparticle system and determine the presence or absence of aggregation. In addition, the PDI value has a range of 0-1. The smaller the PDI value, the more uniform the particle size distribution in the preparation system [23,29]. Based on the table, it can be seen that the polydispersity index (PDI) has a value below one or closer to 0. It indicates that the distribution of nanoemulsion particles is uniform. Therefore, the resulting nanoemulsion formulation has a good particle distribution.

## Zeta Potential Test

The zeta potential value indicates the stability of a system containing dispersed spheres due to the repulsion between particles of the same charge when they are close together. The zeta potential results of nanoemulsion preparations are shown in Table 4. Based on the data in the table, there is no significant difference since the considerable value is 0.89 (p>0.05). Thus, the variation of the concentration of PEG 400 co-surfactant does not affect the nanoemulsion of ethanol extract of Nagasari leaves extract (*M. ferrea* L.) However, the resulting nanoemulsions in formulas 1, 2, and 3 do not follow the

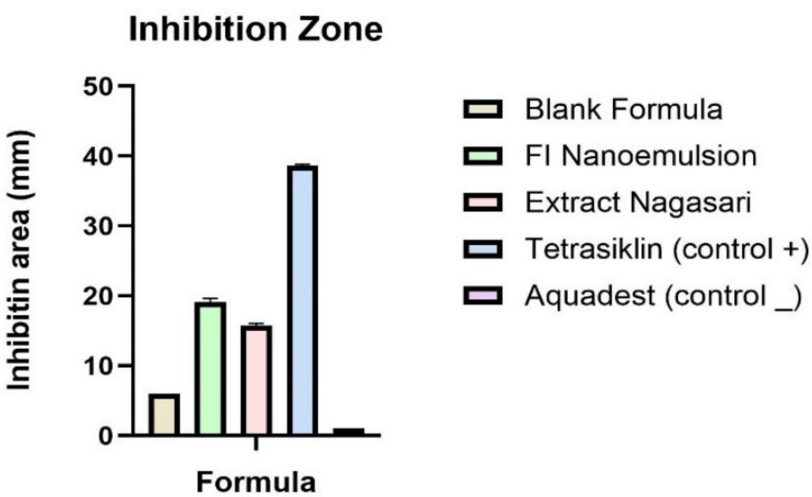
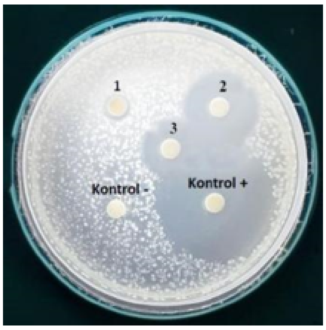



**Figure 3.** Antibacterial Activity against *B.subtilis*.




Explanation:

6. Formula 0 nanoemulsion
7. Formula 1 nanoemulsion
8. Ethanol extract from nagasari leaves
9. Tetrasiklin (control +)
10. Aquadest (control -)

**Figure 4.** The result of antibacterial activity using the disc diffusion method.

good zeta potential range, which is less than -30 mV or more than +30 mV [25,26].

Components in the nanoemulsion, such as Nagasari metabolites, VCO, and surfactants, affect the zeta potential value. The cause of the unsuitable zeta potential can be reviewed from other aspects, such as pH or the possibility of chemical compounds contained in the extract with functional groups that can affect the electron charge or the type of water used in the formulation. Therefore, it tends to decrease the zeta potential value. Most of the particles dispersed in water become negatively charged due to the adsorption that prefers hydroxyl ions [26].

### Evaluation of the Stability of The Freeze-Thaw Method

The freeze-thaw method is carried out for six cycles to see any changes in the appearance of the preparation due to extreme temperature changes during the test process. The test includes organoleptic consistency, colour, odour, and percent transmittance observations. The test results are shown in Table 5.

The results are in Table 5. They show no physical changes, such as no changes in colour, texture, or odor, after the freeze-thaw cycle. The conditions indicate that all formulations in the resulting nanoemulsion preparations are stable.

Furthermore, the statistical calculation of the transmittance percentage before and after the freeze-thaw cycle is not significantly different, with a p-value > 0.05, which is 0.394. Therefore, it shows that the formulation of nanoemulsion preparations of ethanolic extract of Nagasari leaves at F1, F2, and F3 resulted in nanoemulsion preparations with stable organoleptic and transmittance percentages.

### Antibacterial Activity Test

The antibacterial activity test was performed on the selected formula, FI, since it has a zeta potential value closest to -30 mV, which improves the preparation's stability. The results of observing the diameter of the inhibition zone are shown in Table 6.

Antibacterial activity test results show that the inhibition zone measurement in FI nanoemulsion resulted in an inhibition zone diameter of 19.15 ± 0.50 mm against Bacillus subtilis. Thus, the criteria for the inhibition zone are strong since it is in the range of 16.00-20.00 mm [30]. The ethanolic extract of Nagasari leaves has a smaller inhibition zone than FI, which is 15.80 ± 0.28 mm. Meanwhile, the positive control with tetracycline has the strongest inhibition zone, 38.65 ± 0,21 mm.

It is known to contain flavonoid compounds, tannins, and terpenoids based on the research conducted by Chaithanya et al. (2019) on the phytochemical test of the ethanolic extract of Nagasari leaves [3]. These compounds act as antibacterial agents. Moreover, the mechanism of action of flavonoids as antibacterial agents can be carried out in 3 ways: inhibition of nucleic acid synthesis, inhibition of cell membrane function, and inhibition of energy metabolism. In addition, the content of tannin compounds has an antibacterial effect, which is related to its ability to inactivate bacterial adhesives, inhibit enzyme work, and inhibit protein transport in the cell envelope. Meanwhile, terpenoids have an antibacterial mechanism by damaging the bacterial cell membrane [30].

The blank results, the formulation of nanoemulsion without Nagasari leaf extract, show antibacterial activity, including the moderate criteria, i.e. 6.00 ± 0,0 mm. This activity indicates that VCO has potential as an antibacterial agent. It occurs because the nanoemulsion formulation uses virgin coconut oil (VCO) oil phase, which contains saturated fatty acids, including medium chain fatty acid (MCFA) and medium chain triglycerides (MCT) [27]. MCFA in the form of lauric acid has antiviral, antibacterial, and antiprotozoal properties. Lauric acid works by breaking down the peptide chain that makes up peptidoglycan. They weaken the bacterial cell wall and lead to lysis. Bacteria cannot survive external influences without a cell wall and die immediately [26]. The results prove that nanoemulsion preparations can enhance their antibacterial efficacy.

Active ingredient particles in the nano-size range (50-200 nm) more easily penetrate the cell membrane and shed the phospholipid layer of the bacterial cell membrane. This ability increases due to the increased solubility of the particles and the large surface area. These active ingredients then weaken the bacterial cell wall and cause lysis. Bacteria cannot survive external influences without a cell wall and die immediately. Therefore, it can be concluded that the antibacterial activity of Formula 1 nanoemulsion containing ethanolic nagasari leaf extract and VCO is due to the nagasari leaf extract. Additionally, the nanoscale size of the Nagasari leaf extract enables it to penetrate bacterial cells with diameters up to the nanometer scale, thereby inhibiting bacterial growth [27,30].

## Conclusion

Variation of PEG 400 co-surfactant affected the stability and physical properties of the nanoemulsion preparation. Better stability was obtained with the formula (FI). The results of the physical property evaluation of FI nanoemulsion showed stable colour, pH 6.2 ± 0.15, emulsion type: o/w, transmission percentage 94.23 ± 1.18, polydispersity index (PDI) 0.345 ± 0.051, particle size 103.07 ± 51.64, zeta potential -6.47 ± 2.20 and stable in the freeze-thaw test. B. subtilis antibacterial activity test results showed a 19.15 mm inhibition zone.

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