International Conference of Multidisciplinary Cel: Proceeding
1 (1), 2024, 92-100

Available Online: https://proceeding.ressi.id/index.php/IConMC

Antibacterial activity test of n-hexane, ethyl acetate, and water fractions of the flat-top mille graines (*Hedyotis corymbosa L*) herb against the growth of the propionibacterium acnes bacteria

Rosdarni Rosdarni [1] *, Wa Ode Ida Fitriah [1], La Ode Muslimin [1], Yulyuswarni Yulyuswarni [2], Ade Ariska Putri [1]

Universitas Mandala Waluya. Jl. Jend. AH. Nasution, Kota Kendari, 93561, Indonesia
Poltekkes Kemenkes Tanjung Karang. Jl. Soekarno Hatta No.6, Lampung 35145, Indonesia
* Corresponding Author. rosdarni@gmail.com

Received: 2 December 2023; Revised: 12 February 2024; Accepted: 2 April 2024

Abstract: Acne, a chronic obstructive and inflammatory skin condition affecting pilosebaceous units, is often linked to Propionibacterium acnes, a bacterium that disrupts the stratum corneum and stratum germinativum by secreting sebum, leading to damage in the walls of skin pores. This study aims to identify the chemical compounds present in the n-hexane, ethyl acetate, and water fractions of flat-top mille graines or diamond flower, locally known as rumput mutiara (Hedyotis corymbosa L.) herbal extract. Additionally, it seeks to determine the optimal concentration and the fraction exhibiting the most potent antibacterial activity against Propionibacterium acnes, with the aim of inhibiting bacterial growth associated with acne. This study employed laboratory experimental methods. Extraction was conducted through the maceration method using ethanol solvent. Fractionation was achieved through liquid-liquid extraction using n-hexane, ethyl acetate, and water solvents. Phytochemical screening and inhibition tests were performed using Clindamycin as the positive control and DMSO as the negative control. Data analysis involved the One-Way ANOVA method followed by the LSD test for further analysis. Phytochemical screening of the flat-top mille graines (Hedyotis corymbosa L.) fractions revealed positive results for various compounds. The n-hexane fraction contained flavonoid and triterpenoid compounds, the ethyl acetate fraction exhibited tannin, saponin, flavonoid, and steroid compounds, while the water fraction contained alkaloids, tannins, saponins, flavonoids, and steroids. In terms of antibacterial activity against Propionibacterium acnes, the n-hexane fraction at a concentration of 15% displayed a medium inhibition zone diameter of 8.6 mm, whereas the ethyl acetate and water fractions both exhibited strong inhibition zones with diameters of 16.6 mm at the same concentration. The optimal concentration for inhibiting Propionibacterium acnes was found to be 15% for both the ethyl acetate and water fractions. Formulation of the herbal fractions of flat-top mille graines (Hedyotis corymbosa L.) is recommended based on these findings.

Keywords: Flat-Top Mille Grains; Fractions; Propionibacterium.

INTRODUCTION

Acne, commonly known as pimples, is a prevalent skin condition among teenagers and young adults. It is a chronic obstructive and inflammatory disease of the pilosebaceous unit. The primary factors contributing to acne development include heightened sebum production by the skin's oil glands, the accumulation of keratinocytes, and bacterial growth within the pilosebaceous ducts, which are naturally present in healthy skin (Movita, 2013).

Propionibacterium acnes, commonly known as P. acnes, is one of the bacteria responsible for acne. It is a gram-positive, pleomorphic, and aerotolerant anaerobic bacterium that natu-

rally resides on the skin as part of its normal flora. P. acnes contributes to acne development by producing lipase enzymes, which break down free fatty acids from skin lipids, leading to inflammation. This inflammatory response promotes the proliferation of these bacteria and exacerbates inflammatory lesions by stimulating the production of pro-inflammatory cytokines (Fauzi et al., 2014).

Acne is most prevalent between the ages of 16 and 17, affecting approximately 83-85% of women and 95-100% of men. Various factors contribute to acne, including genetic, racial, seasonal, psychological, hormonal, or bacterial factors. However, bacterial infections are generally considered a primary cause of acne, with Propionibacterium acnes being one of the key bacteria responsible (Latifah, S. and Kurniawaty, 2015).

The typical approach to treating acne involves the use of antibiotics, which are medications designed to combat bacterial infections by either killing bacteria (bactericidal) or preventing their multiplication (bacteriostatic) (Indonesian Ministry of Health, 2015). Based on their chemical structure, antibiotics are divided into several groups, including Beta-Lactams, aminoglycosides, tetracyclines, sulfonamides and trimethoprim, macrolides, lincomycin, quinolones, and chloramphenicol. Clindamycin, classified as a macrolide antibiotic, is particularly effective against gram-positive bacteria. Its molecular formula is C18H33C1N2O5S, with a molecular weight of 424.98302. Clindamycin is a semisynthetic antibacterial agent, structurally analogous to lincomycin (Muchtaromah, 2016). The mechanism of action of clindamycin involves reversible binding with the 50S ribosomal subunit, hindering peptide bond formation and thereby inhibiting bacterial protein synthesis. Whether clindamycin exerts a bacteriostatic or bactericidal effect depends on factors such as drug concentration, the type of infection, and the organism involved (Sa'adah & Nurhasnawati, 2015). While clindamycin effectively inhibits protein synthesis in bacteria and targets P. acnes on the skin surface, prolonged antibiotic use can lead to bacterial resistance. Resistance occurs when bacteria no longer respond to antibiotics that were previously effective, rendering them ineffective for clinical use (Mulyani et al., 2016).

The rise of bacterial resistance to antibiotics due to inappropriate use underscores the need for alternative treatments, prompting exploration into the potential antibacterial properties of plants. Among these, flat-top mille graines or diamond flower, locally known as rumput mutiara (Hedyotis corymbosa L.) has garnered attention for its antibacterial potential. Therefore, this study aims to elucidate the inhibitory properties of different fractions and concentrations of flat-top mille graines extract in inhibiting the growth of Propionibacterium acnes.

METHODS

This study involved in five main stages, which included plant identification and preparation, extraction, fractionation, antibacterial testing, and analysis.

Plant Identification and Sample Preparation Stage

The identification process was conducted to confirm that the acquired plants were indeed flat-top mille graines (Hedyotis *corymbosa L.*) specimens, which were located at the Phytochemical Pharmacognosy Laboratory, Mandala Waluya University. Subsequently, the collected samples were dried under sunlight. Once completely dried, the leaves were pulverized into a fine powder using a blender, followed by extraction.

Extraction Stage

A total of 800 grams of flat-top mille graines herb powder was placed into a container along with ethanol solvent using in a ratio of (1:2). The extraction process was conducted over 3

cycles, each lasting 24 hours, employing maceration with stirring. Following the completion of the maceration process, the mixture was filtered using a flannel cloth. Subsequently, the filtrate was evaporated using a rotary evaporator at a temperature of 50°C, followed by further drying in a hairdryer until a thick extract was obtained. Finally, the yield of the flat-top mille graines herbal extract was calculated.

Fractionation Stage of Flat-Top Mille Graines Herbs

This fractionation employed a liquid-liquid partition method, where the dissolved extract was placed into a separating funnel and mixed with solvents based on their polarity. Three solvents were used: n-hexane, ethyl acetate, and water. The process begun by weighing the flat-top mille graines herb extract. Then, 60 grams of the extract was dissolved in 600 mL of water and combined with the n-hexane solvent. After shaking and allowing it to settle for 10-15 minutes, two distinct layers form: the n-hexane layer and the water layer. The n-hexane layer is then separated and collected in an Erlenmeyer flask. Next, the water layer is returned to the separating funnel, and ethyl acetate solvent is added. The mixture is shaken and allowed to settle, resulting in the formation of the ethyl acetate layer and the water layer. These layers are separated and collected in separate Erlenmeyer flasks to obtain the n-hexane, ethyl acetate, and water fractions. The fractions are then concentrated using a rotary evaporator and further dried with a hairdryer until thick fractions of n-hexane, ethyl acetate, and water are obtained (Hepni, 2019).

Fractionation of flat-top mille graines herb Chemical Content of n-Hexane Fraction, Ethyl Acetate, and Water of Flat-Top Mille Graines Herb:

a. Alkaloids represent a group of fundamental compounds characterized by their basic nature, typically comprising one or more nitrogen atoms arranged in cyclic structures. Their presence can be identified through precipitation tests such as the Mayer, Dragendroff, and Bouchardat tests. While the majority of alkaloids exist in solid crystal form, some are liquid at standard room temperature. They exhibit optical activity by rotating the plane of polarization and often possess a bitter taste (Harborne, 1987)
b. Steroid/Triterpenoid The test for steroids and triterpenoids relies on their capacity to produce specific colors when mixed with concentrated H2SO4 in an acetic anhydride solvent. Terpenoids typically exhibit a red-orange or purple coloration, while steroids manifest a blue color. The test for steroids and triterpenoids relies on their capacity to produce specific colors when mixed with concentrated H2SO4 in an acetic anhydride solvent. Terpenoids typically exhibit a red-orange or purple coloration, while steroids manifest a blue color (Simaremare, 2014).
c. Saponins are characterized by having both hydrophilic and hydrophobic components. When shaken, saponins produce foam due to the hydrophilic groups binding to water while the hydrophobic groups bind to air. In the micelle structure, the polar groups faces outward while the non-polar groups faces inward. The presence of saponins is typically confirmed by the formation of stable foam upon shaking. However, in this analysis, the sample did not produce foam, indicating the absence of saponins. Generally, if the result is positive for saponins, the addition of HCl2N is intended to enhance polarity, resulting in more stable bonding of the hydrophilic groups and the formation of stable foam (Simaremare, 2014).
d. Flavonoids Flavonoids can be identified through a test involving the addition of magnesium powder and hydrochloric acid. This reaction typically results in the reduction of flavonoid compounds, leading to a red coloration. However, in this test, the reaction was negative, as the magnesium powder failed to induce the reduction reaction in the

flavonoid compounds, resulting in no change in the color of the test solution (Robinson, 1995).

e. Tannins and Polyphenols Screening for polyphenols/tannins involves the use of iron (III) chloride reagent. When 10% FeCl3 is added, it is expected to result in a dark blue, blackish blue, or greenish-black coloration if polyphenol/tannin compounds are present. However, in this test, no color changes occurred upon the addition of FeCl3, indicating the absence of hydroxyl groups in the tannin compounds.

Antibacterial Activity Testing

Tool Sterilization

The purpose of sterilizing tools and materials is to eliminate microorganisms. This process involved cleaning the tools thoroughly and then drying them. Glassware such as petri dishes, beakers, and test tubes were wrapped in paper and sterilized in an oven at a temperature ranging from 160°C to 180°C for 1-2 hours. On the other hand, media was sterilized using an autoclave at 121°C for 15 minutes.

Nutrient Agar (NA) Media Preparation

The Nutrient Agar (NA) media was prepared by dissolving 3.8 grams of NA powder in 188 ml of distilled water. The solution was heated in an Erlenmeyer flask until completely dissolved. Once dissolved, the flask was sealed with cotton and covered with aluminum foil. Subsequently, the NA media was sterilized using an autoclave at 121°C for 15 minutes, making it ready for the cultivation of *Propionibacterium acnes* bacteria.

Bacterial Suspension Preparation

5 mL of Nutrient Agar (NA) media was dispensed into a test tube. Each pure culture of Propionibacterium acnes bacteria was streaked onto the NA medium in the test tube and tilted at an angle of 30-40°C before allowing it to solidify. The inoculated tubes were then incubated at a temperature of 35-37°C for 24 hours. Following this, a suspension was prepared by extracting 10 mL of NaCl with a sterile syringe and transferring it to a test tube. The bacterial culture was then transferred into the NaCl solution. This bacterial suspension was now ready for use in the antibacterial activity testing.

Positive Control and Negative Control Preparation

The positive control (+) employed was Clindamycin at a concentration of 0.03 mg, which was dissolved in 2 ml of distilled water during preparation. The Negative Control Solution (-) consisted of 21 ml of DMSO solution, with 3 ml aliquoted into each vial.

Antibacterial Activity Testing for Fractions- n-Hexane, Ethyl acetate, and Water of flat-top mille graines (*Hedyotis corymbosa L.*).

3.8 grams of NA media were carefully weighed and then added to an Erlenmeyer flask containing 188 mL of sterile distilled water to dissolve. The mixture was heated using a spirit lamp until it reached boiling point and became uniformly mixed. Subsequently, it was sterilized in an autoclave at 121°C for 15 minutes under pressure of 1 atm (Safitri & Novel S, 2010). Next, a bacterial suspension was prepared by transferring 10 mL of 0.9% NaCl solution into a test tube. Then, one culture of *Propionibacterium acnes* bacteria, which had been rejuvenated in slanted NA media, was transferred into a test tube containing 0.9% NaCl solution. The solution was then thoroughly mixed until a uniform bacterial suspension was obtained.

In the antibacterial activity testing using the well method, the test solution was prepared by dissolving the flat-top mille graines herb fraction in two different concentrations: 10% and 15%, using DMSO solution. DMSO was also utilized as a negative control. To create each concentration series, DMSO solution was added to several grams of the flat-top mille graines herb fraction until the volume reached 3 mL. Additionally, a positive control for the antibacterial test was prepared using clindamycin 0.03 mg dissolved in 3 mL of distilled water. The antibacterial activity test proceeded as follows: Initially, 15 mL of sterile Nutrient Agar (NA) was poured into a test tube, to which 1 mL of bacterial suspension was added and homogenized. The mixture was then transferred to a petri dish and allowed to solidify. Subsequently, an inhibition test was conducted by creating a well in the solidified medium using the tip of a sterile dropper pipette. The test solutions of the n-hexane fraction, ethyl acetate, and water extract of flat-top mille graines (*Hedyotis corymbosa L*.) herb, with concentrations of 10% and 15%, were added into the wells, along with the positive control clindamycin and the negative control DMSO. Each petri dish was then incubated in an incubator at 37°C for 24 hours. After incubation, the petri dishes were removed from the incubator, and observations were made to assess the formation of a clear zone around the well. The diameter of the inhibition zone was measured as an indicator of antibacterial activity.

Processing and data analysis

The One-Way ANOVA analysis test was conducted using SPSS 20 to compare the diameter of the inhibition zone among the positive control, negative control, and all treatments, based on the concentration of n-hexane, ethyl acetate, and water fraction extracts from flat-top mille graines (*Hedyotis corymbosa L*) on the growth of *Propionibacterium acnes* bacteria.

RESULTS AND DISCUSSION

The determination of the Flat-Top Mille Graines Herb (*Hedyotis corymbosa L*.) was conducted at Mandala Waluya University. This determination aimed to confirm the identity of the plant samples used in the research. The results confirmed that the plant utilized in this study was indeed the Flat-Top Mille Graines Herb (*Hedyotis corymbosa L*.).

The simplicia of flat-top mille graines herb (*Hedyotis corymbosa L*) was extracted using the maceration method with ethanol solvent, and evaporated to produce a thick extract weighing 68.8 grams. The percent yield was calculated as follows:

Table 1. Results of Flat-Top Mille Graines (Hedyotis corymbosa L.) Herb Extraction

Sample	Weight of Simplicia (g)	Weight of Extract (g)	Extraction Yield (%) b/b
Flat-top mille graines herb	800 g	68.8 g	8.6%

Yield of n-hexane, ethyl acetate, and water fractions of flat-top mille graines (*Hedyotis corymbosa L*.) herb

Table 2. Yield results of n-hexane, ethyl acetate and water fractions of *Hedyotis corymbosa L*

Sample	Weight of Extract (g)	Weight of Fractions (g)	Extraction Yield (%) b/b
N-hexane fraction		7.5 g	12.5 %
Ethyl acetate fraction	60 g	1.2 g	2 %
Water fraction		22 g	36.6%

The fractionation process from 60 grams of extract yielded 7.5 grams of the n-hexane fraction, accounting for 12.5% of the total yield, 1.2 grams of the ethyl acetate fraction, constituting 2% of the total yield, and 22 grams of the water fraction, representing a total yield

of 36.6%. These values meet the required standards, as the percentage of soaked value with an initial simplicia weight of 800 grams is not less than 3.6%. For an initial weight of 1000 grams, the percentage of soaked value should be no less than 7.2%. Fraction yield is calculated by comparing the final weight (the weight of the produced fraction) with the initial weight (the weight of the extract used), then multiplying by 100%. The yield value is also related to the amount of bioactive content present in the flat-top mille graines herb *(Hedyotis corymbosa L.*) (Dewatisari et al., 2017)

Various solvents employed during the fractionation process influence the quantity of the flat-top mille graines (*Hedyotis corymbosa L.*) fractions yielded, with the water fraction exhibiting the highest yield compared to the ethyl acetate and n-hexane fractions. According to (Tursiman & Nofiani, 2012), the significant yield in the water fraction suggests the presence of numerous bioactive constituents in the flat-top mille graines (*Hedyotis corymbosa L.*) fraction with higher polarity.

Identification of the chemical compound content of fractions in flat-top mille graines (*Hedyotis corymbosa L.*) herb

Table 3. Identification results of the chemical compound content of fractions on the flat-top mille graines (*Hedyotis corymbosa L.*) herb

No.	Reactor	Observation	Fraction results		
			n-hexane	Ethyl Acetate	water
1 Alkaloid 1	Dragendorff	A yellow-red precipitate is formed	-	-	+
Alakaloid 2	Mayer	A white-yellow precipitate is formed	-	-	+
Alakaloid 3	Wagner	A brown precipitate forms and a color change occurs	-	-	+
2 Flavonoids	Concentrated HCl + Mg powder	A red, yellow or orange solution is formed	+	+	+
3 Saponin	Hot Water + HCL2N	10 cm high foam is formed	-	+	+
4 Tanin	FeCl3	A bluish black color is formed	-	+	-
5 Triterpenoid	Concentrated H2SO4	A purple ring is formed	-	-	-
6 Steroid	Glacial acetic acid + sulfuric acid	Greenish blue color change occurs	+	+	+

Information:

\+ : Presence of secondary metabolite compounds.

: Absence of secondary metabolite compounds.

Phytochemical analysis of flat-top mille graines reveals varying compositions across its fractions, with the highest concentration found in the water fraction, followed by the ethyl acetate fraction. In contrast, the n-hexane fraction contains only flavonoids and steroids. This disparity in composition is attributed to the differential solvent capabilities in binding specific compounds contained in each extract.

Average diameter of the inhibitory zone of the fractions in flat-top mille graines (*Hedyotis corymbosa L.*) herb

Antimicrobial testing using *Propionibacterium acnes* bacteria. Antimicrobial testing against Propionibacterium acnes bacteria was conducted on the flat-top mille graines (Hedyotis *corymbosa L.*) fraction at concentrations of 10% and 15% using the well method. This method relies on the diffusion of antimicrobial compounds into the solid medium containing the

inoculated test microbes. Perpendicular holes are made in the solid agar previously inoculated with bacteria to facilitate the testing process.

Table 4. Results of the average diameter of the inhibitory zone of the flat-top mille graines herb fractions against bacteria

Sample	Concentrate	Average observation results (Replications / mm)				Category
		I	II	II	Average	
n-hexane	10%	7.3	7	7	7.1±0.17	Medium
	15%	8.4	9	8.4	8.6±0.34	Medium
	Clindamycin 1%	34	33.3	34	33.7±0.4	Very Strong
	DMSO 3 ml 10%	0	0	0	0	0
Ethyl Acetate	10%	14.4	13.8	14	14.6±0.3	Strong
	15%	16.4	16.4	17	16.6±0.34	Strong
	Clindamycin 1%	34	34.5	34.1	34.2±0.2	Very Strong
	DMSO 3 ml 10%	0	0	0	0	0
Water	10%	14.4	14.1	14.1	14.2±0.1	Strong
	15%	16.4	16.4	17	16.6±0.3	Strong
	Clindamycin 1%	28.5	29	29	28.8±0.28	Very Strong
	DMSO 3 ml	0	0	0	0	0

Information:

10% = Flat-top mille graines herb fractions 10% 15% = Flat-top mille graines herb fractions 15% K+ = Positive control (Clindamycin)

K- = Negative Control (DMSO)

The positive control utilized in this study is clindamycin, a commonly employed topical treatment for acne. Clindamycin works by inhibiting the growth of *Propionibacterium acnes* on the skin surface and reducing the concentration of free fatty acids present in sebum. This reduction in free fatty acid concentration may occur as a result of clindamycin's indirect action, potentially inhibiting the lipase production of *Propionibacterium acnes* in relation to triglycerides and free fatty acids, or directly interfering with the lipase production of Propionibacterium acnes.

Clindamycin acts on Propionibacterium acnes with open comedones by inhibiting its growth and also by other mechanisms such as inhibiting leukocyte chemotaxis, which can suppress inflammation in acne vulgaris *in* vivo (American Society of Health System Pharmacists, 2005: 3341). On the other hand, the negative control in this study is DMSO, as explained by (Setiabudy & Gan, 2007), DMSO lacks antibacterial or antifungal properties and therefore cannot inhibit the growth of either.

Based on the statistical analysis presented in Table 4, there exists a notable disparity in the average diameter of bacterial inhibition zones among the n-hexane, ethyl acetate, and water fractions at concentrations of 10% and 15%, as well as the positive control. Specifically, the n-hexane fraction exhibited average inhibition zone diameters of 7.1 mm (medium), 8.6 mm (medium), and 33.7 mm (very strong) for concentrations of 10% and 15%, respectively. Similarly, the ethyl acetate fraction displayed diameters of 14.6 mm (strong), 16.6 mm (strong), and 34.2 mm (very strong), while the water fraction exhibited diameters of 14.2 mm (strong), 16.6 mm (strong), and 28.8 mm (very strong). These findings suggest that the inhibition zones observed for each fraction, at both 10% and 15% concentrations, are classified as strong, with diameter values falling within the range of 11-20 mm. In comparison, clindamycin (positive control) demonstrated a very strong inhibition zone, with a diameter exceeding 20 mm. Conversely, the negative control, DMSO, exhibited an average inhibition zone diameter of 0 mm.

Variances in the inhibitory diameters formed by each fraction against *Propionibacterium acnes* bacteria indicate variations in the active compounds present in the three fractions of flat-top mille graines herb. These differences in active compounds contribute to the distinct capacities of each fraction to impede the growth of *Propionibacterium acnes*. The ability of the flat-top mille graines herb fractions in restraining bacterial growth was evidenced by the formation of clear zones around the well.

The LSD test results conducted on Propionibacterium acnes bacteria in the flat-top mille graines (*Hedyotis corymbosa L*.) fractions at concentrations of 10% and 15%, as well as the positive control, exhibited variances compared to the negative control, indicating antibacterial activity. Furthermore, comparisons between the 10% and 15% extract groups revealed differences, suggesting that higher concentrations yield enhanced activity. This proves that the increase in concentration is in direct correlation with activity. Additionally, comparisons between all fraction treatment groups and the positive control also exhibited disparities. The ability of the flat-top mille graines (*Hedyotis corymbosa L*.) fraction to inhibit bacterial growth is attributed to its secondary metabolite content, including phenolic compounds, flavonoids, saponins, terpenoids, tannins, and quinones. Analysis of the data using the SPSS application yielded significant results, confirming the antibacterial activity of the flat-top mille graines (*Hedyotis corymbosa L*.) fraction against Propionibacterium acnes bacteria.

CONCLUSION

Based on the findings of the conducted study, it can be concluded that: First, The results of phytochemical screening of the flat-top mille graines (Hedyotis corymbosa L.) fractions reveals positive findings. The n-hexane fraction exhibited the presence of flavonoid and triterpenoid compounds. Meanwhile, the ethyl acetate fraction displayed positive indications for tannin, saponin, flavonoid, and steroid compounds. Additionally, the water fraction demonstrated the presence of alkaloids, tannins, saponins, flavonoids, and steroids. Second, The antibacterial activity of the flat-top mille graines (Hedyotis corymbosa L.) fractions against Propionibacterium acnes bacteria was observed. Specifically, the n-hexane fraction exhibited antibacterial effects at a concentration of 15%, resulting in an inhibitory zone diameter of 8.6 mm, classified as (medium). Meanwhile, the ethyl acetate fraction at a concentration of 15% displayed a diameter of 16.6 mm, classified as (strong). Similarly, the water fraction at a concentration of 15% exhibited a diameter of 16.6 mm, also classified as (strong). These findings demonstrate the efficacy of the flat-top mille graines (Hedyotis corymbosa L.) fractions against Propionibacterium acnes bacteria. Third, The flat-top mille graines herb (Hedyotis corymbosa L.) fractions demonstrated optimal concentration for inhibiting Propionibacterium acnes bacteria. Specifically, the water fraction at a concentration of 15% exhibited a significant inhibitory effect, with a diameter of 16.6 mm, classified as (strong). Similarly, the ethyl acetate fraction at a concentration of 15% also displayed notable antibacterial activity, with a diameter of 16.6 mm, also classified as (strong). These findings highlight the efficacy of the flat-top mille graines herb fractions in combating Propionibacterium acnes bacteria.

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