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Optimization of VCO, TWEEN 80, and PEG 400 Nanoemulsion Formulation of *Gemitir* Flower Extract (*Tagetes Erecta* L.) with *Simplex Lattice Design*

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Abstract

Background: *Tagetes erecta L.* (*Gemitir* flower) is known to have potential antibacterial activity; however, its use in topical preparations is limited by low solubility and stability.
Objective: This study aims to optimize the nanoemulsion formulation of *Gemitir* flower extract using virgin coconut oil (VCO) as the oil phase, Tween 80 as the surfactant, and PEG 400 as the cosurfactant.
Methods: Phytochemical screening, antibacterial activity testing against *Staphylococcus aureus*, total flavonoid determination, and nanoemulsion formulation optimization were conducted using the Simplex Lattice Design method with Design Expert® software version 13 to evaluate the effects of VCO, Tween 80, and PEG 400 combinations on the physical characteristics of the nanoemulsion.
Results: The optimal formulation was obtained with a composition of VCO 0.53 g, Tween 80 8 g, and PEG 400 4 g, with a desirability value of 0.990. The resulting nanoemulsion showed a transmittance value of 97.56%, entrapment efficiency of 1.46%, pH of 5.26, and no phase separation during storage (score = 1). The homogeneity test confirmed a uniform preparation, and the emulsion type test indicated an oil-in-water (O/W) system.
Conclusion: The nanoemulsion formulation of *Gemitir* flower extract using VCO, Tween 80, and PEG 400 optimized through Simplex Lattice Design produced a system with appropriate physical characteristics for topical antibacterial application. However, further studies on particle size, zeta potential, and long-term stability are recommended.

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INTRODUCTION

Indonesia is a country with a large number of flora and fauna species, making it one of the countries with very high levels of biodiversity, including ecosystem, species, and genetic diversity. Based on global biodiversity rankings, Indonesia is placed second after Brazil. In terms of flora, it is estimated that there are approximately 40,000 plant species in Indonesia, and 40% of them are endemic. Meanwhile, around 25,000 to 30,000 of these plants are considered to have potential as

medicinal plants or sources of traditional medicine (Yulisma and Fathiya, 2023). The richness of Indonesian medicinal plants forms the basis for developing novel pharmaceutical preparations with therapeutic potential (Yulisma and Fathiya, 2023).

One of the Indonesian plants that has the potential to become a medicinal plant is the glistening flower. *Gemitir* flowers or also called *Marigold Flower*, is one of the ornamental plants of the type of kenikir that has the potential to be developed because of its many uses (Kurniati, 2021). *Gemitir* flowers are widely cultivated in several regions in Indonesia for medicinal purposes, as ornamental plants and natural food coloring. In Bali, the *Gemitir* plant, especially in the flower, is widely used as a means of daily religious ceremonies and at certain events (Santi, 2021). The ethnopharmacological use of *Gemitir* as a medicinal plant supports further investigation into its bioactive compounds for pharmaceutical formulation development.

Apart from being an ornamental flower, in the health sector, the *Gemitir* flower has the function of being an antioxidant, antibacterial, anti-inflammatory, and anticarcinogenic. The yellow color of the *Gemitir* flower is caused by the presence of two main pigments, namely pigments from the carotenoid group and flavonoids. *Gemitir* flower extract contains about 27% carotenoid pigment and specifically for the crown of the *Gemitir* flower contains about 200 times greater carotenoids than the carotenoids contained by corn (Kurniati, 2021). The antibacterial mechanism of flavonoids involves disruption of bacterial cell membrane integrity, inhibition of enzymatic activity, and interference with nucleic acid synthesis.

Based on a study, the prevalence of acne disease is more experienced by women (69.7%) than men (30.3%). At a young age (16–25 years), more people experience acne with a prevalence of 59.1% (Sari et al., 2023). In research Imasari (2022), prevalence of *Staphylococcus aureus* as the cause of acne reaches 79%, while *Staphylococcus epidermidis* accounts for 21%. *Staphylococcus aureus* are gram-positive bacteria that live in the membranes of the human body, skin surfaces, sweat glands, and intestinal tract. Prevention of antibiotic resistance used to inhibit bacterial growth of *Staphylococcus aureus* can be assisted by using natural or plant-based ingredients with antibacterial chemical content (Sari and Al Basyarahil, 2021). *Gemitir* (*Tagetes erecta* L.) is proven to have antibacterial activity, so it can be used in the treatment of various infectious diseases caused by pathogenic bacteria, including alkaloids, phenolics, flavonoids, and carotenoids. Previous research has proven the antibacterial effect of *Gemitir* flowers on *Streptococcus pyogenes*. In addition, patulitrin, a flavonoid isolated from the *Gemitir* flower, is known to inhibit *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Marsah et al., 2024).

Based on research Afifah (2024) Test of the antibacterial activity of *Gemitir* flower ethanol extract against bacteria *Staphylococcus aureus* obtained from the concentrations of 85%, 90%, 95%, and 100% respectively, the inhibition zones are 30.9 mm, 31.6 mm, 28.8 mm, and 28.6 mm with the category of very strong inhibition. The highest inhibition zone is obtained from the concentration of 90% *Gemitir* flower extract (*Tagetes erecta* L.) against bacteria *Staphylococcus aureus* which is 31.6 mm. Despite its demonstrated antibacterial potential, research on the development of *Gemitir* flower extract into stable pharmaceutical nanoemulsion formulations optimized through experimental design methods remains limited, indicating a significant research gap.

The biological activity of flavonoids, which are derivatives of phenol compounds against bacteria, is caused by the damage to the bacterial cell wall which is composed of lipids and amino acids that react with the alcohol group in phenol compounds. These substances enter the nucleus of bacterial cells as a result of the destruction of the cell wall, interact with the DNA in it, and damage the lipid structure of the bacterial DNA. This causes the bacterial cell nucleus to become lysis. Flavonoids work by altering the microbial cell membrane as well as inhibiting energy metabolism and nucleic acid synthesis.

Flavonoid compounds found in *Gemitir* flowers (*Tagetes erecta* L.) has been shown to have activity as an antibacterial. Quercetin, which is a phenolic flavonoid compound, often has limitations in its low bioavailability which makes it difficult for the body to absorb. In addition, quercetin is also difficult to dissolve in water and has a particle size of more than 300 nm (Rahman *et al*., 2023). According to Santi (2021) compounds contained in the *Gemitir* flower (*Tagetes erecta* L.) is highly susceptible to oxidation and high-temperature heating. One way to increase the bioavailability of *Gemitir* flower extract (*Tagetes erecta* L.) in the body is made into a

nanoemulsion preparation. Nano-sized preparation delivery systems are useful for protecting, transporting and releasing bioactive compounds and increasing the bioavailability of lipophilic compounds in aqueous media. The nanosizing of plant extracts via nanoemulsion systems has been widely demonstrated to improve bioavailability of lipophilic bioactive compounds including flavonoids (Priani et al., 2024).

In today's modern era, nanotechnology is widely used in the delivery system of active substances of medicinal preparations. Particles or globules at the nanometer scale can regulate the release rate of active substances, increase solubility, and increase drug absorption. Nanoemulsion is a form of preparation consisting of an oil phase and a water phase stabilized by a combination of surfactants and cosurfactants with droplets between 100-200 nm in size. The small size of the droplets can prevent sedimentation, flocculation, and fluctuations. The smaller the size of the dispersed particles, the easier it is for the preparation to penetrate the surface of the skin so that it can increase the penetration of the active substance into the skin and increase its effectiveness. Penetration of active substances is the process of entering the active substances into the skin (Firmansyah et al., 2022).

According to Aprilya (2021) Nanoemulsion is a lipid-based drug delivery system that is thermodynamically stable consisting of an oil phase, surfactant, cosurfactant, and water phase that has a droplet size in nanometers. Currently nanoemulsions have been reported to have desirable characteristics such as high drug solubility, significant protection, and thermodynamic stability. Nanoemulsions help lipophilic drugs to be absorbed faster and better compared to oil solutions. The droplet diameter of a nanoemulsion matrix system depends on the type of oil, type of surfactant, and temperature.

In most cases, the use of a single surfactant is not enough to lower the surface tension between oil and water, so cosurfactant assistance is needed to help lower the surface tension. Examples of cosurfactants that are commonly used in nanoemulsion formulations are propylene glycol, PEG 400, and glycerin. The advantage of propylene glycol cosurfactants is that they can help solubilize hydrophilic surfactants in an oil base. PEG 400 cosurfactant is Mid Chain Hydrocarbon which can be placed between the gaps of the nanoemulsion system with the formation of hydrogen chains so that it can maximize the emulsification process in the manufacture of nanoemulsion preparations (Nirmalayanti, 2021).

In this study, Virgin Coconut Oil (VCO) is used as an oil phase. VCO contains Medium Chain Triglyceride (MCT) which can produce nanoemulsion preparations that are more stable and clearer than oils that contain Long Chain Triglyseride (LCT). MCTs dissolve more easily in water than LCTs because they contain more polar groups. So that the oil phase carrier material, MCTs can be better at interacting and connecting with each other. This results in the MCT being able to expand the surface and form a stable emulsion (Zubaydah et al., 2023).

The optimization of the nanoemulsion formula in this study was carried out using the Simplex Lattice Design. This method is found in Software Design Expert to determine the optimal formula of the ingredients used to make the right preparation so that a formula that has optimal physical properties is obtained with the response desired by the consumer. The advantage of this method is that it determines the formula faster and more effectively so that it can be avoided from determining the formula in a systematic manner Trial and error (Dwiputri et al., 2022).

Simplex Lattice Design (SLD) is a mixed design in formulation optimization provided that the total number of ingredients used in the formulation is always constant. Simplex Lattice Design operated with Software called Design Expert. Formulation ingredients are classified as independent variables. Simplex Lattice Design Produce 7-15 formulations with 3 independent variables and 2-3 responses (Akbar et al., 2022). According to Pratiwi (2024), SLD (Simplex Lattice Design) is one of the DE (Experimental Design) methods used for variable mix optimization with the total number of the proportion of the mixture must be one (100%) and consist of at least two types of variables. The concentration of the variables used changes simultaneously but still maintains a fixed final weight of the preparation. Therefore, this method is the right choice for optimizing the mixture of additives.

Based on the description above, this study was conducted with the following aims: (1) to perform phytochemical screening and determine the total flavonoid content of *Gemitir* flower extract (*Tagetes erecta* L.); (2) to evaluate the antibacterial activity of *Gemitir* flower extract

against *Staphylococcus aureus*; and (3) to determine the optimal nanoemulsion formula using Virgin Coconut Oil (VCO) as the oil phase, Tween 80 as surfactant, and PEG 400 as cosurfactant using the Simplex Lattice Design method, in order to produce nanoemulsions with high transmittance, optimal adsorption efficiency, and physical stability suitable for topical antibacterial application.

METHOD

This research was an experimental laboratory study with a quantitative approach and used the Simplex Lattice Design method to optimize a nanoemulsion formulation consisting of Virgin Coconut Oil (VCO) as the oil phase, Tween 80 as a surfactant, and PEG 400 as a cosurfactant. The study was carried out at the Pharmaceutical and Pharmaceutical Technology Laboratory, Bintang Persada Institute of Technology and Health, Denpasar.

Tools

The instruments used in this study included a pH meter, overhead stirrer, centrifuge, UV–Vis spectrophotometer, analytical balance (Kenko), measuring cups (Pyrex), Erlenmeyer flasks (Pyrex), beaker glasses (Pyrex), test tubes (Iwaki), vial bottles, dropper pipettes, volumetric pipettes (Pyrex), stirring rods (Pyrex), and electrodes.

Ingredients

The materials used in this study included ethanol extract of *Gemitir* flower (Tagetes erecta L.) obtained from local farmers in Apuan Village, Baturiti District, Tabanan Regency; Virgin Coconut Oil (VCO) (Bratachem), Tween 80 (Bratachem), PEG 400 (Bratachem), phenoxyethanol, and distilled water (aquadest).

Extraction of *Gemitir* Flower (*Tagetes erecta* L.)

In a sealed container, dried flower powder was macerated with 96% ethanol in a ratio of 1:10 (272 g/2,720 mL). The mixture was left at room temperature for 72 hours to obtain a liquid extract rich in bioactive compounds. After the maceration process was completed, the extract was concentrated using a rotary evaporator at 60°C and 25 rpm until a thick extract was obtained (Marsah et al., 2024). The yield percentage of the extract was calculated as the ratio of the weight of the concentrated extract to the initial dry powder weight, expressed as a percentage.

Phytochemical Screening of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Phytochemical screening of *Gemitir* flower extract (Tagetes erecta L.) was performed to detect flavonoids, tannins, alkaloids, saponins, triterpenoids, and steroids using a modified standard procedure.

Antibacterial Activity Test of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The 96% ethanol extract of *Gemitir* flower was dissolved in 10% DMSO at concentrations of 1%, 1.5%, and 2%. Clindamycin 1% cream (Medi-Klin) was used as a positive control, while 96% ethanol was used as a negative control. *Staphylococcus aureus* suspension was adjusted to McFarland standard 0.5 (1.5×10^8 CFU/mL). The antibacterial activity test was conducted using the well diffusion method on Mueller–Hinton Agar (MHA) medium. A total of 10 µL of extract was placed into a 6 mm diameter well, incubated for 48 hours, and the inhibition zone was then measured. Each treatment was repeated three times.

Optimization of Nanoemulsion Formula of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The optimization of the nanoemulsion formulation of *Gemitir* flower extract (*Tagetes erecta L.*) was carried out using Design Expert® software version 13 with the Simplex Lattice Design method. Variations in the concentrations of extract, VCO (oil phase), Tween 80 (surfactant), and PEG 400 (cosurfactant) were input into the software within defined lower and upper limits, resulting in 13 experimental runs.

Physical quality evaluations included percent transmittance (UV–Vis spectrophotometry at λ 650 nm), pH, adsorption efficiency (centrifugation at 3000 rpm for 15 minutes), and phase

separation test (centrifugation at 3500 rpm for 3 × 10 minutes) (Putri et al., 2024). Additionally, particle size analysis using dynamic light scattering (DLS) with a Malvern Zetasizer instrument was conducted to confirm nanoemulsion droplet size within the range of 100–200 nm and to validate nanoscale characteristics of the formulation.

Based on the four responses (% transmittance, pH, adsorption efficiency, and phase separation), the two primary responses (% transmittance and phase separation) were assigned the highest importance. The optimal formula was selected based on a desirability value close to 1, resulting in Formula 1 with a desirability value of 0.990. The optimal formula was then re-evaluated, including tests for transmittance, pH, adsorption efficiency, phase separation, organoleptic properties, emulsion type, dispersibility, and adhesion.

Data Analysis

The results of physical quality evaluation (% transmittance, pH, adsorption efficiency, and phase separation) from 13 nanoemulsion formulations were analyzed using Design Expert® software version 13 with an ANOVA approach and mathematical models (linear, quadratic, and special cubic). The highest weight of importance was assigned to % transmittance and phase separation parameters.

The optimal formula was selected based on a desirability value close to 1. Verification of the optimal formula was performed using a one-sample test with OpenStat software to compare experimental results with software predictions based on significance (p-value).

RESULTS AND DISCUSSION

Results

Plant Determination Results

The determination of the *Gemitir* flower plant has been carried out at the Materia Medica Herbal Laboratory UPT, located on Jl. Lahor No. 87, Batu City, on April 9, 2025. The following are the results of the determination of the *Gemitir* flower plant (*Tagetes erecta* L.):

Figure 1. *Gemitir* Flower Plants Used in Research
Source: Personal Documentation (2025)

Tribes:	Asteraceae/Compositae
By Name:	Tagetes
Type:	Tagetes erecta L.
Region Name:	Gemini flowers, gumitir, african marigolds, ades, poop kotok, suitable bottles (Sundanese), kenikir (Javanese)
Morphology:	The shrub is small, grows upright, can reach 2 m in height. Hairy, smooth stems. Leaves are ovoid, light green, 2-9 cm long, tip pointed, alternately located, distinctively smelling. Flowers compound, panicle shape, coming out of the leaf armpits, branched, yellowish-white color. The fruit is small, hard, brown

in color. Whitish brown seeds. Propagation by seeds and cuttings

Phytochemical Screening Test Results of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The following results of phytochemical screening conducted in this study include screening of secondary metabolites in the form of alkaloids, flavonoids, tannins and saponins.

Table 1. Phytochemical Screening Results

Compound Groups	Reagents	Observation Results	Test Results
Alkaloids	Dragendorff	There is turbidity/sediment at the bottom of the tube	+
Flavonoids	Heated mg powder and concentrated HCl	No changes	-
	NaOH	There is a change to bright yellow	+
Saponins	Aquadest	There is a stable foam	+
Tannins	$FeCl_3$	Blackish green color	+

Antibacterial Test Results of *Gemitir* Flower Extract (*Tagetes erecta* L)

The following are the results of the antibacterial activity test of 96% ethanol extract of *Gemitir* flower (*Tagetes erecta* L.) against *Staphylococcus aureus* bacteria using the well diffusion *method using* a cork borer tool with a diameter of 6 mm, then each well is filled with a test treatment of 10µL.

Table 2. Antibacterial Activity Test Results

Treatment	Jamming Diameter				
	Replication 1 (mm)	Replication 2 (mm)	Replication 3 (mm)	Average (mm)	Categories
Concentration 1%	8 mm	7.5 mm	7.5 mm	7.6 mm	Medium
Concentration 1.5%	9 mm	10.5 mm	8.5 mm	9.3 mm	Medium
Concentration 2%	16 mm	15.5 mm	15.5 mm	15.6 mm	Strong
(+) Controls	32 mm	34 mm	34 mm	33.3 mm	Very Powerful
Control (-)	0	0	0	0	Weak

Results of Determination of Flavonoid Levels of *Gemitir* Flower Extract

The following are the results of determining the level of flavonoids in *Gemitir* flower extract (*Tagetes erecta* L.). The determination of flavonoid levels of *Gemitir* flower extract begins with the manufacture of a test solution using routine standards. Furthermore, the maximum wavelength measurement was carried out which was measured in the range of 400-800nm. Based on the test results, the maximum wavelength value was obtained which was 445nm.

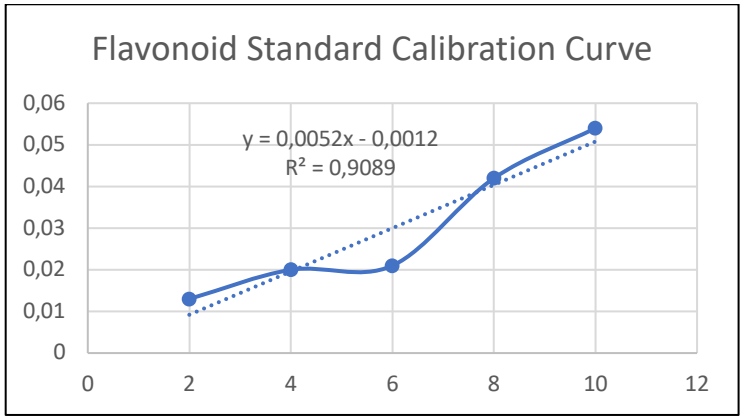

Figure 2. Flavonoid Standard Calibration Curve

Figure 2 shows that the resulting raw curve is linear. The linear standard curve means that when there is an increase in absorbance, it will be followed by a proportional increase in the level. The relationship between the content and absorbance is expressed in r and the absorbance of the sample solution must also be within the absorbance range of the standard curve series.

Optimization of Nanoemulsion Formula of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The optimization of the nanoemulsion formula of *Gemitir* flower extract (*Tagetes erecta L.*) was carried out using software design expert® version 13 by applying the Simplex Lattice Design method. The lower limit and upper limit values are entered in the software so that 13 run formulas are obtained as follows:

Table 3. Nanoemulsion Formula of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Run	***Gemitir* Flower Extract (*Tagetes erecta* L.) (gr)**	**VCO (gr)**	**Tween 80 (gr)**	**PEG 400 (gr)**	**Ethanol phenoxy (gr)**	**Aduadest**
1	0.5	0.5	8	4	0.5	Add 100
2	0.5	1.83	7.43	3.33	0.5	Add 100
3	0.5	1.5	8	3	0.5	Add 100
4	0.5	2.5	7	3	0.5	Add 100
5	0.5	2.5	7	3	0.5	Add 100
6	0.5	1.5	7	4	0.5	Add 100
7	0.5	1.5	8	3	0.5	Add 100
8	0.5	2.5	6	4	0.5	Add 100
9	0.5	2.5	8	2	0.5	Add 100
10	0.5	1.5	8	3	0.5	Add 100
11	0.5	2.5	7	3	0.5	Add 100
12	0.5	1.5	7	4	0.5	Add 100
13	0.5	1.5	7	4	0.5	Add 100

Results of Physical Quality Evaluation of *Gemitir* Flower Extract Nanoemulsion (*Tagetes erecta* L.)

The following are the results of the physical quality evaluation of the formulation of the nanoemulsion of *Gemitir* flower extract (*Tagetes erecta* L.) carried out with 4 responses, namely the %transmitter test, the pH test, the adsorption efficiency test, and the phase separation test.

Figure 3. Result 13 *Run* Nanoemulsion

Table 4. Results of %Transmittant Test, pH Test, Adsorption Efficiency Test, and Phase Separation Test

Run	Test Results			
	%Transmit (%)	**Adsorption Efficiency (%)**	**Phase Separation**	**pH**
1	99,2%	1,7701%	1	5,15
2	99,9%	1,272%	1	6,74
3	99,4%	1,4121%	1	6,64
4	89,9%	1,4026%	0,9	6,36
5	90,1%	1,3923%	0,9	6,36
6	100%	1,5742%	1	6,26
7	99,1%	1,4569%	1	6,64
8	80,6%	0,8481%	0,85	6,53
9	97,7%	1,0644%	1	6,86
10	99,7%	1,5178%	1	6,64
11	90,1%	1,5332%	0,9	6,36
12	100%	1,3351%	1	6,26
13	99,2%	1,7085%	1	6,26

Optimal Formula of Nanoemulsion of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Based on the optimization of the nanoemulsion formula of mourning flower extract (*Tagetes erecta* L.) with four responses (% transmitter, pH, adsorption efficiency, and phase separation), the two main responses (transmitter and phase separation) were given the highest level of importance (+++++). Of the six recommended formulas, formula 1 was chosen as the optimal formula because it has a desirability value closest to 1, which is 0.990.

Table 5. Optimal Formula of Nanoemulsion of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Formula	**VCO**	**Tween 80**	**PEG 400**	***Desirability***
1	**0,529**	**7,971**	**4**	**0,990**

Results of Physical Quality Evaluation of the Optimal Formula of *Gemitir* Flower Extract Nanoemulsion (*Tagetes erecta* L.)

The following are the results of the evaluation of the physical quality of the optimal formula of the nanoemulsion of *Gemitir* flower extract (*Tagetes erecta* L.).

Table 6. Results of Physical Quality Evaluation of Optimal Formula of *Gemitir* Flower Extract Nanoemulsion (*Tagetes erecta* L.)

Yes	Physical Quality Evaluation	Test Results
1	% Transmitter (%)	97,5%
2	Adsorption Efficiency (%)	1,46223%
3	Phase Separation	1
4	pH	5,26
5	Organoleptis	Yellow Color, Distinctive Smell of Sparkling Flowers, Liquid Form
6	Emulsion Type	Oil in water
7	Homogeneity	Homogeneous
8	Spreadability	5.4 cm
9	Adhesive	0.20 seconds

Discussion

Phytochemical Screening Results of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Phytochemical screening aims to identify the presence of secondary metabolites in 96% ethanol extracts of *Gemitir* flowers (*Tagetes erecta L.*) to determine compounds with potential antibacterial activity (Putri and Lubis, 2020). The alkaloid test using Dragendorff's reagent showed positive results, characterized by the formation of a brownish-orange precipitate, indicating the presence of alkaloid compounds in the extract.

Meanwhile, the flavonoid test was conducted by adding magnesium powder (Mg) and concentrated HCl, followed by heating. The result was negative, as no red, orange, or purplish-red coloration was observed. The absence of flavonoids detected by the Mg-HCl method may be attributed to low flavonoid content or incomplete extraction of polar or glycoside-bound flavonoid compounds (Permatasari, 2020). However, the presence of flavonoids was confirmed through a positive reaction with NaOH solution, which produced a distinct yellow coloration due to its interaction with hydroxyl groups in the flavonoid structure.

The saponin test, performed using the foam formation method with hot distilled water, showed positive results, indicated by stable foam formation exceeding 1 cm in height. The tannin test using $FeCl_3$ solution produced a blue-black or dark green discoloration, indicating a positive result. These outcomes are influenced by compound solubility in ethanol and their reactivity with specific reagents under controlled conditions. Collectively, these secondary metabolites, flavonoids, alkaloids, saponins, and tannins, contribute to the antibacterial potential of *Gemitir* flower extract against pathogenic bacteria (Marsah et al., 2024).

Antibacterial Test Results of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The antibacterial activity of *Gemitir* flower extract (*Tagetes erecta L.*) was evaluated using the well diffusion method. This method was selected due to its ability to clearly visualize inhibition zones and facilitate the measurement of antibacterial activity. However, the well diffusion method has limitations, as results depend on the diffusion capacity of active compounds in the medium; compounds with low diffusion ability may produce small inhibition zones despite strong antibacterial activity.

The test bacterium used in this study was Staphylococcus aureus, obtained from the Microbiology Laboratory, Udayana University, with proof of receipt documented in Attachment 2. Antibacterial activity was indicated by the formation of inhibition zones around the wells in the inoculated medium.

In this study, a 96% ethanol extract of *Gemitir* flowers was formulated into three concentration series: 1%, 1.5%, and 2%. These variations were designed to identify and compare the effectiveness of the extract in inhibiting the growth of Staphylococcus aureus. Dimethyl sulfoxide (DMSO) 10% was used as the solvent for dissolving the extract. The medium used was Mueller Hinton Agar (MHA). Clindamycin (150 mg capsules) was used as the positive control, while 96% ethanol served as the negative control. Wells were created using a cork borer with a diameter of 6 mm.

This research was conducted in three replications to obtain reliable and consistent data and to improve the accuracy of the results. The antibacterial activity of the ethanol extract of *Gemitir* flower (*Tagetes erecta L.*) was indicated by the formation of clear inhibition zones around the wells in the test medium at concentrations of 1%, 1.5%, and 2%.

The results showed that the 96% ethanol extract of *Gemitir* flower (*Tagetes erecta L.*) exhibited the most effective inhibitory activity against the growth of Staphylococcus aureus at a concentration of 2%.

This study evaluated the antibacterial activity of 96% ethanol extract of *Gemitir* flower (*Tagetes erecta L.*) against Staphylococcus aureus using the well diffusion method. The test was conducted at extract concentrations of 1%, 1.5%, and 2%, with clindamycin as a positive control and 96% ethanol as a negative control. Antibacterial activity was indicated by the formation of clear inhibition zones around the wells. Inhibition zones were also observed in wells containing clindamycin, while no clear zones were observed in wells containing 96% ethanol, indicating the absence of antibacterial activity of the solvent.

The diameter of the inhibition zone was measured along two perpendicular axes (vertical and horizontal) using a ruler, and the results were expressed in millimeters (mm). The average inhibition zone diameter was then calculated and analyzed to assess the antibacterial potential of the extract.

Each treatment is replicated three times to ensure data reliability and reduce the possibility of error factor influence, so that the results obtained reflect the effects of the treatment consistently (Yusri, 2020). The evaluation of antibacterial inhibition was carried out by comparing the diameter of the inhibition zone to the standard category, namely >21 mm (very strong), 11–20 mm (strong), 6–10 mm (medium), and <5 mm (weak), to determine the level of antibacterial effectiveness of each extract concentration (Fitri et al., 2024).

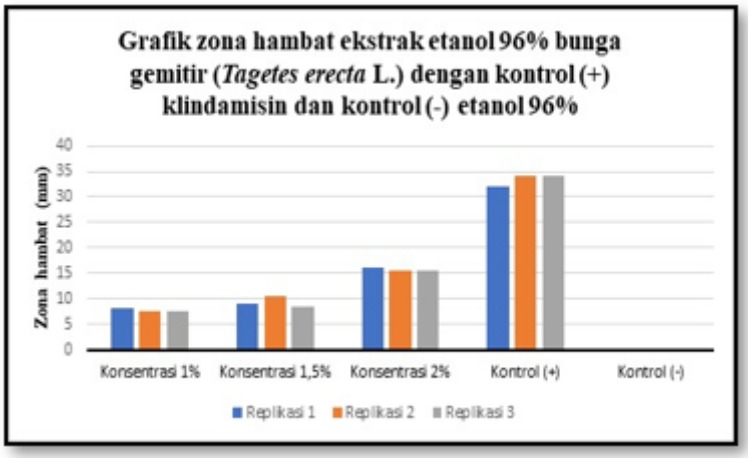

Figure 4. Graph of the bridge zone of 96% ethanol extract bunge gamitir (Tagetes erecta L.) with (+) clindamycin control and 96% ethanol (-) control

The graph illustrates the relationship between the concentration of 96% ethanol extract of *Gemitir* flowers (Tagetes erecta L.) and its antibacterial activity against Staphylococcus aureus, as indicated by the diameter of the inhibition zone. Based on the results of three replications, it is observed that increasing extract concentrations of 1%, 1.5%, and 2% correspond to larger inhibition zones. The 1% concentration produced an average inhibition zone of 7.6 mm (medium category), the 1.5% concentration produced 9.3 mm (medium category), and the 2% concentration produced 15.6 mm (strong category). These findings indicate that the antibacterial activity of the extract is concentration-dependent, where higher extract levels are directly proportional to increased bacterial growth inhibition.

This antibacterial effect is closely associated with the bioactive compounds present in *Gemitir* flower extract, particularly flavonoids. Flavonoids contain hydroxyl (-OH) groups and aromatic structures that play a significant role in antibacterial activity. The hydroxyl groups facilitate interactions with proteins and lipids in the bacterial cell membrane, while the aromatic

rings support penetration into microbial cell membranes. In addition to flavonoids, *Gemitir* flowers also contain alkaloids, saponins, and tannins; however, flavonoids are the primary compounds contributing to the extract's strong antibacterial inhibition (Puspitasari and Dari, 2022).

The mechanism of action of flavonoids in inhibiting Staphylococcus aureus involves several pathways. Flavonoids disrupt the structural integrity of bacterial cell membranes, induce leakage of intracellular contents, and inhibit essential enzymes required for bacterial metabolism. In addition, flavonoids interfere with nucleic acid synthesis and inhibit cell wall formation, ultimately suppressing bacterial growth and leading to cell death (Tridesianti et al., 2025).

Results of Determination of Flavonoid Levels of *Gemitir* Flower Extract (*Tagetes erecta* L.)

The determination of total flavonoid content of *Gemitir* flower extract was carried out using the UV-Vis spectrophotometry method with reference standards. Maximum wavelength determination of a standard solution at 10 µg/mL within the range of 400–800 nm resulted in a peak absorption at 445 nm. The addition of 10% $AlCl_3$ aims to induce a bathochromic effect, namely a shift in wavelength to a higher region so that it falls within the UV-Vis range, while forming a stable complex between Al^{3+} and the carbonyl group at C-4 and the hydroxyl group at C-3 or C-5 of flavonols. Subsequently, 1 M sodium acetate was added as a reaction stabilizer, and the solution was allowed to stand for 30 minutes to ensure that the complexation reaction occurred completely.

The calibration curve was constructed using concentrations of 2, 4, 6, 8, and 10 µg/mL, measured at a wavelength of 445 nm. The resulting linear regression equation is $y = 0.0052x - 0.0012$, with a correlation coefficient (r) of 0.9089. The coefficient of determination (R^2) is approximately 0.826, indicating that around 82.6% of the absorbance variation can be explained by concentration changes. This demonstrates an acceptable linear relationship between standard concentration and absorbance. However, further validation of the calibration curve using a wider concentration range and stricter standard solution preparation procedures is recommended to improve analytical accuracy (Haresmita and Pradani, 2022).

Formulation of *Gemitir* Flower Extract Nanoemulsion Preparation (*Tagetes erecta* L.) with *Simplex Lattice Design*

In the optimization of the nanoemulsion formulation of *Gemitir* flower extract using Design Expert software, three components were selected, namely VCO, Tween 80, and PEG 400. The minimum and maximum limits of each component were determined based on the range of the previous extended study, resulting in 13 formulation runs. The preparation of nanoemulsions was carried out using a low-energy emulsification method through spontaneous emulsification with a magnetic stirrer at an appropriate speed. The concentration of the *Gemitir* flower extract used was set at 0.5 grams because at this concentration the extract can be well dispersed in the oil phase without causing a significant increase in viscosity, while also producing a homogeneous mixture, high transmittance, droplet size <200 nm, and good physical stability. It should be noted that several runs (4, 5, 11 and 6, 12, 13) appear to have identical or near-identical component compositions. This is inherent to the Simplex Lattice Design, where replicate points at mixture vertices and centroids are intentionally included to assess the repeatability of responses and improve the statistical robustness of the model (Akbar et al., 2022).

Based on the results of the analysis using Simplex Lattice Design on the percent transmittance response, the special cubic model showed a significant influence (p-value < 0.0001). The coefficients for VCO (A) are negative (-19.23), while Tween 80 (B) and PEG 400 (C) are positive (+18.50 and +55.57). This indicates that an increase in the proportion of VCO tends to decrease the clarity of the nanoemulsion, whereas an increase in Tween 80 or PEG 400 increases the transmittance value. Two formulas (run 4 and 8) did not meet the transmittance limit of 90–100% because they were suspected of containing a relatively higher VCO proportion than the optimal formula. The ANOVA results confirmed the significance of the model ($p < 0.0001$) for the transmittance response. Complete ANOVA tables for all four responses are presented in the supplementary data, showing that the special cubic model provided the best fit across all evaluated physical quality parameters.

In the absorption efficiency response, the three components made a positive contribution (A = +8.94; B = +7.25; C = +8.30), with their combined interaction showing the strongest effect in enhancing active compound uptake. For the phase separation test, the largest coefficient was shown by PEG 400 (C = +1.22), followed by Tween 80 (B = +0.9247) and VCO (A = +0.4244), indicating that PEG 400 was the most dominant factor in maintaining stability against gravitational phase separation. Meanwhile, the pH response was most strongly influenced by the interaction between VCO and PEG 400, with coefficients A = +2.46, B = +7.46, and C = +5.08.

Evaluation of the Physical Quality of the Optimal Formula of Nanoemulsion of *Gemitir* Flower Extract (*Tagetes erecta* L.)

Based on the highest desirability value of 0.990, Formula 1 was selected as the optimal formula among the six recommendations generated by the Design-Expert software. The results of triple replication showed that the organoleptic properties of the preparation were consistent, the emulsion type formed was oil-in-water (O/W), and homogeneity was confirmed without the presence of coarse particles. Verification between the Design-Expert prediction and observational data using OpenStat software revealed a significant difference in percent transmittance response ($p = 0.009$) and absorption efficiency ($p = 0.049$), indicating possible process-related variability during nanoemulsion preparation. These significant deviations suggest limitations in the predictive accuracy of the model for these parameters, which may be attributed to inconsistencies in processing conditions. Such discrepancies should be addressed in future optimization studies through tighter process control and improved experimental standardization.

Meanwhile, no significant difference was observed for pH response ($p = 0.215$), indicating that the model's prediction for pH was consistent with the experimental results. The optimal formula showed an average dispersion diameter of 5.4 cm, which still met the required range (5–7 cm). However, the adhesion test results demonstrated a very low average value of 0.20 seconds, failing to meet the desired adhesion range (2–4 seconds). This indicates that the optimized formulation exhibits insufficient skin adhesion, requiring further modification to improve its bioadhesive properties without compromising other optimized characteristics. The inadequate adhesion performance represents a critical formulation limitation. Future studies should consider incorporating bioadhesive polymers such as Carbopol or HPMC to enhance skin residence time while maintaining the stability and performance of the nanoemulsion system.

CONCLUSION

Based on the research findings, it can be concluded that the 96% ethanol extract of *Gemitir* flower (*Tagetes erecta L.*) possesses promising potential as an antibacterial agent and can be successfully formulated into a nanoemulsion system. Phytochemical screening confirmed the presence of alkaloids, flavonoids, saponins, and tannins, while the total flavonoid content was determined using UV-Vis spectrophotometry at a wavelength of 445 nm, yielding an acceptable calibration curve ($r = 0.9089$; $R^2 \approx 0.826$).

The antibacterial activity test demonstrated a concentration-dependent inhibitory effect against Staphylococcus aureus, with inhibition zones of 7.6 mm, 9.3 mm, and 15.6 mm at extract concentrations of 1%, 1.5%, and 2%, respectively. The strongest antibacterial activity was observed at the 2% concentration, which falls into the strong inhibition category. This activity is likely associated with the synergistic action of flavonoids, alkaloids, saponins, and tannins contained in the extract.

Furthermore, nanoemulsion optimization identified the optimal formulation consisting of 0.53 g VCO, 8 g polysorbate 80 (Tween 80), and 4 g PEG 400, achieving a desirability value of 0.990. The optimized formulation exhibited favorable characteristics, including 97.56% transmittance, high UV–Vis absorbance performance (corrected from "adsorption efficiency"), a pH value of 5.26, homogeneous distribution, no phase separation, and an oil-in-water (O/W) emulsion type. However, the adhesion test result of 0.20 seconds did not meet the required standard of 2–4 seconds, indicating the need for further formulation improvement to enhance topical retention.

Future studies should focus on evaluating particle size distribution, zeta potential, long-term stability, and the incorporation of adhesion-enhancing excipients to support the

development of this nanoemulsion as an effective topical antibacterial delivery system.

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AUTHOR CONTRIBUTION STATEMENT

Putu Ayu Ratih Listiani contributed to the conceptualization of the study, methodology development, investigation, data collection, formal analysis, and preparation of the original manuscript draft. Putu Ika Indah Indraswari contributed to research design, methodological validation, supervision, and manuscript review and editing. Made Dwike Swari Santhi was responsible for laboratory experimentation, data curation, and interpretation of research findings. Sri Suwarni contributed through supervision, validation of the results, scientific guidance, and critical revision of the manuscript. Gusti Ayu Oviani contributed to data analysis, visualization, manuscript editing, and final manuscript preparation. All authors have read and approved the final version of the manuscript and agree to be accountable for the accuracy and integrity of all aspects of the work.

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