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The Effect of Different Soxhlet Extraction Solvents Towards Anti-*Propionibacterium acnes* Activity of Balik Angin Leaves (*Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz)

(*Pengaruh Perbedaan Pelarut Ekstraksi Soxhlet Terhadap Aktivitas Anti-Propionibacterium acnes dari Daun Balik Angin (Alphitonia incana (Roxb.) Teijsm. & Binn. ex Kurz)*)

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ABSTRACT

Background: Acne is a skin infection that may be induced by *Propionibacterium acnes*. Treatment of acne infection from natural ingredients native to Kalimantan such as Balik Angin or *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz can be an alternative therapy. Prior research indicates that leaves macerated with methanol and 70% ethanol solvents can inhibit the growth of *P.acnes* in the medium category with a Minimum Inhibitory Concentration (MIC) of 3.2%. **Objectives:** The research purpose is to compare the effect of the different solvents of soxhlet extraction on the antibacterial activity of Balik Angin leaves in inhibiting the growth of *P.acnes*. **Material and Methods:** Balik Angin leaves were extracted using the Soxhlet with two different solvents, which were methanol and 70% ethanol, then followed by phytochemical screening. The antibacterial activity assay was carried out using well diffusion with each sample concentrations of 25.6%; 12.8%; 6.4%; 3.2%; 1.6%; 0.8%; 0.4%; and 0.2%. **Results:** Both extracts contained the same phytochemical groups, which were phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids. The antibacterial activity test results showed that both extracts had the same MIC of 0.8%. however, the 70% ethanol extract of Balik Angin leaves was able to provide a larger inhibition diameter average of 9.61±0.35 mm compared to the methanol extract of 9.20±0.22 mm. Nonetheless, there was no significant difference ($p>0.05$) between both concentrations, when compared to the range of 1.6-25.6%. **Conclusions:** The conclusion of this research is that differences in Soxhlet extraction solvents in Balik Angin leaves can affect its activity as an anti-*Propionibacterium acnes* agent

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INTRODUCTION

Acne vulgaris is a disease that often occurs in teenagers and young adults. It is characterized 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). One of the bacteria that play an active role in the formation of acne is *Propionibacterium acnes* (Ramadhan et al, 2022). The mechanism by which *P. acnes* forms acne is by damaging the stratum corneum and stratum germinate and as a result it can destroy the pore walls, resulting in inflammation, the oil glands become blocked and harden, then inflammation occurs which leads to the formation of acne (Gerung et al, 2021).

Acne can be treated using several antibiotics such as clindamycin, erythromycin, and tetracycline. This drug can function in inhibiting and killing *P. acnes* bacteria. Long-term use of these drugs can cause skin irritation, resistance, organ damage, and immune hypersensitivity (Agistia et al, 2021; Fitriyanti et al, 2023). Natural ingredients are an alternative treatment to overcome this problem, considering that empirical factors have been the basis for their use from generation to generation. A natural ingredient that empirically has potential as an antibacterial is Balik Angin or *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 and Wahyudi, 2013; Dodo et al, 2016).

The Balik Angin plant is empirically used by the Dayak tribe as a natural bath soap for skin care. This plant is also used by the community to treat skin infections such as tinea versicolor and itching as well as inflammation by kneading it and then rubbing it on the sick part of the body (Wardah and Sundari, 2019; Forestryana et al, 2022). Based on this ethnomedicine, previous research carried out antibacterial testing on Balik Angin leaves which were extracted with methanol and 70% ethanol using the maceration method, each of which had the same Minimum Inhibitory Content (MIC) value, which is 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 methanol extract with an average inhibition zone diameter of 2.550 ± 0.850 mm which was classified as weak category (Ramadhan et al, 2023). This research prompted further research to be carried out to find out the effect of differences in Soxhlet extraction solvents (methanol and 70% ethanol), on the content of secondary metabolites and the antibacterial activity of Balik Angin leaves in inhibiting *P. acnes* bacteria growth.

MATERIAL AND METHODS

Materials

This research used Balik Angin leaves sourced from Mount Tahura, Banjar Regency, South Kalimantan in January 2023.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).

Methods

Extraction of Balik Angin Leaves

Balik Angin leaves simplicia was obtained from the processing of fresh, mature Balik Angin leaves, 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: 0.1 g of extract was dissolved in 2 mL of each solvent, then droped 1 mL of 10% $FeCl_3$ solution. The appearance of green, red, blue, purple, or black color formation indicates that it was positive for phenolic content (Ramadhan et al, 2020).
2. Flavonoid test: 0.1 g of extract 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: 0.1 g of extract was added with 5 mL of HCl, then divided into three tubes, in tube 1 was added Mayer's reagent, tube 2 was added with Dragendorff's reagent, and tube 3 was added with Wagner's reagent. 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: 0.1 g of extract was dissolved in 2 mL of each solvent, then 1% gelatin solution was added, and a white precipitate was formed indicating the presence of tannin compounds (Ramadhan et al, 2023).
5. Saponin test: 0.1 g of extract was added to 5 mL of hot water and shaken vigorously for approximately 10 seconds. If stable foam forms for approximately 10 minutes and after adding one drop of 2N HCl, the foam does not disappear (Ramadhan et al, 2020).
6. Steroid-triterpenoid test: 0.1 g of extract was added with 2-3 mL of chloroform 10 drops of acetic anhydrous and two-three drops of H_2SO_4 (Liebermann-Burchard's reagent) through the tube wall. If a blue to green color forms, it was positive for containing steroids, whereas if a red or purple color forms, it was 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 implanted with a suspension of *P. acnes* bacteria using a sterile cotton swap. Methanol and 70% ethanol extracts of Balik Angin leaves were each made to a concentration of 0.2%; 0.4%; 0.8%; 1.6%; 3.2%; 6.4%; 12.8%; and 25.6%, then 20 µL of various concentrations were added to each well of the MHA media. Clindamycin antibiotic disc was used as a positive control and Na-CMC of 0.5% (extract solvent) as a negative control. The culture was placed in the refrigerator at 2-8°C for 14-18 hours so that the compound can diffuse in the media. The resulting diffusion culture was incubated at 37°C for 24 hours. The diameter of the inhibition zone formed was measured using a caliper and classified based on category (Fitriyanti et al, 2020; Ramadhan et al, 2020; Ramadhan et al, 2023).

Data Analysis

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

RESULTS AND DISCUSSION

Balik Angin leaves were sourced from Mount Tahura, Banjar Regency, South Kalimantan, and were identified at LIPI, Biological Research Center, Cibinong, Bogor, with the scientific 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 of 3,100 g are made into simplicia and pollination until the weight of

simplicia is 585.70 g with a yield value of 18.89%. The Soxhlet extraction process was carried out until a cycle was obtained which showed that the methanol and ethanol solvents became clear during the circulation process. This indicates that the active compounds extraction process was running optimally. Extraction using a Soxhlet apparatus has the principle of repeated condensation due to the heating of the solvent, resulting in indirect immersion of the sample to filter the active substances. The Soxhlet method can influence the level of solubility of secondary metabolites contained in Balik Angin leaves, where high temperatures will increase the solubility of active substances in solvents because heating will open the tissue of the plant so that it can attract some of the secondary metabolites which cannot be extracted only at room temperature. In addition, the increasing temperature can cause the diffusion process to become optimal, so that the extraction process will also run faster (Pamungkas et al, 2017).

Table 1. Comparison of the yield of Balik Angin leaves extract from extraction with Soxhlet.

No	Solvent	Weight of Powder (g)	Weight of Extract (g)	Yield (%)
1	Methanol	50	19.73	39.46
2	Ethanol of 70%	50	9.3	18.6

The research results showed that the yield of methanol extract was greater than the 70% ethanol extract of Balik Angin leaves as seen in Table 1. In previous research, Ramadhan et al, (2023) stated that the yield of maceration results of Balik Angin leaves was 26.22% for methanol extract and 49.91% for 70% ethanol extract. Based on the comparison with previous research, this study shows that Soxhlet extraction using methanol solvent on Balik Angin leaves produces a higher yield than maceration. However, Soxhlet extraction using 70% ethanol on Balik Angin leaves produces a lower yield value than maceration in the previous research. Many factors may affect the content of compounds and their bioactivity in the extraction process of plant material. These factors include the type of solvent, solvent concentration, solvent pH, material-to-solvent ratio, extraction method, extraction duration, agitation, pressure, and the temperature used for extraction. Solvents are known to directly affect the extraction efficiency of phytochemicals from plant materials. The range of solvents from polar to nonpolar can extract various compounds with corresponding polarities, depending on the polarities of the solvents and target compounds. Typically, a combination of polar (methanol) and less polar (ethanol) solvents is more effective in extracting phytochemicals from plant materials (Al Ubeed et al., 2022).

The phytochemical screening identified in both the methanol extract and the 70% ethanol extract of Balik Angin leaves did not show any differences in the content of secondary metabolite compounds

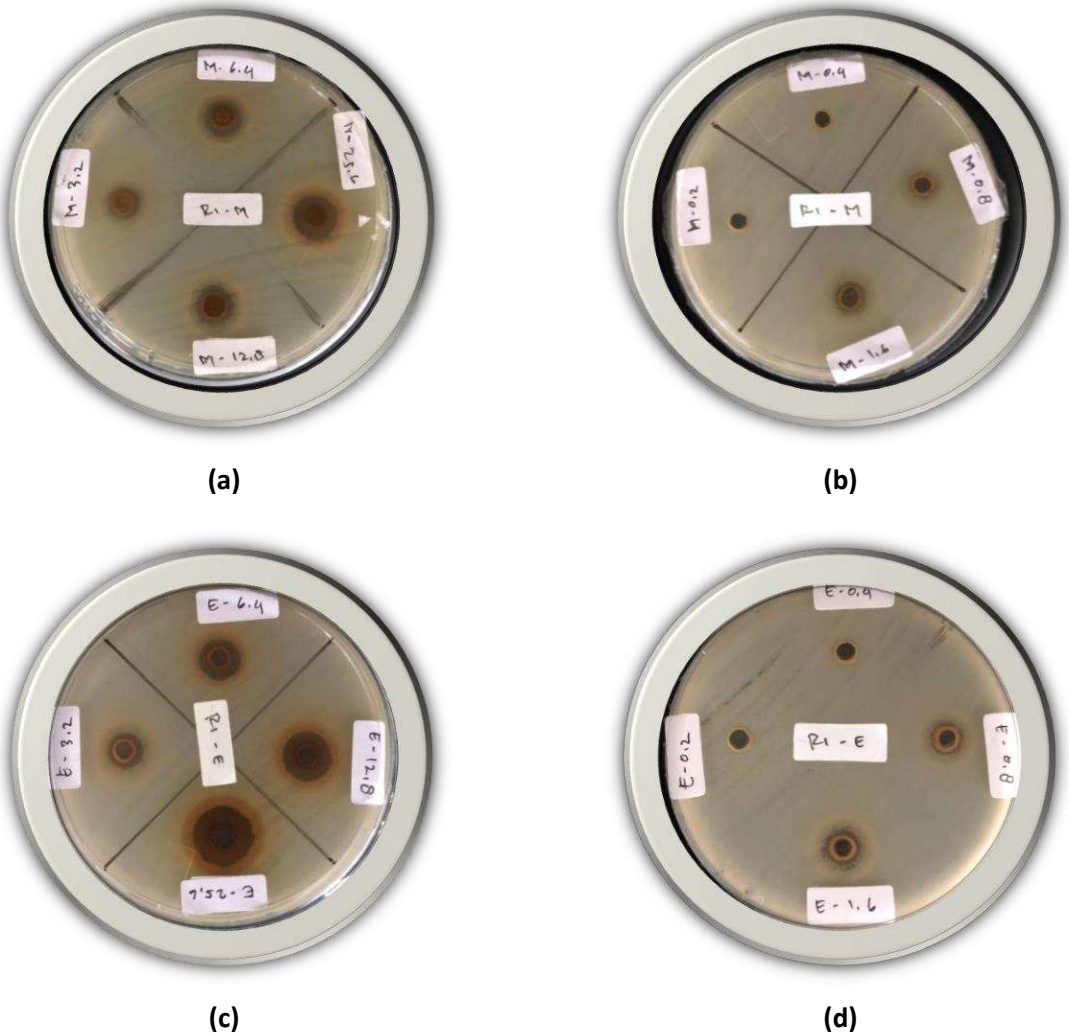
including phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids as in Table 2. Secondary metabolite compounds identified in Balik Angin leaf extract as playing a role in producing antibacterial activity. Phenolic compounds, flavonoids, and tannins can initiate protein denaturation of bacterial cells by forming hydrogen bonds, thereby affecting the permeability of cell walls and cytoplasmic membranes, causing macromolecules and ions imbalance in cells which results in bacteria becoming lysed. Flavonoids can also inhibit nucleic acid synthesis, which is the B-ring of flavonoids that can intercalate or form hydrogen bonds with the base structure of nucleic acids, thus causing inhibition of DNA and RNA synthesis in bacteria. Apart from that, flavonoids are also able to inhibit the use of oxygen by bacteria, trough preventing the formation of energy in the cytoplasmic membrane and inhibiting bacterial motility (Kumar and 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 through their reaction with porins (transmembrane proteins), forming strong polymer bonds, thereby disrupting the permeability of the cell wall, resulting in bacteria lacking nutrition and their growth will being stunted or die (Amalia et al, 2017). Meanwhile, alkaloid compounds disrupt peptidoglycan so that the cell wall layer of bacteria unforms perfectly. 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).

Table 2. Comparison of phytochemical screening results of Balik Angin leaves extract.

No	Secondary Metabolite	Reagent	Methanol extract	70% Ethanol extract	Information
1.	Phenol	$FeCl_3$ of 10%	(+)	(+)	A blue-black color is formed.
2.	Flavonoid	Mg powder & HCl + Amyl Alcohol	(+)	(+)	A red color forms.
3.	Alkaloid	H_2SO_4 & Mayer's reagent	(+)	(+)	A white precipitate is formed.
		H_2SO_4 & Dragendorff's reagent	(+)	(+)	A red precipitate is formed.
		H_2SO_4 & Wagner's reagent	(+)	(+)	A brown precipitate forms.
4.	Tannin	Gelatin of 1%	(+)	(+)	A white precipitate is formed.
5.	Saponin	Distilled water & HCl of 2N	(+)	(+)	A stable foam is formed.
6.	Steroid	Chloroform & Liebermann-Burchard's reagent	(-)	(-)	No green-blue color is formed.

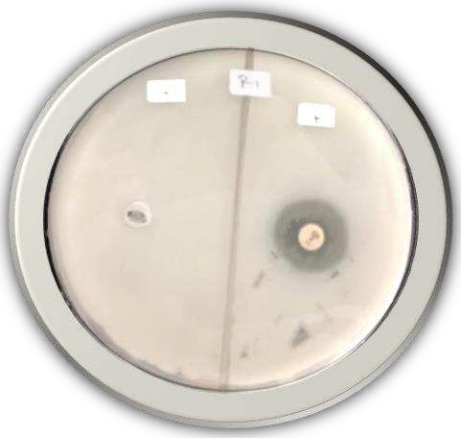
Triterpenoid	Chloroform & Liebermann-Burchard's reagent	(+)	(+)	A red color forms.

The antibacterial mechanism of the secondary metabolites in the methanol and 70% ethanol extracts results in the high antibacterial potential of Balik Angin leaves. The anti-*Propionibacterium acnes* 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 as a positive control and 0.5% Na-CMC as a negative control. The antibacterial test results using the Well diffusion method show that only 6 concentrations (1.6%-25.6%) of each extract can inhibit the growth of *P. acnes* bacteria, meanwhile, concentrations of 0.2% and 0.4% do not show inhibitory power against bacteria. An inhibitory zone diameter of *P. acnes* bacteria growth can be seen as a clear zone area around the sample Well that indicates the extract can inhibit the growth of *P. acnes* (Figure 1).



(a) **(b)**

(c) **(d)**



(e)

Figure 2. Anti-Propionibacterium acnes activity test results from (a. 3.2-25.6%; b. 0.2-1.6%) methanol extract and (c. 3.2-25.6%; d. 0.2-1.6%) 70% ethanol extract of Balik Angin leaves compare to (e) Clindamycin 2 µg/disk and 0.5% Na-CMC.

Research indicates that the methanol and 70% ethanol extracts of Balik Angin leaves exhibit identical minimum inhibitory concentrations for bacterial growth, measured at 0.8%, categorizing them as antibacterial agents. Table 3 shows that the 70% ethanol extract of Balik Angin leaves is able to provide a larger average inhibitory zone diameter of 9.61 ± 0.35 mm compared to methanol extract with an average inhibitory zone diameter of 9.20 ± 0.22 mm. Meanwhile, at a concentration of 25.6% to 1.6%, it is categorized as a strong antibacterial but still can not go beyond potent when compared to the positive control clindamycin which has a very strong antibacterial category. The results of this research were compared with the anti-*Propionibacterium acnes* activity test of macerated Balik Angin leaves using methanol and 70% ethanol , as reported by Ramadhan et al. (2023), indicating potential for development as an alternative therapy for acne infections. At the same concentration, which was 3.2%, the results of extraction with Soxhlet are able to provide a larger inhibition zone diameter as an antibacterial with a strong category, but in Balik Angin leaves 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 can prove that Balik Angin leaves extracted using Soxhlet with methanol and 70% ethanol solvents have more potential as anti-*Propionibacterium acnes* than extraction using maceration. The extraction process using the soxhlet apparatus will run continuously with a pure solvent resulting from condensation, causing the pressure difference to be more optimal between inside and outside the cell, to break down its walls and membranes, which ultimately results in more active substances being extracted (Mukhrani, 2014).

Table 3. Comparison of Inhibition Zone Diameters from Variation of Concentrations of Balik Angin Leaves Extract.

Concentration	Mean ± SD (mm)		Category
	Methanol Extract	70% Ethanol Extract	
25.6%	14.11 ± 0.38	19.46 ± 0.71	Strong
12.8%	12.83 ± 0.18	15.66 ± 0.32	Strong
6.4%	11.83 ± 0.22	13.01 ± 0.77	Strong
3.2%	11.03 ± 0.02	11.56 ± 0.27	Strong
1.6%	10.13 ± 0.20	10.45 ± 0.13	Strong
0.8%	9.20 ± 0.22	9.61 ± 0.35	Medium
0.4%	0	0	-
0,2%	0	0	-
Positive control	23.36 ± 0.71		Very Strong
Negative control	0		-

Data analysis using SPSS version 24 went through stages of Normality test. The methanol extract data showed two sig values, which are (<0.05) and (>0.05), while the 70% ethanol extract obtained a sig value (>0.05). Meanwhile, in the Test of Homogeneity, a sig value was obtained (<0.05) in the methanol extract data and 70% ethanol extract data. The analysis test was continued with non-parametric tests, which are the Kruskal-Wallis Test and the Mann-Whitney Test. Based on the results of the Kruskal-Wallis test, a value was obtained (Asymp. Sig. 0.002 < 0.05) for the methanol extract data and 70% ethanol extract data. Meanwhile, the results of the Mann-Whitney Test on 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) show 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 the positive control, as well as the 70% ethanol extract data using the same extract concentration, variation shows a significant difference to the positive control with a significance value of no more than 0.050.

Statistical analysis was also carried out to determine the comparison of methanol extract with 70% ethanol of Balik Angin leaves at each concentration using the Mann-Whitney Test. At the smallest concentration of 0.8%, it shows a significance value of 0.184, which means that at this concentration the methanol and 70% ethanol extracts do not have a significant difference. Meanwhile, at concentrations of 1.6% to 25.6%, a significance value of 0.046 to 0.050 was obtained, which means that the average diameter of the inhibition zone produced by each of the same concentrations of methanol extract compared to 70% ethanol extract had a significant difference. Based on the analysis of this data, it can be assumed that the antibacterial activity produced by the 70% ethanol extract of Balik Angin leaves has more potential to inhibit the growth of *P. acnes* than the methanol extract, so its potential needs to be explored more deeply, especially against other acne-causing bacteria, such as

Staphylococcus aureus and *Staphylococcus epidermidis*, so that can strengthen the potential of Balik Angin leaves as an alternative treatment for acne.

CONCLUSION

This research concludes that the uses of different solvents which are methanol and ethanol in the soxhlet extraction of Balik Angin leaves were effectively extracted equivalent groups of secondary metabolite compounds including phenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids. Meanwhile, the differences in Soxhlet extraction solvents can influence the antibacterial activity of Balik Angin leaves, as evidenced by test concentrations; 70% ethanol extract of Balik Angin leaves has more powerful activity with a larger average diameter of inhibition zones against the growth of *Propionibacterium acnes* compared to the the methanol extract.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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