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RESEARCH ARTICLE

Comparison study of methanol and ethanol extract of the leaves of *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz as anti-*Staphylococcus epidermidis*

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Abstract

Background: The treatment of acne infections using natural ingredients from Kalimantan, such as Balik Angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz), offers an alternative therapy. Research shows that leaves from the plant, when soxhleted with methanol and ethanol, can inhibit the growth of *Propionibacterium acnes*. **Objective:** This research compared the effects of different solvents on the Soxhlet extraction of Balik Angin leaves in inhibiting the growth of *Staphylococcus epidermidis*. **Method:** Balik Angin leaves were extracted using Soxhlet with methanol and 70% ethanol, followed by phytochemical screening. Antibacterial activity was assessed using the well diffusion method with sample concentrations ranging from 25.6% to 0.2%. **Result:** Both extracts contained the same phytochemicals: phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids. The methanol extract had a MIC of 0.4% with weak antibacterial activity, whereas the 70% ethanol extract had a MIC of 0.8% and demonstrated stronger activity, resulting in an inhibition zone of 15.08±0.15 mm, compared to 7.30±0.16 mm for the methanol extract. Statistical analysis showed a significant difference in inhibition zones for methanol ($p < 0.05$), but not for ethanol extracts ($p > 0.05$). **Conclusion:** The use of different Soxhlet extraction solvents for Balik Angin leaves affects their activity as an anti-S. *epidermidis* agent.

Introduction

Acne vulgaris is a common condition that often affects teenagers and young adults. It is characterised by the presence of blackheads, papules, pustules, and nodules, which occur due to excessive oil gland production, resulting in blockages in the hair follicle ducts and skin pores (Syahputra *et al.,* 2021). Bacterial infections caused by Staphylococcus epidermidis, Staphylococcus aureus, and Propionibacterium acnes can lead to acne irritation (Abdulatif *et al.,* 2023). The mechanism by which bacteria cause acne involves damaging the stratum corneum and stratum granulosum, which can compromise the integrity of pore walls, leading to inflammation. The oil glands become blocked and hardened, leading to inflammation, which in turn causes the formation of acne (Gerung *et al.,* 2021).

Acne can be treated with several antibiotics, including clindamycin, erythromycin, and tetracycline. This drug can function in inhibiting and killing acne bacteria. Antibiotic use over a prolonged period can lead to skin irritation, antibiotic resistance, organ damage, and immune hypersensitivity (Agistia *et al*., 2021; Ramadhan *et al*., 2022; Fitriyanti *et al*., 2023). Natural ingredients offer an alternative treatment to address this problem, as empirical factors have been the basis for their use across generations. A natural ingredient that has been empirically shown to have potential antibacterial properties is Balik Angin, also known as Alphitonia incana (Roxb.). Teijsm. & Binn. ex Kurz, which is one of the efficacious endemic plants from the island of Kalimantan. The Balik Angin plant has Latin synonyms including *A. excelsa, A. moluccana,* and *A.*

Philippinensis (Sarifudin & Wahyudi, 2013; Dodo *et al*., 2016).

The Dayak tribe traditionally uses the Balik Angin plant for skin care as a natural soap for bathing. The community also uses this plant to treat tinea versicolor infections and reduce itching and inflammation by kneading it and then applying the extract to the affected part of the body (Wardah & Sundari, 2019; Forestryana *et al.,* 2022). Based on this ethnomedicine, previous research carried out antibacterial testing on Balik Angin leaves, which were extracted using the maceration method with methanol and 70% ethanol, yielding the same Minimum Inhibitory Content (MIC) value of 3.2% for P. acnes bacteria. However, the mean diameter of the inhibition zone produced was greater in the 70% ethanol extract of 9.475 ± 0.311 mm, which was classified as medium activity, compared to the methanol extract with an average inhibition zone diameter of 2.550 ± 0.850 mm, which was classified as weak category (Ramadhan *et al.,* 2023a).

Further research showed that Balik Angin leaves were soxhleted with methanol and ethanol solvents can inhibit the growth of P. acnes in the medium category with a Minimum Inhibitory Concentration (MIC) of 0.8% which has a higher diameter of the inhibition zone of 9.2 ± 0.229 mm (methanol extract) and 9,6 ± 0,351 mm (ethanol extract) (Ramadhan *et al*., 2023b). The result showed that Soxhlet extraction optimally attracts the antibacterial active compounds from Balik Angin leaves compared to maceration extraction. Based on this research, it is necessary to compare the effect of different solvents used in Soxhlet extraction (methanol and ethanol) on Balik Angin leaves in inhibiting the growth of *Staphylococcus* epidermidis, a bacterium that causes acne.

A previous study stated that *Staphylococcus epidermidis* was the most commonly isolated microorganism from acne patients and grew well under aerobic conditions, in approximately 50.5% of all cultures. Bacterial susceptibility testing results indicate that, out of a total of 91 specimens, 46 specimens exhibited growth of S. *epidermidis*, 10 specimens showed growth of P. *acnes*, and 7 specimens demonstrated growth of S. aureus. *S. epidermidis* was more resistant to erythromycin, but more sensitive to minocycline. The resistance patterns of *P. acnes* and *S. epidermidis* were similar, with both being resistant to erythromycin. The susceptibility of *S. epidermidis* against doxycycline (89.1%) was slightly lower than that of *P. acnes* (100%). However, erythromycin resistance was higher in *S. epidermidis* (65.2%) than in *P. acnes* (10%). The research also found that *S. epidermidis* was resistant to clindamycin (52.2%) and tetracycline (32.6%). Resistance genes in *S. epidermidis* are more easily obtained and transmitted. These genes have an essential role as reservoirs of antibiotic resistance (Sitohang *et al.,* 2019).

The use of clindamycin and erythromycin should be limited, as most S. *epidermidis* isolates are resistant to both agents. Choosing the best acne treatment, especially for sensitive skin, can be challenging because topical antibiotic medications and benzoyl peroxide may cause skin irritation. Traditional Methodologies utilise nature-derived substances to mitigate acne symptoms and may be the choice of acne treatment for sensitive skin, minimising irritation. Alternative systemic therapies for acne encompass various natural remedies with purported efficacy, reputed for their effectiveness, have been reported in acne management (Jaiswal *et al*., 2024).

Methods

Chemicals

The materials used in this research include Balik Angin leaves obtained from Mount Tahura, Banjar Regency, South Kalimantan in February 2024, methanol (Onemed), 70% ethanol (Onemed), $FeCl_3$ (Merck), anhydrous acetic acid (Merck), chloroform (Merck), magnesium (Mg) powder (Merck), HCl (Merck), Mayer's reagent (Nitra Kimia), Wagner's reagent (Nitra Kimia), Dragendorff's reagent (Nitra Kimia), gelatin (Merck), Na-CMC (Himedia), H_2SO_4 (Merck), Nutrient Agar powder (Merck), Muller Hinton Agar powder (Oxoid), distilled water (Onemed), sterile 0.9% NaCl solution (PT Widatra Bakti), *Propionibacterium acnes* ATCC 1223 bacterial culture, and Clindamycin (2µg/disc) (Oxoid).

Extraction of Balik Angin leaves

Balik Angin leaves simplicia are obtained from the processing of fresh, mature Balik Angin leaves, which are collected, washed, wet-sorted, and dried for four days at room temperature. The dried samples were subjected to dry sorting, then ground and sieved with a 40 mesh size (Cock, 2020; Fuentes *et al.*, 2020), then extracted using the Soxhlet method with an initial weight of 50 g of Simplicia and solvent (methanol and 70% ethanol) respectively of 358 mL, then evaporated at < 60ºC and concentrated using a water bath at 50ºC (Ahmed *et al*., 2019).

Phytochemical screening

1. Phenolic test: A sample of 0.1 g was dissolved in 2 mL of each solvent, then 1 mL of 10% FeCl3 solution was added. The appearance of green, red, blue,

purple, or black colour formation indicates that it is positive for phenolic content (Ramadhan *et al.,* 2020).

2. Flavonoid test: A sample of 0.1 g was dissolved in 2 mL of each solvent, then 2 mg of Mg powder and 1 mL of concentrated HCl were added, after which amyl alcohol was added and a red, yellow, or orange color was formed indicating the presence of flavonoid compounds (Ramadhan *et al*, 2021).
3. Alkaloid test: A sample of 0.1 g was added to HCl (5 mL), then divided into three tubes. In tube 1, Mayer's reagent was added, in tube 2, Dragendorff's reagent was added, and in tube 3, Wagner's reagent was added. Until an orange-yellow precipitate was formed in tube 1, a white precipitate in tube 2, and a red-brown precipitate in tube 3. This colored precipitate indicated the presence of alkaloid compounds (Ramadhan *et al*, 2020).
4. Tannin test: A sample of 0.1 g was dissolved in 2 mL of each solvent, and then a 1% gelatin solution was added. A white precipitate formed, indicating the presence of tannin compounds (Ramadhan *et al*., 2023).
5. Saponin test: A sample of 0.1 g was added to 5 mL of hot water and shaken vigorously for approximately ten seconds. If stable foam forms for approximately ten minutes and after adding one drop of 2N HCl, the foam does not disappear (Ramadhan *et al*., 2020).
6. Steroid-triterpenoid test: A sample of 0.1 g was added with 2-3 mL of chloroform, ten drops of acetic anhydride, and two to three drops of H_2SO_4 (Liebermann-Burchard's reagent) through the tube wall. If a blue to green colour forms, it is positive for containing steroids, whereas if a red or purple colour forms, it is positive for containing triterpenoids (Ramadhan *et al*., 2020).

Antibacterial assay

The antibacterial activity assay of Balik Angin leaves extract was carried out using sterile Muller Hinton Agar (MHA) media and then inoculated with a suspension of S. *epidermidis* bacteria using a sterile cotton swab. Methanol and 70% ethanol extracts of Balik Angin leaves were prepared at concentrations of 0.2%, 0.4%, 0.8%, 1.6%, 3.2%, 6.4%, 12.8%, and 25.6%. Then, 20 µL of each concentration was added to each well of the MHA media. A clindamycin antibiotic disc was used as a positive control, and Na-CMC of 0.5% (extract solvent) as a negative control. The culture is placed in the refrigerator at 2-8°C for 14-18 hours, allowing the compound to diffuse into the media. The resulting diffusion culture was incubated at 37°C for 24 hours. The diameter of the inhibition zone formed is measured using a calliper and classified based on category (Fitriyanti *et al*., 2020; Ramadhan *et al*., 2020; Ramadhan *et al*., 2023).

Statistical analysis

The data obtained is the diameter of the inhibition zone. The data was analysed using SPSS to see whether there were differences between each test group with clindamycin and Na-CMC. If the data obtained is regular and homogeneous, a One-Way ANOVA test is carried out with a 95% confidence level, followed by a Post hoc Tukey HSD Test with a 95% confidence level. If the data is not normally distributed or homogeneous, the Kruskal-Wallis and Mann-Whitney tests are carried out (Ramadhan *et al*, 2023a).

Results

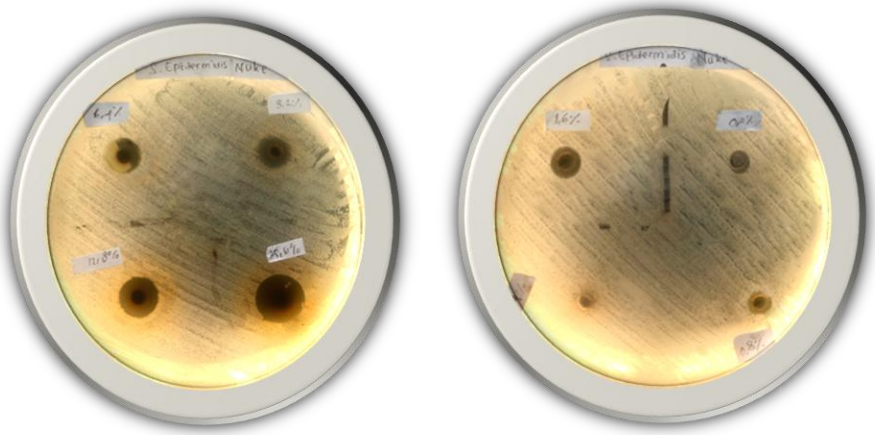
The results of this study showed that the methanol extract has the same number of secondary metabolite groups as the ethanol extract based on phytochemical screening. Both extracts contain phenolics, flavonoids, alkaloids, tannins, saponins, and triterpenoids, which are shown in Table I.

Table I: Phytochemical screening result of Balik Angin leaves extract

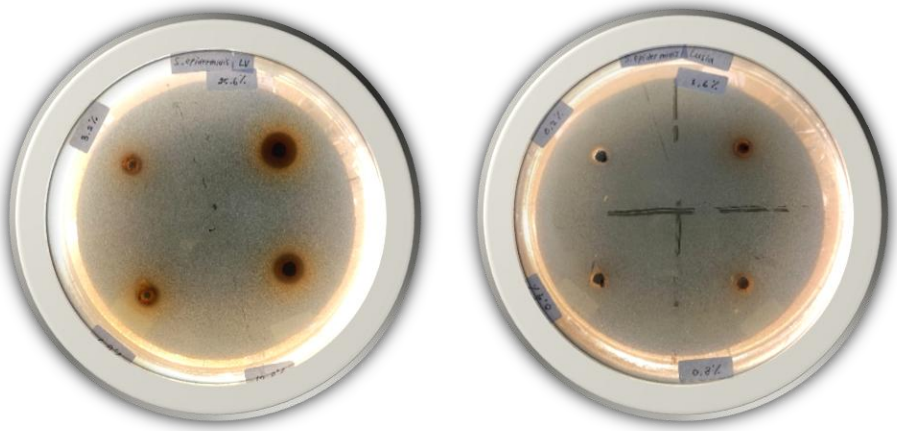
Compounds	Extract of	
	Methanol	Ethanol
Phenols	+	+
Flavanoids	+	+
Alkaloids	+	+
Tannins	+	+
Saponins	+	+
Steroids	-	-
Triterpenoids	+	+

The anti-*Staphylococcus epidermidis* activity assay of Balik Angin leaves extract utilised concentration variations of 25.6%, 12.8%, 6.4%, 3.2%, 1.6%, 0.8%, 0.4%, and 0.2%, with the positive control being Clindamycin at 2 µg/disk and the negative control being 0.5% Na-CMC. The test results show that only six concentrations of ethanol extract can provide an inhibitory zone diameter for the growth of *S. epidermidis* bacteria from 0.8%-25.6%, while concentrations of 0.2% and 0.4% do not show inhibitory power against bacteria, but methanol extract

showed a Minimum Inhibitory Concentration (MIC) of 0.4%. However, the positive control showed better antibacterial activity than all test concentrations, as it produced a larger inhibitory zone diameter and was categorised as very strong. The results are shown in Figure 1.



(a)



(b)

(c)

Figure 1: The result of anti-*Staphylococcus epidermidis* activity assay of (a) methanol extract of Balik Angin leaves, (b) ethanol extract of Balik Angin leaves, and (c) Clindamycin 2 µg/disk and 0.5% Na-CMC.

Based on the research that has been carried out, it can be said that the methanol and 70% ethanol extracts of Balik Angin leaves have different MIC (Minimum Inhibitory Concentration) values, which is methanol extract has MIC of 0.4 % with the medium category as antibacterial and ethanol extract has MIC of 0.8% with the strong category as shown in Table II. The high concentration (25.6%) of Balik Angin leaves extract can provide a larger average inhibitory zone diameter and is categorized as a strong antibacterial but still can not go beyond potent when compared to the positive control clindamycin with an average inhibitory zone diameter of 23.38 ± 0.33 mm which has a very strong antibacterial category.

Table II: The result of inhibition zone diameters of anti-*Staphylococcus epidermidis* of Balik Angin leaves extract

Concentrations (%)	Diameters of inhibition zone (mm)	
	Methanol extract	Ethanol extract
25.6	12.13±0.15	17.34±0.40
12.8	11.25±0.10	16.46±0.20
6.4	10.28±0.23	15.62±0.29
3.2	9.3±0.24	15.52±0.29
1.6	8.3±0.14	15.21±0.43
0.8	7.3±0.16	15.08±0.15
0.4	4.5±0.10	-
0.2	-	-

Discussion

Balik Angin Leaves were obtained from Mount Tahura, Banjar Regency, South Kalimantan. Based on the results of the determination at the LIPI Biological Research Centre, Cibinong, Bogor, it has the Latin name Alphitonia incana (Roxb.). Teijsm. & Bin. ex Kurz with certificate number B-208/V/DI.05.07/1/2022. The green and fresh Balik Angin leaves, weighing 3,100 g, are made into simplicia. The stages of making simplicia begin with leaf collection, wet sorting, chopping, drying, dry sorting, and pollination, culminating in a simplicia weight of 585.70 g with a yield value of 18.89%. The extraction process for Balik Angin leaves was carried out using the Soxhlet apparatus until the cycle was complete (indicating that methanol and 70% ethanol are optimal solvents for extracting the active compounds), and a liquid extract was obtained. The liquid extract obtained is then concentrated to get a thick extract. Extraction using a Soxhlet apparatus is based on the principle of repeated condensation, which occurs when the solvent is heated, allowing for the indirect immersion of the sample to filter out the active substances.

The Soxhlet method can affect the solubility of secondary metabolites contained in Balik Angin leaves. High temperatures increase the solubility of active substances in solvents, as heating opens the plant tissue, allowing some compounds that cannot be extracted at room temperature to be extracted. Additionally, the increasing temperature can enhance the diffusion process, allowing the extraction process to run faster (Pamungkas *et al*., 2017). The research results showed that the yield of methanol extract was greater (39.46%) than that of the ethanol extract (18.6%). In previous research, Ramadhan *et al*. (2023) reported that the yield of maceration from Balik Angin leaves was 26.22% for the methanol extract and 49.91% for the 70% ethanol extract. This indicates that Soxhlet extraction using a methanol solvent on Balik Angin leaves yields a greater amount than maceration; however, this condition does not apply to the 70% ethanol extract.

The phytochemical screening of both the methanol extract and the 70% ethanol extract of Balik Angin leaves revealed no differences in the content of secondary metabolite compounds, including phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids, as shown in Table I. The similarity of phytochemical compounds in both extracts may be due to the phytochemical polarity index and its association with the solvent polarity index. A similar polarity index containing solvents can dissolve phytochemicals that have a similar or closely related polarity index. Methanol has a polarity of 5.1, while ethanol has a polarity of 4.3 (Wakeel *et al*., 2019). This value influences the similarity of the phytochemical groups contained in the methanol and 70% ethanol extracts of Balik Angin leaves.

Secondary metabolite compounds identified in Balik Angin leaf extract contribute to its antibacterial activity. Phenolic compounds, flavonoids, and tannins can initiate protein denaturation of bacterial cells. The compounds can form hydrogen bonds with cell walls and cytoplasmic membranes, thereby affecting their permeability. Finally, bacteria become lysed because the balance of macromolecules and ions within the cells is disrupted. The B ring of flavonoids can also inhibit bacteria's DNA and RNA synthesis by intercalating with the base structure of nucleic acids. Apart from that, flavonoids are

also able to disturb the bacteria's oxygen circulation by preventing the formation of energy in the cytoplasmic membrane and inhibiting bacterial motility (Kumar & Pandey, 2013; Nomer *et al*., 2019). Tannins also act as antibacterials by inhibiting the RNA reverse transcriptase enzyme and DNA topoisomerase, thereby inhibiting bacterial replication. Triterpenoid compounds can act as antibacterials by interacting with porins (transmembrane proteins), forming strong polymer bonds that disrupt the bacterial cell wall and alter its permeability, resulting in bacteria lacking nutrition and exhibiting stunted or dying growth (Amalia *et al*., 2017). Meanwhile, alkaloid compounds disrupt the peptidoglycan, allowing the cell wall layer of bacteria to form imperfectly. The mechanism of saponin is to increase the cell membrane's permeability and change its structure, thereby disrupting bacterial metabolic processes (Fitriyanti *et al*., 2020; Pariury *et al*., 2021).

The antibacterial mechanism of the secondary metabolites contained in the methanol and 70% ethanol extracts results in the high antibacterial potential of Balik Angin leaves. The anti-*Staphylococcus epidermidis* activity assay of Balik Angin leaves extract used a concentration variation of 25.6%; 12.8%; 6.4%; 3.2%; 1.6%; 0.8%; 0.4%; and 0.2% with Clindamycin 2 µg/disk and 0.5% Na-CMC. Based on the research that has been carried out in Table II, it can be said that the ethanol extracts have more antibacterial potential than methanol extract because in same concentration value of 0.8% shows that the ethanol extract can provide a larger average inhibitory zone diameter of 15.08 ± 0.15 mm compared to methanol extract with an average diameter of the inhibitory zone which is 7.3 ± 0.16 mm. The different antimicrobial activities of both extracts may be due to the varying types and quantities of biological compounds present in these extracts. The role of solvent polarity in determining the amount and quality of secondary metabolites and their associated biological activities has been previously reported. Soraya (2022) reported that the total phenolic contents (TPC) of the ethanol extract (438.3 ± 0.122 µg GAE/mg extract) were found to be higher than those of the methanol extract (25.23 ± 0.15 µg GAE/mg extract) from the research of Ahmed *et al*. (2019). Both studies also reported that the total flavonoid content (TFC) from the ethanol extract was 11.64 ± 0.039 µg QE/mg extract, which was higher than the value for the methanol extract, 9.84 ± 0.06 µg QE/mg extract. However, the MIC values of these extracts exhibit lower anti-Staphylococcus epidermidis activity compared to standard antibiotics used for acne treatment, such as Clindamycin.

The results of this research were compared with those of the anti-Propionibacterium acnes activity test using the macerated extract of Balik Angin leaves with methanol and 70% ethanol, as reported by Ramadhan *et al*. (2023a), which showed better potential for development as an alternative therapy for acne infections. At the same concentration, which was 3.2%, the results of extraction with Soxhlet can provide a larger inhibition zone diameter as an antibacterial with the medium to the strong category, but in the Balik Angin leaves extract from maceration extraction only produce an inhibition zone diameter which is included in the weak to the medium category as an antibacterial in both methanol extract (2.550 ± 0.850 mm) and 70% ethanol extract (9.475 ± 0.311 mm). These results suggest that Balik Angin leaves extracted using Soxhlet with methanol and 70% ethanol solvents have more potential as an antiacne agent than those extracted using maceration. The extraction process using the Soxhlet apparatus will run continuously with a pure solvent resulting from condensation, causing the pressure difference between inside and outside the cell to be more optimal. This pressure difference breaks down the walls and membranes of the cell, ultimately resulting in more active substances being extracted (Mukhrani, 2014).

This research also provides new evidence that Balik Angin leaves have great potential as an anti-acne agent caused by *S. epidermidis* compared to other natural ingredients such as green tea leaves. In 2020, Azizah *et al*. reported that green tea ethanol extract at a high concentration of 100% showed an inhibition zone diameter of 19.86 ± 0.850 mm against S. *epidermidis*, while Balik Angin leaves ethanol extract at 25.6% concentration provided an inhibition zone diameter of 17.34 ± 0.40 mm. Pramiastuti *et al*. (2024) also reported in the recent study of 0.5% single ethanol extract of green tea leaves obtained inhibition zones of 5.17mm and the combination of cherry leaf extract and green tea leaves with a different ratio obtained results of around 8.5-9 mm which showed antibacterial activity in the moderate category, while 0.8% single ethanol extract of Balik Angin show a strong category of 15.08 ± 0.15 mm against *S. epidermidis*.

Data analysis using SPSS version 26 proceeded through stages, including a Test of Normality. The data from both extracts showed the same sig values, which are obtained (> 0.05). Meanwhile, in the Test of Homogeneity, a significant value was obtained (> 0.05) in both the methanol extract data and the 70% ethanol extract data. The analysis test was continued with parametric tests, which included the One-Way ANOVA Test. The one-way ANOVA result showed a value ($p < 0.05$) indicating a significant difference in the inhibition zone between each concentration of methanol extracts. In contrast, the value for ethanol extracts ($p > 0.05$) showed no significant differences. Meanwhile, the results also showed methanol extract data using variations in concentration were 25.6%, 12.8%, 6.4%, 3.2%, 1.6%, and

0.8% compared to the positive control (Clindamycin) showing the significance value is no more than 0.050, so it can be concluded that the variation in extract concentration has a significant difference to Clindamycin, as well as the 70% ethanol extract data using the same extract concentration, variation shows a substantial difference to the positive control with a significance value of no more than 0.050. Based on the analysis of this data, it can be assumed that the antibacterial activity produced by the Balik Angin leaves ethanol extract has more potential to inhibit the growth of *Staphylococcus epidermidis* than the methanol extract, so its potential needs to be explored more deeply, especially against other acne-causing bacteria, such as *Staphylococcus aureus*, so that can strengthen the potential of Balik Angin leaves as an alternative treatment for acne.

Conclusion

This research concludes that the differences in solvents used in the Soxhlet extraction of Balik Angin leaves do not affect the classes of secondary metabolite compounds contained, which include phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids. Differences of Soxhlet extraction solvents in Balik Angin leaves can influence their antibacterial activity against the growth of *Staphylococcus epidermidis* by producing different average diameters of inhibition zones, which are methanol extract of 4.5 ± 0.10 mm is included in the weak category with a MIC of 0.4% and 70% ethanol extract of 15.08 ± 0.15 mm are included in the strong category with a MIC of 0.8%.

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