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Research Article

Comparison of Extraction Solvents Towards Anti-*Propionibacterium acnes* activity of *Alphitonia incana* (Roxb). Teijsm. & Binn. ex Kurz Leaves

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Abstract

Alphitonia incana (Roxb.) Teijsm. & Binn. ex Kurz locally known as Balik Angin in Borneo Island is one of the endemic plants that have the potential to be an alternative antibacterial agent. Balik Angin leaves contain secondary metabolites that have to play a role to inhibit the growth of Gram-positive bacteria. This study aimed to compare the different extraction solvents which are methanol and ethanol on the maceration of Balik Angin leaves in producing antibacterial activity against *Propionibacterium acnes*. Antibacterial assay was carried out by the Well diffusion method. The results showed Minimum Inhibitory Concentration (MIC) of ethanol extract which is 3.2% has a diameter of clear zone of 9.475±0.311 mm. Meanwhile, the methanolic extract with a similar MIC has a lower diameter of clear zone of 2.55±0.85 mm. The results correlate with the number of secondary metabolites groups that were identified based on phytochemical screening in ethanol extract containing alkaloid, phenolic, flavonoid, tannin, saponin, and phytosteroid, however, methanol extract not containing alkaloid. The conclusion is ethanol solvent more effective to extract secondary metabolites from Balik Angin leaves compare to methanol solvent, so that can produce more powerful anti-*Propionibacterium acnes* activity.

Keywords: *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz, Balik Angin leaves, ethanol extract, methanol extract, anti-*Propionibacterium acnes*.

Perbandingan Pelarut Ekstraksi Terhadap Aktivitas Anti-*Propionibacterium acnes* dari Daun *Alphitonia incana (Roxb)*. Teijsm. & Binn.ex Kurz

Abstrak

Alphitonia incana (Roxb.) Teijsm. & Binn.ex Kurz secara lokal dikenal sebagai Balik Angin di Pulau Kalimantan merupakan salah satu tumbuhan endemik yang berpotensi sebagai alternatif antibakteri. Daun Balik Angin mengandung senyawa metabolit sekunder yang berperan dalam menghambat pertumbuhan bakteri Gram positif. Penelitian ini bertujuan untuk membandingkan perbedaan pelarut ekstraksi yaitu metanol dan etanol pada maserasi daun Balik Angin dalam menghasilkan aktivitas antibakteri terhadap *Propionibacterium acnes*. Uji antibakteri dilakukan dengan metode difusi sumuran. Hasil penelitian menunjukkan Konsentrasi Hambat Minimum (KHM) ekstrak etanol sebesar 3,2% memiliki diameter zona bening sebesar 9,475±0,311 mm. Sedangkan ekstrak metanol dengan KHM yang sama memiliki diameter zona bening yang lebih rendah yaitu 2,55±0,85 mm. Hasil tersebut berkorelasi dengan jumlah golongan metabolit sekunder yang teridentifikasi berdasarkan skrining fitokimia pada ekstrak etanol yang mengandung alkaloid, fenolik, flavonoid, tanin, saponin, dan fitosteroid, sedangkan ekstrak metanol tidak mengandung alkaloid. Kesimpulannya adalah pelarut etanol lebih efektif untuk mengekstrak metabolit sekunder dari daun Balik Angin dibandingkan dengan pelarut metanol, sehingga dapat menghasilkan aktivitas anti-*Propionibacterium acnes* yang lebih kuat.

Kata Kunci: *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz, daun Balik Angin, ekstrak etanol, ekstrak metanol, anti-*Propionibacterium acnes*.

1. Introduction

Propionibacterium acnes is a gram-positive bacterium, rod-shaped, non-spore-forming, and involved in the pathogenesis of acne. *P. acnes* is the main pathogen microorganism which is found on the skin that caused most of the symptoms of acne[1,2]. These bacteria prefer areas with high sebum production, which are more often found in hair follicles, preferring anaerobic conditions[3]. The mechanism of acne formation by *P. acnes* bacteria is by damaging the stratum corneum, the outermost and thinnest layer of skin on the eyelids, cheeks, and forehead, as well as the stratum germinativum, a layer of keratinocytes located at the base of the epidermis just above the dermis, by secreting chemicals that destroy pore wall. This condition can cause inflammation, fatty acids, and skin oils to get clogged and then harden. The inflammation will expand if a pimple is touched, so the fatty acid solids and hardened skin oils will enlarge, causing pimples[4].

Treatment of acne in each patient depends on several factors. These factors include the clinical grade of acne, the size and number of lesions, the severity of inflammation, the oil content of the skin, the presence of hyperandrogenism in women, and the presence of scar tissue[5]. Clindamycin and erythromycin antibiotics effectively use to treat pustular and inflammatory acne. Clindamycin belongs to the macrolide group because it has a macrocyclic chemical compound structure that is bacteriostatic and can be bactericidal depending on the concentration of the drug. However, the use of synthetic antibiotics in acne treatment often causes serious problems such as hypersensitive side effects, and can also lead to bacterial resistance[2,6]. This encourages research on alternative acne therapies to develop more widely, especially alternative treatments from natural ingredients[7]. Ethnomedically, one of the plants used to prevent acne comes from Alphitonia species[8].

Alphitonia incana (Roxb.) Teijsm. & Binn. ex Kurz locally known as Balik Angin in Borneo Island is one of the endemic plants that are efficacious as traditional medicine[8]. Balik Angin is a type of tree that belongs to the lesser-known species and has the potential to be developed. The plant is a member of the Rhamnaceae family synonyms of *A. excelsa, A. moluccana,* and *A. philippinensis*. This type is classified as a fast-growing species, a pioneer, adaptive to the environment, and known as a "soap tree"[9,10]. The leaves usually use daily as a natural bath soap for skin care and were used to treat skin diseases, such as tinea versicolor and itching by rubbing it on the affected parts of the body[11,12].

The Balik Angin plant has the potential for the prevention and treatment of infection, so that have the potential to be an alternative antibacterial agent[13]. Balik Angin leaves contain secondary metabolites that have to play a role to inhibit the growth of Gram-positive bacteria[14]. The main flavonoid compound is alphitonin. This plant also contains quercetin derivatives and isorhamnetin derivatives. Isolated triterpenoids were lupeol, ceanothic acid, betulinic acid, alphitolic acid which have been shown to have antimicrobial activity[11,15,16].

The previous study stated the macerated methanol extract of Balik Angin leaves had antibacterial activity against *S. aureus* and *S. epidermidis*[14]. According to the antibacterial assay by Cock[14] showed the leaf extract has the ability to inhibit the acne-causing bacteria which were *S. aureus* and *S. epidermidis* with Minimum Inhibitory Concentration (MIC) of 927 µg/ml and 1428 µg/ml which show inhibition zone diameters of 7.5 mm and 7 mm. There have not been many studies examining the testing of antibacterial activity on *Propionibacterium acnes* from methanol extract or ethanol extract of balik angin leaves, so their potential as an antibacterial is not yet known. The study aimed to compare the different extraction solvents which are methanol and ethanol on the maceration of Balik Angin leaves in producing antibacterial activity against the main pathogen of acne which was *Propionibacterium acnes*. This research is an *in vitro* experimental study using the Post Test Only Control Group Design method. The procedures begin with the extraction of Balik Angin leaves as the

sample with two different kinds of extraction solvents. The results of the antibacterial activity assay against *Propionibacterium acnes* are observed after treatment.

2. Method

2.1. Instruments

The instruments used in this research is macerator, rotary evaporator (IKA RV 10, Germany), hot plate stirrer (Thermo Fisher Scientific, US), water bath (Memmert, Germany), analytical balance (Pioneer PA214C, Ohaus, New Jersey, US), autoclave (All American 50X 120V, US), oven (Memmert, Germany), incubator (Memmert, Germany), 10-100 µL volume micropipette (Socorex Acura 825, Switzerland), 100-1000 µL (Socorex Acura 825, Switzerland), blue and yellow micropipette tips, Corck Borrer, petri dish, and other glassware (Pyrex).

2.2. Materials

All materials including reagents and medium of the antibacterial assay were obtained from Merck KGaA, Germany, except solvents (70% ethanol and methanol) for extraction were technical grade from Indonesia. *Propionibacterium acnes* ATCC 11827 and Clindamycin 2 µg/disc (Oxoid, Indonesia) were used for the antibacterial assay. The mature leaves of Balik Angin were collected from Mount Tahura, Banjar Regency, South Kalimantan, and the plant specimens were identified with the Latin name *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz in Organisasi Riset Ilmu Pengetahuan Hayati, Pusat Riset Biologi, Cibinong, Bogor (Number of certificate: B-208/V/DI.05.07/1/2022).

2.3. Procedure

2.3.1. Preparation of Extract

The collected leaves were cleaned up with water, cut into small pieces, and dried for a week at room temperature. The dried samples were powdered and sifted with a size 40 mesh. The 50 g of powders were macerated for 24 h using methanol and ethanol respectively with the comparison between sample and solvent which was 1:10 with modification according to Cock[14] and Sutomo et al.[17]. Remaceration was carried out two times. The solvent was evaporated using a rotary evaporator and a water bath at 50^{o}C[17,18].

2.3.2. Phytochemical Screening

Secondary metabolite qualitative testing on both extract of Balik Angin leaves involved several tests as reported by Ramadhan et al.[19]. Before treatments, 0.1 g of each sample was dissolved in 25 mL of their solvent.

Test of flavonoids: All samples of 2 mL were added 2 mg of Mg powder and 1 mL of concentrated HCl then the mixture was shaken. Positive samples containing flavonoids if any cause red, yellow, or orange color changes after added 0.4 mL of amyl alcohol.

Test of polyphenols: Addition of several drops of 10% (w/v) Ferric chloride solution to all samples produce a blue, blue-black, or blue-green color reaction showed the presence of polyphenols.

Test of tannins: The gelatin-salt test procedure was used for the detection of tannins which is all samples added a 1% NaCl and 5% gelatin solution. The formation of a precipitate in treatment suggests the presence of tannins.

Test of alkaloids: The sample of 2 mL was added 5 mL of HCl, then divided into three test tubes. Several reagents used to precipitate alkaloids were Mayer reagent solution produced a white to yellowish precipitate after being added to the first tube, Dragendorff reagent solution in acid solutions could appear an orange-brownish precipitate after being added to the second tube, and Wagner reagent solution in the third tube showed a brown precipitate in acidic solutions suggests the presence of alkaloids.

Test of steroids triterpenoids: Liebermann-Burchard test solution (a combination of 1 mL acetic anhydride and 2-3 mL chloroform and one drop concentrated H_2SO_4) is added into all samples, either in the solid form or in chloroform solution, blue, green, red, or orange colors that change with time indicated a steroid positive reaction. If a reddish-brown ring formed at the interface

indicates the presence of terpenoids.

Test of saponins: a Froth method was used for this test which is 2 mL of all samples being taken and agitated in 2 mL of water. When shaken, an aqueous solution of a saponin-containing sample produces 1 cm of the foam layer, which is stable for 15 min or more even after being added with 1 mL of 2N HCl.

2.3.3. Antibacterial Activity Assay

The antibacterial assay was carried out by the Well diffusion method. *Propionibacterium acnes* suspension that was made according to Ramadhan et al.[20] was inoculated and spread exactly 100 µL on twenty plates of Mueller Hinton Agar (MHA) media which were each eight plates for Balik Angin leaves methanol extract and ethanol extract. Each plate is given some holes which will be filled with samples test (various concentrations which were 0.05; 0.1; 0.2; 0.4; 0.8; 1.6; 3.2; 6.4; 12.8; and 25.6%), four plates placed with negative control (0.5% Na-CMC) and positive control (Clindamycin). All cultures were diffused at 2-8^{o}C for 14-18 h and then incubated at 37^{o}C for 24 h. After incubation, Furthermore, observations were made by measuring the diameter of the clear zone which showed a zone of inhibition of the growth of *P. acnes* bacteria. All treatments were carried out in quadruplicate independently[14,21].

2.3.4. Data Analysis

Data analysis to determine differences in producing anti-*Propionibacterium acnes* activity was tested using SPSS Version[18]. The normality test was tested using Levene's Test. If the data is met, it is continued with One Way ANOVA analysis. If the normality and homogeneity tests are not met, then the data is analyzed using the Kruskal-Wallis test[22].

3. Result

Yields: This research was a preliminary study to identify secondary metabolites in Balik Angin leaves as an alternative drug for the therapeutical use of infectious diseases, so it was necessary to study further for the use of optimal solvents and extraction methods in extracting the compounds which can produce antibacterial activity. Based on the research conducted, showed that the use of methanol solvent on the maceration for Balik Angin leaves can produce 26.22% yields of extract. This value was significantly different when compared to the 70% ethanol extract of Balik Angin leaves with the same extraction method which produced high yields of 49.91%.

Phytochemical Screening: The phytochemicals that were investigated in this study used specific color reactions. The presence of phytochemicals based on Table 2. showed Balik Angin leaves ethanol extract containing much more phytochemical compounds than methanol extract which is not containing alkaloids and triterpenoids.

Antibacterial activity assay: The results of the study in Table 3 and Figure 1 show that the use of ethanol solvent in the extraction of Balik Angin leaves can produce more powerful anti-*Propionibacterium acnes* activity compared with maceration using methanol solvent. The positive control used was Clindamycin 2µg/disc which produced a clear zone diameter of 19.5 ± 0.5 mm, and no inhibition zone from the negative control 0.5% Na-CMC.

4. Discussion

The Balik Angin plant was an endemic plant in some countries and can be found in Indonesia, Malaysia, Phillippines, Papua New Guinea, China, and the Solomon Islands[23]. The Balik Angin with the Latin name *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz potentially as traditional medicine based on an ethnopharmacology study[8]. The diverse efficacy of Balik Angin leaves is very promising to be developed as a traditional medicine as well as an active ingredient in anti-acne cosmetics. The extraction of Balik

Table 1. The yield of the Balik Angin leaves extracts.

Macerated solvent	Powder weight (g)	Extract weight (g)	Yield (%)
Methanol	50	13.11	26.22
Ethanol	50	24.95	49.91

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

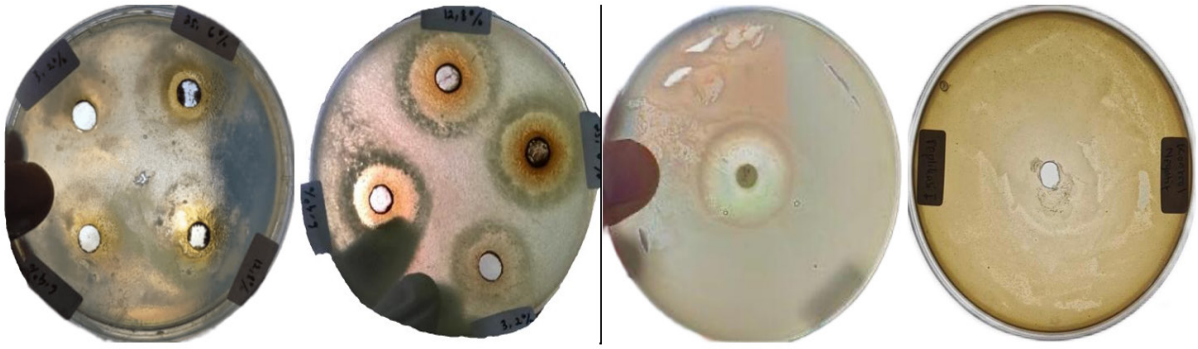
Secondary Metabolites	Macerated solvents			
	Methanol	Result*	Ethanol	Result*
Phenolic		+		+
Flavanoids		+		+
Alkaloids**				
1.		-		+
2.		-		+
3.		-		+
Tannins		+		+
Saponins		+		+
Steroids		+		+
Triterpenoids		-		-

*(+) containing and (-) not containing secondary metabolite, **(1) Dragendorff test, (2) Mayer test, and (3) Wagner test

Angin leaves was carried out by maceration with two different solvents, which are methanol and ethanol. The extraction method greatly affects the extraction results, this is because the extraction method affects the concentration or therapeutic effect of the sample because some compounds are not stable and thermolabile (can be damaged by heating)[24]. This research uses the maceration method because this method is the most common method used. The reason for choosing the maceration extraction method is that it has many advantages compared to other extraction methods especially can protect nature compounds from decomposition because of heat. Cold extraction allows many compounds to be extracted[25]. Based on the results of the research that has been carried out, the extract yields from both extracts, where the extract using the methanol solvent

Table 3. Inhibition zone diameters of anti-*Propionibacterium acnes* assay.

Concentrations (%)	Diameters of clear zone (mm)	
	Methanol extract	Ethanol extract
25.6	5.150±0.773	13.875±0.432
12.8	3.950±0.940	12.725±0.383
6.4	3.550±1.064	11.500±0.332
3.2	2.550±0.850	9.475±0.311
1.6	-	-
0.8	-	-
0.4	-	-
0.2	-	-
0.1	-	-
0.05	-	-



(a) (b) (c) (d)

Figure 1. The Inhibition Zone of (a) methanolic extract (25.6%; 12.8%; 6.4%; 3.2%), (b) ethanolic extract (25.6%; 12.8%; 6.4%; 3.2%), (c) the Clindamycin 2µg/disc as the positive control against *Propionibacterium acnes,* and (d) negative control (0.5% Na-CMC).

is 26.22%, while the highest yield of the extract using the ethanol solvent is 49.91%. The difference in the yield of this extraction can be caused by differences in the solvents polarity[26].

The phytochemicals that were investigated in this study were seven groups of secondary metabolites. The reaction of phenolic compounds with $FeCl_3$ will form a complex compound (iron (III) hexaphenolate) which results in a color change to black-green because the ion from Fe^{3+} undergoes orbital hybridization so that it has six empty orbitals filled by oxygen atoms electron pair donors from phenolic compounds[27,28]. The reaction that occurs in the identification of flavonoids is the hydrolysis of the glycoside structure by HCl to become its aglycone and undergo reduction by Mg to produce complex compounds that are red or orange in color[28]. In the identification of alkaloids using the Dragendorff, Mayer, and Wagner reagents, a reaction occurs between potassium salt from reagents and nitrogen atoms from the alkaloids to form coordinate covalent bonds, resulting in a precipitate which is a potassium alkaloid[28]. Tannins can react with proteins to form copolymers which precipitate and are insoluble in water. This precipitate occurs because of hydrogen bonds between tannins and proteins in gelatin[29]. Saponins are glycosides that are polar so they can dissolve in solvents such as water. The foam appears because the glycosides are hydrolyzed in water to form non-polar glycones and aglycones. The foam produced in the saponin test is caused by the presence of glycone which forms miscellanea[28]. In the steroid and terpenoid tests using Liebermann-Burchard which contains acetic anhydride and sulfuric acid. The addition of acetic anhydride aims to form acetyl derivatives. The reaction that occurs between steroids and acetate anhydride is an acetylation reaction of the hydroxyl group on steroids which will produce an acetyl steroid complex. The addition of concentrated sulfuric acid is to hydrolyze water which will react with acetyl derivatives to form a green ring so that the sample is tested positive for steroids but does not contain triterpenoids[30].

The research that has been done shows that Balik Angin leaves extract with 70% ethanol solvent can attract compounds more optimally than the methanol solvent. The results of this study showed that Balik Angin leaves ethanol extract containing phenolic, flavonoid, tannin, alkaloid, saponin, and phytosteroid, however, methanol extracts not containing alkaloids. The mechanism of action of alkaloids as antibacterials is by interfering with the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is not formed intact and causes cell death[4]. Al Omar et al.[31] stated *A. excelsa* contains a flavonoid compound which was alphitonin and a phenolic compound which was salicylic acid. They also stated the similar name of Balik Angin which was *A. philippinensis* contains antimicrobial and antibacterial agents such as isorhamnetin derivatives, quercetin derivatives (flavonoid), β-sitosterol (steroid), and stigmasterol (steroid). Phenolic compounds, flavonoids,

and tannins can denature bacterial cell proteins by forming hydrogen bonds, thereby affecting the permeability of cell walls and cytoplasmic membranes, causing macromolecular and ion imbalances in cells which result in bacteria becoming lysed. Flavonoids can also inhibit nucleic acid synthesis, namely the B ring of flavonoids can intercalate or form hydrogen bonds with the basic arrangement of nucleic acids, causing inhibition of DNA and RNA synthesis in bacteria. In addition, flavonoids are also able to inhibit the use of oxygen by bacteria, namely by preventing the formation of energy in the cytoplasmic membrane and inhibiting bacterial motility[32,33]. The mechanism of action of steroids as an antibacterial in inhibiting bacterial growth is related to the lipid membrane and sensitivity to steroid components which cause leakage in bacterial liposomes[34].

The antibacterial activity results correlate with the number of secondary metabolites groups that were identified in both extracts based on phytochemical screening. The results of the study show that the ethanol solvent in the extraction of Balik Angin leaves produces more powerful anti-*Propionibacterium acnes* activity compared with maceration using methanol solvent. The Minimum Inhibitory Concentration (MIC) of ethanol extract which is 3.2% has a diameter of clear zone of 9.475±0.311 mm. Meanwhile, the methanolic extract with a similar MIC has a lower diameter of clear zone of 2.55±0.85 mm, but not strong enough if compared with Clindamycin (19.5±0.5 mm). The mechanism of action of Clindamycin is similar to flavonoids, which was inhibiting protein synthesis from microbes by binding to the 50S subunit[35]. Based on statistical analysis using ANOVA, it can be concluded that there was a significant difference in inhibition between groups, which was sig value <0.05, except for the 3.2% ethanol extract and 6.4% methanol group and the methanol group at concentrations of 3.2%; 6.4%; and 12.8% which had a sig value > 0.05 so that there is no significant difference in antibacterial activity, this indicates that the 3.2% ethanol extract has almost the same inhibitory power as the 6.4% and 12.8% methanol extract so that in terms of antibacterial inhibition.

These antibacterial activities were less effective in inhibiting the growth of *P. acnes* if compared with the previous research by Cock[14] who mentioned methanol extract of Balik Angin with a concentration of 1% more sensitive against *S. aureus* and *S. epidermidis* with an approximate diameter of the clear zone which was 7-8 mm. The activity of the ethanol extract from Balik Angin leaves is more potent in inhibiting the growth of Gram-positive bacteria such as *P. acnes* compared to Gram-negative bacteria, which was *E. coli*. This is shown by the research of Sandra et al.[36] which states that the methanol extract of Balik Angin leaves at a concentration of 3.2% has a lower clear zone diameter and is still classified as a medium inhibition category, which was 8.28 mm. Various methods have been adopted to evaluate the antibacterial activities of natural compounds, such as the Well diffusion method or cup plate technique. This method was chosen because it is able to obtain a larger diameter of the inhibition zone compared to the disc diffusion method. This is because, in the Well method, there is a higher osmolarity process of extract concentration into the media compared to the disc diffusion method. In the Well method, each hole is filled with an extract concentration so that the osmolarity occurs more thoroughly and is more homogeneous, and the resulting extract concentration is higher and stronger to inhibit bacterial growth[37].

5. Conclusion

Based on the research that has been done, it can be concluded that the results of the study showed that the ethanol solvent is more effective to extract secondary metabolites from Balik Angin leaves compare to methanol solvent, so that can produce more powerful anti-*Propionibacterium acnes* with the 3.2% Minimum Inhibitory Concentration (MIC) of ethanol extract has a higher diameter of clear zone of 9.475±0.311 mm than methanolic extract with a lower diameter of clear zone of 2.55±0.85 mm. The activity of each extract correlated with the number of secondary

metabolite contents so the Balik Angin leaves ethanol extract has more potential as a natural antiacne drug.

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