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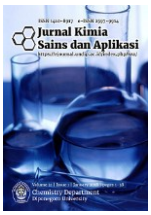

Copigmented Butterfly Pea Flower Extract (*Clitoria ternatea* L.) as an Antibacterial Agent against *Propionibacterium acnes* and Its Formulation in Microneedle Patches

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Abstract

Acne vulgaris is a common skin condition often caused by a bacterial infection with *Propionibacterium acnes*. The extract of butterfly pea flower (*Clitoria ternatea* L.) contains anthocyanins and other bioactive compounds that have potential as antibacterial agents. Anthocyanins are unstable to heat and light; thus, copigmentation with 1.2% citric acid was performed to maintain their stability. This study aims to evaluate the antibacterial potential of the copigmented extract and to characterize the hyaluronic acid- based microneedle patch formulation as a transdermal delivery system, including organoleptic tests, mechanical tests, and morphology using Scanning Electron Microscopy (SEM), as well as the antibacterial potential of the formulation. The results showed an extract yield of 55.45% with an anthocyanin content of 2.9668 mg/L (expressed as cyanidin-3-glucoside equivalents in the analytical solution), with an inhibition zone classified as strong (10–20 mm), and ANOVA analysis indicated significant differences among concentrations ($p = 0.032$). The microneedle patch formulation exhibited needle deformation following a standardized 32 N compression test (from 395 ± 1.67 µm to 291 ± 17.56 µm), but showed antibacterial activity with an inhibition zone of 12.82 ± 0.07 mm, classified as strong, and was significantly different from the negative control ($p = 0.032$).

1. Introduction

Acne vulgaris is a common issue experienced by adolescents and adults, with a high prevalence among individuals aged 15–49 years, especially among teenage boys and girls (>90%) [1]. Acne typically appears on the face and consists of various types, such as blackheads, common pimples, and cystic acne, which are caused by excessive oil production, dead skin cells, clogged pores, and an infection with *Propionibacterium acnes* [2]. Although antibiotics are effective in treating bacterial infections, long-term use can lead to resistance. In Indonesia, *P. acnes* shows the highest resistance to clindamycin (43%) and erythromycin (32%), followed by minocycline (23%) and tetracycline (16%), with less than 10% resistance to doxycycline [3]. Globally, increasing resistance of *P. acnes* to macrolides and lincosamides has been reported, thereby highlighting the need for alternative antibacterial agents derived from natural products.

An alternative treatment for acne, apart from antibiotics, is the use of natural materials, one of which is butterfly pea flower (*Clitoria ternatea L.*), which contains anthocyanins and other bioactive compounds with potential antibacterial properties [4]. Anthocyanins act as antibacterial agents by forming hydrogen bonds to disrupt bacterial cell walls [5]. In the study by Ganis *et al.* [6], butterfly pea flower extract was shown to effectively inhibit *Staphylococcus aureus*, *Escherichia coli*, and *Propionibacterium acnes*, with strong inhibitory activity at 15% [7]. However, anthocyanins are susceptible to degradation by heat and light. Therefore, copigmentation was employed to enhance their stability, with citric acid selected as a copigment due to its effectiveness in improving anthocyanin stability and biological activity [5]. The 1.2% citric acid concentration was selected based

on literature reporting optimal anthocyanin stabilization [8].

Acne medications are generally available in topical formulations. Transdermal patches are an innovative new approach for acne treatment. Patch formulations are an effective alternative for treating acne because they pose minimal risk and result in fewer lesions. However, for cases with more extensive acne, the use of patches becomes less efficient, as they can affect appearance when worn [9].

Transdermal patches are topical formulations that can deliver active ingredients directly to the affected area. One such formulation is the Microneedle patch, which is effective because it can deliver active ingredients directly through the targeted skin layers using a minimally invasive approach via micro-sized needles on its surface [10]. This formulation offers greater benefits and greater effectiveness than conventional transdermal patches [2]. The creation of the extract in patch form is an innovation in transdermal formulations that has been modified to improve compliance, safety, and user comfort. Additionally, the patch can also function to protect the acne-prone area from bacterial contamination [9].

Based on the background, this study aims to determine the antibacterial potential of the anthocyanin extract from butterfly pea flowers obtained through maceration with 1.2% citric acid copigmentation against *P. acnes* growth and to characterize its properties in the form of a microneedle patch formulation, as well as to evaluate the activity of the formulation against *Propionibacterium acnes* (*P. acnes*) bacteria.

2. Experimental

2.1. Materials

The materials used in this study included butterfly pea flower (*Clitoria ternatea* L.), technical ethanol (Merck, 96%), citric acid (Merck), distilled water, hydrochloric acid (Merck), sodium hydroxide (Merck), magnesium (Mg) (Merck), toluene (Merck), filter paper (Whatman), Muller Hinton Agar (MHA) (Micromaster), Nutrient Agar (NA) (Oxoid), barium chloride ($BaCl_2$) (Merck), sulfuric acid (H_2SO_4) (Emsure), 0.9% NaCl (Otsu), DMSO (dimethyl sulfoxide) (Emsure), *Propionibacterium acnes* (ATCC-6919), doxycycline pharmaceutical grade, and hyaluronic acid (Rebteck).

2.2. Instrumentation

The equipment used in this study included an analytical balance (Ohaus), blender (Cosmos), maserator, rotary evaporator (IKA RV10 3 V), glassware (Pyrex), porcelain dish, water bath (Digital Thermostatic), filter paper (Whatman), crucible, crucible tongs, oven (Memmert UN 55), desiccator (Duran), furnace (Wisetherm), petri dish (Normax), inoculating needle, 66 mm filter paper discs (Macherey-Nagel), hot plate (Stirring Hotplate HS0707V2), incubator (Memmert), autoclave, LAF (Laminar Air Flow), glass rod spreader, caliper (Tricle), micropipette (SOCOREX), sonicator (GT Sonic R9), microneedle mold (Micropoint Technologies Pte Ltd., PDMS material, patch size 8 mm × 8 mm, array size 10 × 10), digital microscope, hardness tester (Nanbei), SEM (Scanning Electron Microscope), and UV-Vis spectrophotometer (Genesys).

2.3. Determination

The taxonomy of butterfly pea flower (*Clitoria ternatea L.*) was confirmed through plant identification at the Plant Taxonomy Laboratory, Faculty of Biology, Universitas Padjadjaran, Bandung.

2.4. Sample Preparation

The butterfly pea flower (*Clitoria ternatea L.*) was obtained from Tawangmangu, Karanganyar, Central Java, in the form of dried simplicia. The simplicia was ground in a blender until it formed a powder, then stored in a clean, sealed container.

2.5. Extraction Preparation

Butterfly pea flower was extracted using the maceration method with two remaceration steps. A total of 500 g of butterfly pea flower powder was placed in a macerator, followed by the addition of 2.5 L of a solvent mixture consisting of 96% ethanol and 1% HCl (9:1), along with a 1.2% citric acid copigment solution until the simplicia powder was completely immersed. The citric acid concentration (1.2%) was selected based on previous studies reporting its effectiveness in enhancing anthocyanin stability through copigmentation and increasing extract yield [8]. The mixture was stored in a dark place and stirred occasionally. After 24 hours, the extract was filtered, and the residue was remacerated twice using the same solvent system. The overall extraction time was 72 hours. The combined filtrates were then concentrated by evaporation to obtain a thick extract.

2.6. Extract Yield

The yield obtained was calculated based on the weight percentage (w/w) using Equation 1.

$$\%\ Yield = \frac{Extract\ weight}{Simplicia\ weight} \times 100\% \quad (1)$$

2.7. Phytochemical Screening of Simplicia and Extract

2.7.1. Flavonoids

The simplicia powder or copigmented butterfly pea flower extract was heated with 10 mL of distilled water and subsequently filtered. Five milliliters of the filtrate was mixed with 0.1 g of magnesium powder and 1 mL of concentrated HCl, followed by the addition of 2 mL of amyl alcohol. The mixture was shaken and allowed to stand until two layers formed. A positive result was indicated by the appearance of a red, yellow, or orange coloration in the amyl alcohol layer [11].

2.7.2. Anthocyanins

The simplicia powder or copigmented butterfly pea flower extract was heated with 2 M HCl at 100°C for 5 minutes and then gradually titrated with 2 M NaOH. The presence of anthocyanins was indicated by a color change from red under acidic conditions to a bluish-green color upon alkalization [8].

2.8. Quality Control Parameter of Simplicia and Extract

2.8.1. Loss on Drying

Two grams of simplicia powder or copigmented butterfly pea flower extract were placed in a pre-weighed crucible. The sample was evenly distributed and dried in an oven at 105°C until a constant weight was obtained. The crucible was then cooled to room temperature in a desiccator before weighing. The percentage loss on drying was calculated using Equation 2.

$$Loss\ on\ drying\ (\%) = \frac{(Initial\ weight - Final\ weight)}{Sample\ weight} \times 100 \quad (2)$$

2.8.2. Moisture Content

Moisture content was determined using the azeotropic distillation method with toluene. A mixture of toluene and distilled water was first distilled for 2 hours to saturate the toluene. After cooling, the water volume in the graduated receiver was recorded. Subsequently, 5 g of simplicia powder or copigmented butterfly pea flower extract was placed in a distillation flask containing saturated toluene and heated gently for 15 minutes. Distillation was then carried out at a rate of 2–4 drops per second until no more water was collected, followed by additional heating for 5 minutes. After cooling and complete phase separation of the toluene-water phase, the volume of water was measured. The moisture content (% v/w) was calculated using Equation 3.

$$Moisture\ content\ (\%v/w) = \frac{Measured\ volume\ of\ water}{Sample\ weight} \times 100 (3)$$

Where, the measured volume of water (mL) is the volume of water collected during distillation, and the sample weight (g) is the initial weight of the sample used for analysis.

2.8.3. Total Ash Content

Two grams of simplicia powder or copigmented butterfly pea flower extract were placed in a pre-weighed porcelain crucible. The sample was incinerated gradually at 500–600°C until all carbonaceous matter was removed and a constant weight was obtained. The crucible was then cooled in a desiccator and weighed. The total ash content was calculated as a percentage of the sample weight using Equation 4 [11].

$$Total\ ash\ content\ (\%) = \frac{Ash\ weight\ (g)}{Sample\ weight\ (g)} \times 100 \quad (4)$$

2.8.4. Water-Soluble Ash Content

The ash from the simplicia powder or copigmented extract was heated with 25 mL of water for 5 minutes, then filtered to separate the insoluble portion. The residue was washed with hot water and heated for 15 minutes at a maximum temperature of 450°C until a constant weight was reached. The weight difference was used to determine the water-soluble ash content [8].

2.8.5. Acid-Insoluble Ash Content

The ash from the total ash content determination was heated with 25 mL of dilute HCl for 5 minutes. The insoluble portion was filtered using ash-free filter paper, then washed with hot water. The residue and filter paper were heated at 500–600°C until a constant weight was achieved [11].

2.8.6. Water-Soluble Extract Content

A total of 2.5 g of simplicia powder or copigmented butterfly pea flower extract was placed in a stoppered flask, and 50 mL of chloroform-saturated water was added. The mixture was shaken occasionally during the first 6 hours and then allowed to stand for 18 hours. Subsequently, the solution was filtered, and 20 mL of the filtrate was transferred to a pre-weighed evaporating dish and evaporated to dryness. The residue was further dried at 105°C until a constant weight was obtained. The water-soluble extract content was calculated using Equation 5.

$$Water - Soluble\ extract\ content\ (\%) = \frac{(B-A) \times 5}{W} \times 100 \quad (5)$$

Where, A is the weight of the empty evaporating dish (g), B is the weight of the evaporating dish containing the dried residue after heating to constant weight (g), and W is the initial sample weight (g).

2.8.7. Ethanol-Soluble Extract Content

A total of 2.5 g of simplicia powder or copigmented butterfly pea flower extract was placed in a stoppered flask, and 50 mL of ethanol was added. The mixture was shaken occasionally during the first 6 hours and then allowed to stand for 18 hours. Subsequently, the solution was filtered, and 20 mL of the filtrate was transferred to a pre-weighed evaporating dish and evaporated to dryness. The residue was further dried at 105°C until a constant weight was obtained. The ethanol-soluble extract content was calculated using Equation 6.

$$Ethanol - Soluble\ extract\ content\ (\%) = \frac{(B-A) \times 5}{W} \times 100 (6)$$

Where, A is the weight of the empty evaporating dish (g), B is the weight of the evaporating dish containing the dried residue after heating to constant weight (g), and W is the initial sample weight (g).

2.9. Determination of Total Anthocyanin Content by the pH Differential Method

Total anthocyanin content was determined using the pH differential method by measuring the absorbance of the sample under two pH conditions, pH 1.0 and pH 4.5. One gram of the thick extract was dissolved in 25 mL of solvent, and a 5 mL aliquot was used for analysis. For the pH 1.0 measurement, the aliquot was mixed with potassium chloride (KCl) buffer (pH 1.0) and allowed to stand for 15 minutes. For the pH 4.5 measurement, the aliquot was mixed with sodium acetate buffer (pH 4.5) and allowed to stand for 5 minutes. The absorbance of each solution was measured at 544 nm and 700 nm using the corresponding buffer as a blank [8, 12].

The total anthocyanin content was calculated using the pH differential method based on the molecular weight (449.2 g/mol) and molar absorptivity (26,900 $L \cdot mol^{-1} \cdot cm^{-1}$) of cyanidin-3-glucoside (C3G), as shown in Equation 7. The results were expressed as mg/L cyanidin-3-glucoside equivalents (C3G).

$$Total\ anthocyanin\ (mg/L) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times b} \quad (7)$$

Where:

$$A = (A_{\lambda max} - A_{700})_{pH1.0} - (A_{\lambda max} - A_{700})_{pH4.5}$$

where A represents the absorbance difference between the sample solutions at pH 1.0 and pH 4.5, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, ε is the molar absorptivity coefficient of cyanidin-3-glucoside (26,900 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$), and b is the optical path length of the cuvette (1 cm).

2.10. Antibacterial Test of Copigmented Butterfly Pea Flower Extract Against *P. acnes*

2.10.1. Sterilization of Equipment and Materials

The equipment used was washed and dried, then wrapped in umbrella paper along with the test media MHA and NA, and sterilized using an autoclave at 121°C for 15 minutes. The LAF was sterilized using UV light for 30 minutes and sprayed with 70% alcohol before and after use.

2.10.2. Bacterial Culture Stock Preparation

The slanted NA media was used to prepare the bacterial culture stock by dissolving 5 g of NA in 250 mL of distilled water, boiling for approximately 40 minutes, and sterilizing in an autoclave. The media was then allowed to solidify at a 30° angle. A pure culture of *P. acnes* was streaked onto the solidified NA media and incubated at 36–37°C for 18–24 hours [13].

2.10.3. Bacterial Suspension Preparation

A McFarland standard solution was prepared by mixing 0.5 mL of 1% $BaCl_2$ solution with 9.95 mL of 1% H_2SO_4 solution to form a cloudy solution, which served as the turbidity standard for bacteria. Then, the *P. acnes* bacterial suspension was prepared by dissolving the colonies from the NA media culture into 10 mL of sterile 0.9% NaCl solution until the turbidity matched the McFarland standard of 108 CFU/mL. The solution was then checked using a UV-Vis spectrophotometer at 600–625 nm [14].

2.10.4. Antibacterial Activity Test

The antibacterial activity of *P. acnes* against the copigmented butterfly pea flower extract was tested using the disk diffusion method. The extract was tested in varying concentrations of 20, 25, 30, 35, and 40% (w/v), with doxycycline 1% as the positive control and sterile distilled water as the negative control. A total of 20 mL of MHA media was poured into petri dishes and allowed to solidify. Subsequently, 100 µL of the bacterial suspension was evenly spread on the media using a rod spreader and left for 1–5 minutes. Sterile paper discs were impregnated with 20 µL of each extract concentration and allowed to stand for 15 minutes before being placed on the inoculated MHA plates. The plates were then incubated under anaerobic conditions in an anaerobic jar equipped with a gas-generating kit. Antibacterial activity was determined by measuring the diameter of the inhibition zone formed around each disc after incubation.

2.11. Microneedle Patch Preparation

The microneedle patch was prepared by first dissolving the copigmented butterfly pea flower extract in 5 mL of distilled water (mixture 1). Separately, hyaluronic acid was dissolved in hot distilled water under continuous stirring until a homogeneous solution was obtained, followed by sonication for 30 minutes to remove entrapped air bubbles (mixture 2). A total of 500 mg of hyaluronic acid was used in mixture 2, after which 500 µL of mixture 1 was added and stirred until homogeneous.

The resulting mixture was poured into a microneedle mold and subjected to vacuum treatment for 3 hours to facilitate cavity filling and remove trapped air. Subsequently, a backing layer consisting of the base material alone was added, followed by an additional 2 hours of vacuum treatment. After the vacuum process, more base material was added, and the mold was placed in a desiccator for 20 hours to allow complete drying. The formed microneedle patch was carefully removed from the mold and stored in a tightly sealed container until further use [2].

2.12. Evaluation of Microneedle Patch Characteristics

2.12.1. Organoleptic Test

Organoleptic evaluation was performed by observing the color, odor, and surface characteristics of the microneedle patch [15].

2.12.2. Mechanical Test

The mechanical test aimed to evaluate the compression strength and deformation of the microneedles to ensure effective skin penetration. The test was conducted using a digital hardness tester, where the microneedle patch, coated with parafilm on its surface, was placed on the device and subjected to a pressure of 32 Newtons applied to the entire microneedle patch array [16]. The needle morphology was then observed using SEM, and the needle size (height and width) was measured using the ImageJ application.

2.12.3. Scanning Electron Microscopy (SEM) Analysis

SEM analysis was performed to evaluate the morphology and structural characteristics of the microneedles. The analysis was conducted at the Integrated Physics Laboratory, Institut Teknologi Bandung (ITB), using magnifications of 80× and 200×.

2.13. Antibacterial Activity Test of Microneedle Patch Formulation

The antibacterial activity of the microneedle patch formulation containing copigmented butterfly pea flower extract was evaluated using the disk diffusion method. All equipment and culture media, including MHA and NA, were sterilized in an autoclave at 121°C for 15 minutes. Three microneedle patches containing 40% extract were cut into small pieces and individually dissolved in 1.5 mL of DMSO. Each solution was vortexed for 15 minutes and sonicated until completely dissolved. The resulting solutions were used to impregnate sterile paper discs for

15 minutes. Doxycycline 1% and DMSO served as positive and negative controls, respectively.

Sterile MHA medium was poured into Petri dishes and allowed to solidify. Subsequently, 100 µL of bacterial suspension (10^8 CFU/mL) was evenly spread over the agar surface, and the impregnated paper discs were placed on the inoculated medium. The plates were incubated under anaerobic conditions using an anaerobic jar equipped with gas-generating kits. Antibacterial activity was determined by measuring the diameter of the inhibition zone formed around each disc after incubation.

2.14. Data Analysis

The inhibition zone diameter data obtained from the antibacterial activity tests of the extract and microneedle patch formulations were analyzed using IBM SPSS Statistics version 25. Data normality and homogeneity were evaluated prior to statistical analysis. Differences among treatment groups were assessed using one-way analysis of variance (ANOVA), followed by an appropriate post hoc tests.

3. Results and Discussion

3.1. Determination

Plant identification conducted at the Jatinangor Herbarium, Plant Taxonomy and Botany Laboratory, Universitas Padjadjaran, confirmed that the sample used in this study was *Clitoria ternatea* L. (Fabaceae), as documented in the determination certificate No. 29/HB/05/2025.

3.2. Extraction

The powdered butterfly pea flower simplicia exhibited a homogeneous appearance with the characteristic bluish-purple color of butterfly pea flowers. Extraction of 500 g of powdered simplicia yielded 277.27 g of thick extract, corresponding to an extraction yield of 55.45%. Extraction yield is a parameter used to evaluate the efficiency of the extraction process in recovering soluble compounds from plant materials [17]. The extraction yield obtained in the present study was higher than that reported in a previous study, which yielded 40.72% using 95% ethanol as the extraction solvent [18].

The relatively high extraction yield observed in this study may be attributed to several factors, including the type and composition of the solvent, extraction duration, and the remaceration process. The use of acidified ethanol and the addition of 1.2% citric acid as a copigment may improve anthocyanin stability during extraction, thereby reducing the degradation of active compounds [8]. The remaceration process performed for 3 × 24 hours also provided prolonged contact between the simplicia and the solvent, allowing greater diffusion of bioactive compounds into the extraction medium. Increased contact time between the plant material and solvent enables more compounds to be dissolved until equilibrium is reached [19]. Differences in extraction yield compared with previous studies may also be influenced by variations in raw material characteristics, particle size, solvent-to-material ratio, and extraction conditions [19]. These findings indicate that the extraction conditions applied in the present study were effective in obtaining butterfly pea flower extract with a relatively high yield.

3.3. Quality Control Parameters of Simplicia and Extract

The quality control parameters were tested to ensure that the simplicia and butterfly pea flower extract met the standards outlined in the Indonesian Herbal Pharmacopoeia 2017. However, the standards for butterfly pea flower are still general due to the absence of a specific monograph for *Clitoria ternatea* [11]. The quality parameters tested included loss on drying, moisture content, total ash content, acid-insoluble ash, water-soluble ash, and the extractable content soluble in ethanol and water.

The purpose of the loss on drying test is to determine the amount of water and volatile compounds that evaporate during heating at 105°C until a constant weight is reached. This temperature is used to ensure that water and volatile compounds evaporate without damaging the active compounds [20]. The test results showed that the simplicia experienced a loss on drying of 6.28%, which is within the standard of the Indonesian Herbal Pharmacopoeia (≤10%), indicating the amount of water, volatile compounds, and solvents lost during heating [11]. However, the extract's loss on drying exceeded the specified limit, reaching 12.62%. This is likely due to the hygroscopic nature of the citric acid content in the extract, which absorbs more water from the environment, leading to a higher weight loss during evaporation.

Table 1. Quality control parameter results of simplicia and extract

Parameter	Result (%) ± SD		Standard (%) (FHI edition II)
	Simplicia	Extract	
Loss on drying	6.28 ± 0.1201	12.62 ± 0.1693	≤ 10.00
Moisture content	3.99 ± 0.000	9.98 ± 0.0047	≤ 10.00
Total ash content	5.42 ± 0.0216	4.80 ± 0.0410	≤ 10.00
Acid-insoluble ash content	0.41 ± 0.0081	0.30 ± 0.0047	≤ 0.7
Water-soluble content	1.72 ± 0.0124	0.77 ± 0.0169	≤ 10.00
Water-soluble extract content	51.48 ± 1.2719	24.72 ± 0.5875	≥ 32.2
Ethanol-soluble extract content	41.93 ± 0.6695	65.49 ± 0.9700	≥ 20.5

The purpose of the moisture content test is to establish the minimum water content in both simplicia and extracts [20]. The test results showed a moisture content of 3.99% in the simplicia and 9.98% in the extract, both of which comply with the Indonesian Herbal Pharmacopoeia standard (≤10%). Other studies have also mentioned that the acceptable moisture content in thick extracts ranges from 5–30% [21]. Excessive water content can accelerate microorganism growth and cause degradation of active compounds, thus reducing the stability of the extract during storage [22].

The examination of ash content parameters in simplicia and extract includes total ash, acid-insoluble ash, and water-soluble ash. The total ash content indicates the amount of inorganic materials or minerals remaining after the incineration process at approximately 600°C, where organic compounds are destroyed and evaporated, while minerals remain [23]. The test results showed that the total ash content in the simplicia was 5.42% and in the extract was 4.80%, both of which comply with the Indonesian Herbal Pharmacopoeia standard (≤10%).

The determination of acid-insoluble ash content aims to identify the amount of contaminants from outside the simplicia or extract that do not dissolve in hydrochloric acid [8]. The results showed that the acid-insoluble ash content in the simplicia and extract was 0.41% and 0.30%, respectively, both of which are within the Indonesian Herbal Pharmacopoeia standard (≤0.7%).

The determination of water-soluble ash content aims to measure the inorganic compounds in the simplicia or extract that are soluble in water [8]. The results showed 1.72% for the simplicia and 0.77% for the extract, both of which comply with the Materia Medica Indonesia standard (≤10%).

The determination of extract content in the simplicia and butterfly pea flower extract includes both water-soluble extract content and ethanol-soluble extract content. Water-soluble extract content aims to give an initial indication of the amount of polar compounds that can be extracted in water. The results showed 51.48% for the simplicia and 24.72% for the extract, both of which meet the Indonesian Herbal Pharmacopoeia standard (≥16.0%).

The ethanol-soluble extract content increased from 41.93% in the simplicia to 65.49% in the extract, indicating successful enrichment of ethanol-soluble phytochemicals during extraction. These results also meet the Indonesian Herbal Pharmacopoeia requirement, which specifies that the ethanol-soluble extract content must be greater than 20.5%.

3.4. Qualitative Test for Flavonoids and Anthocyanins

Qualitative tests for flavonoids and anthocyanins in the simplicia and butterfly pea flower extracts were conducted to detect their presence.

Table 2. Results of qualitative tests for flavonoids and anthocyanins

Compound	Result	
	Simplicia	Extract
Flavonoid	Red color on the amyl alcohol layer (+)	Red color on the amyl alcohol layer (+)
Anthocyanin	Color change from red (acidic) to green/blue (alkaline), gradually fades (+)	Color change from red (acidic) to green/blue (alkaline), gradually fades (+)

The flavonoid test was conducted by adding magnesium metal, concentrated HCl, and amyl alcohol. Magnesium and HCl reduce the benzopyrone ring in flavonoids, forming flavylium salts that appear red or orange. The result was positive due to the formation of a red color in the amyl alcohol layer [11].

The anthocyanin test was conducted by adding HCl and NaOH. HCl hydrolyzes the glycosidic bonds, converting the anthocyanins into a more stable aglycone form, while NaOH was used to identify anthocyanins through a color change. The result was positive due to the color change from red under acidic conditions to green or blue under alkaline conditions, which gradually faded [8].

3.5. Determination of Total Anthocyanin Content by the pH Differential Method

The determination of total anthocyanin content in the copigmented butterfly pea flower extract was carried out using the pH differential method (pH 1 and pH 4.5) based on Giusti and Wrolstad [24]. At pH 1, anthocyanins exist in the flavylium cation (oxonium) form, which is colored, while at pH 4.5, they change into the colorless carbinol and hemiketal forms [25]. The absorption spectrum was measured in the range of 400–700 nm. The wavelength of 400 nm was used because the main peak of anthocyanins lies within the range of 400–535 nm, while the wavelength of 700 nm was used to correct for interference from non-anthocyanin compounds [8]. The measurement results showed a peak at 544 nm, indicating that the absorbance maximum falls within the reported range for cyanidin-3-glucoside (515–545 nm) [26].

Table 3. Results of anthocyanin content in copigmented butterfly pea flower

Sample	Anthocyanin content (mg/L)	Average (mg/L)
Copigmented butterfly pea flower extract	2.9891	2.9668
	2.9724	
	2.9390	

Based on the calculation results, the average total anthocyanin content in the copigmented butterfly pea flower extract was 2.9668 mg/L, expressed as cyanidin-3-glucoside equivalents (C3GE). This result is higher compared to the study by Dewi and Yusri [26], which reported a total anthocyanin content of 1.666 mg/L. This increase indicates that the addition of citric acid as a copigment effectively enhances the absorbance of anthocyanins. This is because citric acid can lower the pH of the solution, thereby allowing more anthocyanins to exist in the colored flavilium cation form [25]. The use of cyanidin-3-glucoside parameters represents an approximation, as butterfly pea anthocyanins are mainly ternatins. Consequently, the application of the cyanidin-3-glucoside molar absorptivity coefficient may result in minor quantitative deviation when estimating anthocyanin levels in ternatin-rich extracts. Nonetheless, the pH differential method remains a widely accepted and standardized approach for the comparative quantification of total anthocyanin content.

3.6. Antibacterial Activity of Copigmented Butterfly Pea Flower Extract Against *P. acnes*

The antibacterial activity of the copigmented butterfly pea flower extract was tested using the disk diffusion method at concentrations of 20, 25, 30, 35, and 40%. Doxycycline was used as the positive control due to its high effectiveness against *P. acnes*, while sterile distilled water was used as the negative control. The results of the antibacterial activity test of the copigmented butterfly pea flower extract are shown in Figure 1 and Table 4.

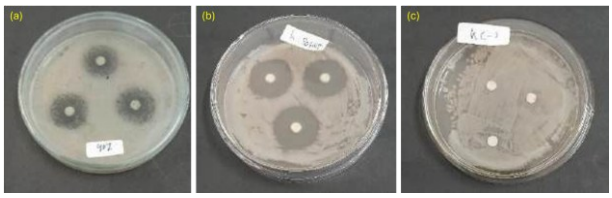

Figure 1. Representative inhibition zones of (a) copigmented butterfly pea flower extract (40%), (b) positive control, and (c) negative control against *P. acnes*

Table 4. Inhibition zone diameter and antibacterial activity of copigmented butterfly pea flower extract against *P. acnes*

Concentration (%)	Inhibition zone (mm)	Antibacterial activity
20	17.45 ± 0.02	Strong
25	18.57 ± 0.23	Strong
30	19.19 ± 0.28	Strong
35	20.75 ± 0.24	Strong
40	21.43 ± 0.16	Very strong
Control (+)	29.67 ± 0.94	Very strong
Control (-)	0	No inhibition

The antibacterial activity test results show that as the concentration of the copigmented butterfly pea flower extract increases, the diameter of the inhibition zone also increases. The 40% concentration produced an inhibition zone of 21.43 mm, classified as very strong (>20 mm), while the 20%–35% concentrations were classified as strong (10–20 mm). The positive control (doxycycline) showed the highest inhibition zone of 29.67 mm, while the negative control (sterile distilled water) showed no antibacterial activity. This antibacterial activity is attributed to the flavonoid and anthocyanin content in the extract. Flavonoids act by damaging bacterial cell membranes through binding with cell wall proteins, while anthocyanins break down the cell wall via hydrogen or hydrophobic interactions. Additionally, citric acid may contribute to the antibacterial activity of the extract, as previous studies have reported antimicrobial effects of organic acids [5].

Statistical analysis was performed to support the results of the antibacterial activity test. The Shapiro-Wilk normality test showed that most of the data were normally distributed (P>0.05). The homogeneity test showed a P-value <0.05, indicating that the variations between groups were not homogeneous. Therefore, the analysis continued using ANOVA and post hoc Games-Howell tests. The ANOVA results showed a significance value of p = 0.032, indicating a significant difference in the antibacterial activity of the copigmented butterfly pea flower extract against *P. acnes*. The post hoc Games-Howell test showed that all concentrations of the extract differed significantly compared to the negative control (p = 0.032). Additionally, the higher concentrations (35% and 40%) also differed significantly from the lower concentrations (20% and 25%). This confirms that increasing the concentration is directly proportional to the antibacterial activity produced.

3.7. Characterization of Microneedle Patch Formulation of Copigmented Butterfly Pea Flower Extract

The microneedle patch containing copigmented butterfly pea flower extract was prepared using the solvent-casting method, which involves dissolving and mixing the formulation components, followed by molding [9]. A 40% extract concentration was selected based on its demonstrated very strong antibacterial activity. Hyaluronic acid was used as the polymer matrix because it can form microneedles with high mechanical strength, enabling effective skin penetration while remaining readily dissolvable after application [27]. The resulting microneedle patch formulation is presented in Figure 2.

Figure 2. Microneedle patch formulation of butterfly pea flower extract

3.7.1. Organoleptic Test

The organoleptic examination was conducted to visually observe the formulation, assisted by a digital microscope and Hiview software. Parameters evaluated included color, aroma, and morphological characteristics. The results showed that the microneedle patch containing butterfly pea flower extract exhibited a purple color and a characteristic aroma. Microscopic observation further revealed that the needles were intact, uniformly distributed, and exhibited a consistent morphology across the patch surface.

3.7.2. Scanning Electron Microscopy (SEM) Analysis

The morphology of the microneedles was characterized using SEM, while ImageJ software was employed to determine needle dimensions, including height and base width. SEM observations were conducted at magnifications of 80× and 200×, and the resulting micrographs are presented in Figure 3. SEM analysis revealed that the microneedles were uniformly arranged and exhibited smooth, homogeneous surfaces. The top-view image obtained at 80× magnification showed that the microneedles possessed a pyramidal or quadrangular-based conical geometry. The tilted-view image acquired at 200× magnification (30° angle) indicated an average needle height of 395 ± 1.67 µm, sufficient to penetrate the stratum corneum (10–20 µm). The average base width was 203 ± 3.32 µm, falling within the commonly reported range of 50–250 µm for microneedle applications. In addition, the average interneedle spacing was 204 ± 2.61 µm, which is within the recommended range of 100–500 µm and is considered adequate to minimize pain during skin insertion [28].

3.7.3. Mechanical Test

The mechanical strength test was conducted to evaluate the compression resistance and deformation behavior of the microneedles, which are critical parameters for ensuring effective skin penetration [29]. Mechanical testing using a hardness tester revealed structural deformation at the needle tips, characterized by bending and partial damage. To further assess these morphological changes, the microneedles were examined using SEM at magnifications of 80× and 500×. The SEM micrographs obtained after mechanical testing are presented in Figure 4.

Based on the study conducted by Chen *et al.* [30], hyaluronic acid-based microneedles showed a slight decrease in mechanical strength but were still able to penetrate the skin effectively. However, under high humidity conditions, the insertion strength significantly decreased, resulting in inefficient penetration. This deformation may be associated with the hygroscopic nature of hyaluronic acid and citric acid, which can promote moisture absorption and reduce the mechanical integrity of microneedles. As a result, the formulation readily absorbed moisture from the environment, causing deformation at the needle tips after the mechanical test, with a reduction in needle height to 291 ± 17.56 µm. Environmental humidity during testing was not controlled and may affect mechanical properties.

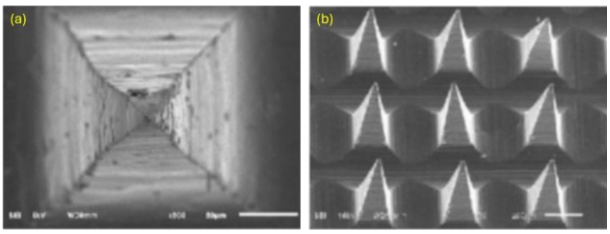

Figure 3. SEM images of the microneedle patch: (a) 200× and (b) 80× magnification

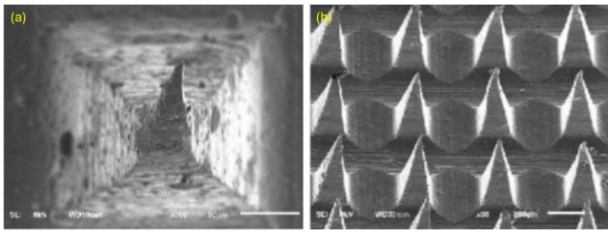

Figure 4. SEM images of microneedles after mechanical testing: (a) 500× and (b) 80× magnification

3.8. Antibacterial Activity Test of Microneedle Patch Formulation of Copigmented Butterfly Pea Flower Extract

The antibacterial activity test of the microneedle patch was conducted to evaluate the inhibitory effect of the copigmented butterfly pea flower extract patch formulation against *P. acnes*. The test was performed in triplicate using 1% doxycycline as the antibiotic comparator. The results of the antibacterial activity test of the formulation are presented in Table 5. Based on the antibacterial activity assay, the microneedle patch formulation containing copigmented butterfly pea flower extract exhibited strong antibacterial activity against *P. acnes*, with an average inhibition zone diameter of 12.82 ± 0.07 mm and a relative standard deviation (RSD) of 0.57%. According to the inhibition zone classification proposed by David and Stout (1971), as cited by Mayasari [31], inhibition zones of <5 mm, 5–10 mm, 10–20 mm, and >20 mm are categorized as weak, moderate, strong, and very strong, respectively. Therefore, the antibacterial activity of the microneedle patch formulation was classified as strong.

A previous study by Balapadang *et al.* [32] reported that a microneedle patch containing 40% *Kalanchoe pinnata* (cocor bebek) leaf extract exhibited very strong antibacterial activity. In the present study, however, the inhibition zone produced by the butterfly pea flower extract microneedle patch was substantially smaller than that of the positive control (1% doxycycline), which generated an inhibition zone of 30.51 ± 0.81 mm and was categorized as very strong. The lower antibacterial activity of the microneedle patch compared with the extract alone may be attributed to the limited diffusion of active compounds from the hyaluronic acid matrix during the disk diffusion assay. Furthermore, although a 40% (w/v) extract stock solution was used during formulation, the final concentration of the extract incorporated into the microneedle patch was approximately 2% (w/w), which may have further contributed to the reduced inhibitory effect.

Table 5. Antibacterial activity results of the microneedle patch formulation

Sample	Inhibition zone diameter (mm)	Antibacterial activity
Positive control (Doxycycline 1%)	30.51 ± 0.81	Very strong
Negative control (DMSO)	0	No inhibition
Microneedle patch of copigmented butterfly pea flower extract	12.82 ± 0.07	Strong

Statistical analysis of the antibacterial activity data demonstrated a trend similar to that observed for the extract alone. Normality testing indicated that most datasets were normally distributed ($p > 0.05$), whereas the homogeneity test revealed unequal variances among groups ($p < 0.05$). Consequently, one-way ANOVA followed by the Games–Howell post hoc test was performed. The ANOVA results showed a significant difference among treatment groups ($p = 0.032$), indicating that the antibacterial activities differed significantly.

The Games-Howell post hoc test showed no significant differences among the 40% microneedle patch groups (P1, P2, and P3), but there were significant differences between the microneedle patch groups and the negative control (DMSO), confirming that the microneedle patch formulation of copigmented butterfly pea flower extract exhibits significant antibacterial activity against *P. acnes*. The absence of a blank patch control is acknowledged as a limitation. The post hoc test also indicated a significant difference between the microneedle patch groups and the positive control (1% doxycycline), as the positive control demonstrated much stronger antibacterial activity.

4. Conclusion

This study demonstrates that the butterfly pea flower (*Clitoria ternatea L.*) extract, copigmented with 1.2% citric acid, has potential as an antibacterial agent against *Propionibacterium acnes*. Copigmentation successfully increased the extract yield to 55.45% and the total anthocyanin content to 2.9668 mg/L. The extract exhibited strong to very strong antibacterial activity, with the largest inhibition zone of 21.43 ± 0.16 mm at 40% concentration, and demonstrated significant inhibitory activity compared to lower concentrations, as determined by ANOVA and Games–Howell post hoc tests. The hyaluronic acid-based microneedle patch formulation maintained antibacterial activity, while exhibiting needle deformation under supra-maximal compression due to the hygroscopic properties of the materials, with an inhibition zone of 12.82 ± 0.07 mm (strong category), and showed significant antibacterial activity against *P. acnes* according to ANOVA and Games–Howell post hoc tests. Therefore, the microneedle patch formulation of copigmented butterfly pea flower extract has the potential to be developed as an alternative topical antibacterial therapy for acne.

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