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Antibacterial activity of water spinach herbs against acne-inducing bacteria

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ABSTRACT

Acnes is commonly caused by infection by *Propionibacterium acnes* when the sebum is overproduced from the body. Based on previous research, water spinach extract had antimicrobial activity. The purpose of this study was to determine the antibacterial activity of water spinach (*Ipomoea aquatic* Forsk.) herbs extract against bacteria *Propionibacterium acnes.* Extraction was carried out by the reflux method using three different polarity solvents. The extracts were evaporated using a rotary evaporator. Antibacterial activities were assessed by using disc diffusion, microdilution, and equality to reference antibiotics. All three extracts of water spinach herbs had better antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. The ethanol-water spinach herbs extract had the best antibacterial activity against *P. acnes*. The minimum inhibitory concentration (MIC) value of ethanol-water spinach herbs extract on *P. acnes* was 1280 μg/ml while the minimum bactericidal concentration (MBC) value was > 5120 μg/ml. The equivalency of ethanol-water spinach herbs extract to tetracycline hydrochloride presented 1163.87 μg ethanol extract (with density 1% extract: 0.780) equal to 1 μg tetracycline hydrochloride. All three extracts of water spinach herbs had antibacterial activity against *P. acnes*. The ethanol extract had the best antibacterial activity against *P. acnes* among all three extracts.

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INTRODUCTION

Acne vulgaris or often known as acne is a dermatological disorder which is commonly occurred on teenagers in puberty. This data was supported by the American Academy of Dermatology, which stated that there are at least 50 million cases of acne in the United States each year, and about 85% are teenagers (Zaenglein *et al.*, 2016). Acne is commonly caused by infection by *P. acnes* when the sebum is overproduced from the body (Zaenglein *et al.*, 2016; Dipiro *et al.*, 2008; Achermann *et al.*, 2014). *P. acnes* is an aerotolerant anaerobic, Gram-positive, non-spore forming, pleomorphic rod belonging to the phylum Actinobacteria, class Propionibacteriales (Achermann *et al.*, 2014; Vorobjeva, 1999). This infection disease is often treated by antibiotics that can inhibit or kill *P. acnes*. The limitation of treatment with antibiotics is the emergence of resistance problems, especially for the first-line antibiotics such as clindamycin, erythromycin, so there is a need for alternative therapy for acne (Swanson, 2003). Medicinal plants were

commonly researched for this alternative therapy nowadays (Shetty *et al.*, 2015).

Water spinach (*Ipomoea aquatic* Forsk.) is a perennial herb that belongs to family Convolvulaceae and can be found throughout mostly on Asia (Dalimartha, 2007; Austin, 2007). The plant can grow in both plateau and lowland, especially in moist soil (Austin, 2007). The previous study stated (Manvar and Desai, 2013; Shamli and Chandra, 2015; Malakar and Choudhury, 2015) that water spinach many pharmacological activities such as antidiabetic, anti-inflammatory, diuretic, anticancer, antiseptic. Water spinach herbs contain many compounds such as alkaloid; flavonoid such as quercetin, myricetin, luteolin; tannin; saponin; phenolic compound; carbohydrate; protein; lipid; nicotinic acid; alpha-tocopherol; ascorbic acid; β-carotene; xanthophyll (Manvar and Desai, 2013; Malakar and Choudhury, 2015; Sahid and Kalpana, 2016; Igwenyi *et al.*, 2011). Previous studies (Sivaraman *et al.*, 2010; Ahmad *et al.*, 2015; Bhakta *et al.*, 2008) stated that water spinach had antimicrobial activity, so it had the potential to become alternative therapy for infectious disease. There has been no research on the effects of antibacterial activity of water spinach herbs against acne-inducing bacteria.

The goal of this study was to determine the antibacterial activity of water spinach herbs extracts against the bacteria *P. acnes*. The antibacterial activity would be shown by MIC, MBC value, and equality to reference antibiotic tetracycline hydrochloride.

MATERIALS AND METHODS

Materials

Tetracycline hydrochloride was purchased from Bratachem. Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), Tryptic Soy Agar (TSA), Nutrient Agar (NA) were microbiological grade. Dimethyl sulfoxide, ethanol, n-hexane, ethyl acetate was analytical grade.

Microbial strains

Propionibacterium acnes ATCC 11827, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8939. All bacterial strains were maintained at 4ºC on NA medium.

Preparation of sample

Water spinach herbs was collected from Ciwidey, Bandung, West Java-Indonesia, were thoroughly washed with tap water, wet sortation, cut, dried, and grinded into powder.

Extraction

About 300 g of powdered samples were extracted by reflux using three different polarity solvents. Extraction using n-hexane was repeated 3 times. The remaining residue was then extracted 3 times by ethyl acetate. Finally, the remaining residue was extracted 3 times using ethanol. All of three extracts then were concentrated by using a rotary evaporator.

Characterization of extracts

The three extracts were characterized by phytochemical screening and density determination. Alkaloid, flavonoid, tannin, quinone, steroid triterpenoid groups were determined by using Farnsworth's method (Farnsworth, 1966) while saponin was determined by using Indonesia Materia Medica method (Ministry of Health Republic of Indonesia, 1989).

The viscous extract was made until 1% w/v extract and put into the pycnometer up to the boundary mark. The pycnometer containing 1% w/v extract was then weighed, and the density was calculated based on pycnometer's weight difference containing the extract with the weight of an empty pycnometer to the pycnometer's volume.

Preparation of bacterial culture

Bacterial culture of *Propionibacterium acnes*, *Staphylococcus aureus, Escherichia coli* were streaked on the NA medium and incubated at 37ºC for 18-24 h. The turbidity of the culture of bacteria was adjusted with 0.9% sodium chloride sterile to obtain an absorbance of 0.08-0.13 using ultraviolet (UV)-visible (Vis) spectrophotometer Beckman Coulter DU720 at 625 nm wavelength. Sterile sodium chloride 0.9% was used as blank. The absorbance of 0.08-0.13 is equal to 0.5 McFarland or colonies of test bacteria as much as 1-2 x 10^8cfu/ml (CLSI, 2012).

Total plate count test

Standardized bacterial suspension to 0.5 McFarland was diluted with 0.9% sterile sodium chloride with a 10^{-1} to 10^{-8} dilution factor. Each of these dilutions was taken 1 ml and inoculated into a 15 ml MHA medium in a petri dish then incubated at 37ºC for 24 h. The bacterial growth colonies were calculated, and colonies should be within the range of 30-300 colonies.

Sensitivity test

Fifteen ml sterile MHA medium was poured into sterile petri dishes, and then 100 μl of standardized bacterial suspensions to the turbidity of 0.5 McFarland were added on each petri dish (Balouiri *et al.*, 2016). The agar medium which had been

Table 1: Antibacterial activity of water spinach herbs extract to Gram-positive and negative bacteria

Sample	Diameter of inhibition zone (mm)	
	Staphylococcus aureus	*Escherichia coli*
Ethanol extract 20% w/v	22.78 ± 0.51	7.00 ± 0.00
Ethyl acetate extract 20% w/v	29.56 ± 0.51	-
N-hexane extract 20% w/v	-	-
DMSO 2.5% v/v	-	-
Ethyl acetate	-	-
N-hexane	-	-

(-)means there was no inhibition zone

Table 2: Antibacterial activity of water spinach herbs extract to *P. acnes*

Sample	Diameter of inhibition zone (mm)
Ethanol extract 20% w/v	25.00 ± 0.33
Ethyl acetate extract 20% w/v	27.67 ± 0.33
N-hexane extract 20% w/v	13.00 ± 0.00
Tetracycline hydrochloride 0.1% w/v	28.83 ± 0.24
Tetracycline hydrochloride 0.01% w/v	19.83 ± 0.24
DMSO 2.5% v/v	-
Ethyl acetate	-
N-hexane	-

(-)means there was no inhibition zone

Table 3: MIC and MBC value of ethanol extract to *P. acnes*

Sample	MIC (μg/ml)	MBC (μg/ml)
Ethanol extract	1280	> 5120
Tetracycline hydrochloride	2	128

Table 4: Antibacterial activity of tetracycline hydrochloride to *P. acnes*

Concentration (μg/ml)	Log concentration	Diameter of inhibition zone (mm)
128	2.11	17.89 ± 0.38
64	1.81	16.44 ± 0.51
32	1.51	14.89 ± 0.19
16	1.20	12.67 ± 0.33
8	0.90	11.22 ± 0.19
4	0.60	9.67 ± 0.33
2	0.30	7.67 ± 0.33

mixed with bacterial suspension was homogenized by shaking. Four pieces of 6 mm sterile disc paper were placed on a medium. All three extracts were reconstituted by using dimethyl sulfoxide (DMSO) 2.5 % v/v for ethanol extract, ethyl acetate for ethyl acetate extract, and n-hexane for n-hexane extract to obtain 20% w/v extract. Each paper disc was impregnated with 10 μl of reconstituted extract. The test was performed triplicate for each extract. Tetracycline hydrochloride was used for positive control and DMSO 2.5 % v/v, ethyl acetate, and n-hexane solvent, which were used for reconstituting extract;, therefore, they were used as blank. Preincubation was performed at room temperature for 1 h and continued with incubation at 37ºC for 18-24 h. Inhibition zone was measured, and extract, which had

the best antibacterial activity, continued for MIC and MBC determination.

MIC value determination

Standardized bacterial suspension to 0.5 McFarland was diluted 1:20 to reach a concentration of 5 x 10^6cfu/ml. Each well on 96-wells microplate was filled with 100 μl MHB medium except the first well as a sterility control was filled with 100 μl MHB medium and the second well as a positive control was filled with 100 μl medium MHB and 10 μl bacterial suspension. The twelfth well was filled with 100 μl extract until the final concentration of 5120 μg/ml. Serial two-fold dilution was done by transferring 100 μl to the next well. The third to twelfth wells were added 10 μl of bacterial suspension to obtain the final bacterial suspension concentration of 5 x 10^5cfu/ml. The microplate was incubated at 35 $\pm$ 2ºC for 16-20 h, and MIC value was determined by observing the turbidity that occurred after incubation. Tetracycline hydrochloride was used as a positive control. The test for the extract was performed triplicate (CLSI, 2012).

MBC value determination

About 5 μl of clear solutions on (MIC, 2009) determination were streaked on petri dishes containing 15 ml sterile MHA medium. The medium was allowed to be solidified and then incubated at 37ºC for 24 h. MBC value was determined based on the lowest concentration values that had no bacterial growth on the petri dish after 24 h incubation.

Equivalency of extract to tetracycline hydrochloride

Equivalency test was done by agar disc-diffusion method with various concentrations of tetracycline hydrochloride. The diameter of the inhibition zone was measured, and the standard equation calculated for the relationship between the logarithm of the tetracycline hydrochloride concentration and the diameter of the inhibition zone. The equivalency of water spinach herbs extract to tetracycline hydrochloride was done by inserting the diameter of the inhibition zone by extract into the equation.

RESULTS AND DISCUSSION

Characterization of extracts

The ethanol extract had positive results for flavonoid, phenolic compound, tannin, saponin, and steroid/triterpenoid. Ethyl acetate extract had positive results for alkaloid, flavonoid, phenolic compound, and steroid/triterpenoid, meanwhile, n-hexane extract had an only positive result for steroid/triterpenoid group. The density of 1% n-hexane, ethyl acetate, and ethanol-water spinach herbs extracts were 0.787, 0.7950,780 g/ml, respectively.

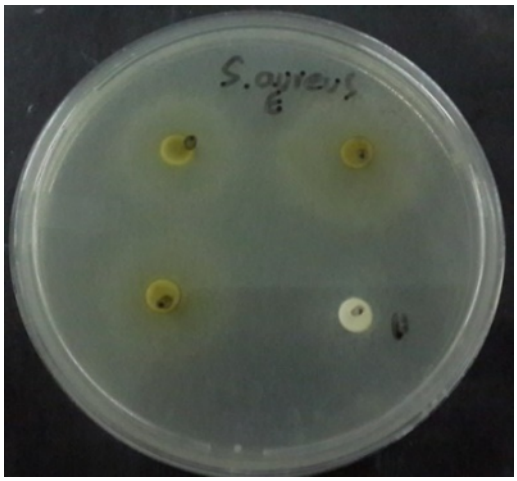

Figure 1: Inhibition zone of ethanol extract to *Staphylococcus aureus*

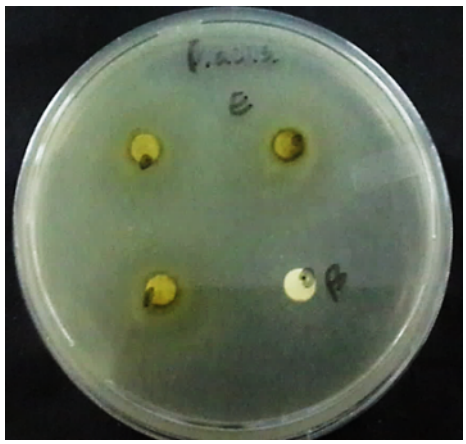

Figure 2: Inhibition zone of ethanol extract to *Propionibacterium acnes*

Total plate count

Based on total plate count, standardized *Propionibacterium acnes* suspension to the turbidity of 0.5 McFarland was equal to 2.87 x 10^8cfu/ml.

Sensitivity test to Gram-positive and negative bacteria

The present of the inhibition zone means there was an antibacterial activity of extract to the chosen bacteria. The biggest value of the diameter of the inhibition zone means the highest antibacterial activity.

The biggest diameter of inhibition zone to *Staphylococcus aureus* was given by ethyl acetate extract (29.56 mm) while the smallest for n-hexane extract, which had no inhibition zone (Table 1). Extract that had an antibacterial activity to *Escherichia coli* was ethanol extract (7.00 mm) could be seen inFigure 1. All solvents that be used to dissolve the extract had

no antibacterial activity to both Gram-positive and negative bacteria.

Sensitivity test to Propionibacterium acnes

The biggest diameter of inhibition zone was given by ethyl acetate extract (27.67 mm), while the smallest for n-hexane extract (13.00 mm). The ethanol extract, which had a diameter of inhibition zone (25.00 mm), produced the clearest inhibition zone among all three extracts that were tested (Table 2). The higher concentration of tetracycline hydrochloride gave the bigger inhibition zone (Figure 2). All solvents that be used to dissolve the extract showed no antibacterial activity to both Gram-positive and negative bacteria.

MIC and MBC determination of selected extract

The MIC value of ethanol extract was 1280 μg/ml. Meanwhile, the MIC value of tetracycline hydrochloride was 2 μg/ml (Table 3). All concentrations of ethanol extract that had been tested, there was no any concentration that could kill *P. acnes,* so MBC value of ethanol extract was concluded as > 5120 μg/ml which was the highest concentration of ethanol extract that was tested, while MBC value of tetracycline hydrochloride was 128 μg/ml.

Equivalency of extract to tetracycline hydrochloride

The equivalency of antibacterial activity of ethanol extract (Table 4) was reported in terms of tetracycline hydrochloride equal using the standard curve equation y = 5.6793x+6.0829, R^2=0.9975. The water spinach herbs ethanol extract 2.5% w/v produced 13.67 mm inhibition zone.

The previous studies exposed that water spinach had antibacterial activity. The part of the water spinach that had been tested was leaves part only and tested to some bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*¸ *Salmonella typhi* (Sivaraman *et al.*, 2010; Ahmad *et al.*, 2015; Bhakta *et al.*, 2008). There was no research regarding the antibacterial activity of water spinach herbs against all bacteria, especially to *Propionibacterium acnes*.

After extraction, all three extracts (ethanol, ethyl acetate, and n-hexane) were characterized by phytochemical screening and density determination. Phytochemical screening was examined to determine secondary metabolite compound which contained in water spinach herbs. The ethanol extract had positive results for flavonoid, phenolic compound, tannin, saponin, and steroid/triterpenoid. Ethyl acetate extract had a positive result for alkaloid, flavonoid, phenolic compound, and steroid/triterpenoid, while n-hexane extract had an only positive result for steroid/triterpenoid group. From the data, it can be concluded that water spinach herbs contained flavonoid, alkaloid, phenolic compound, tannin, saponin, and steroid/triterpenoid. The present research had the same results as the previous studies (Manvar and Desai, 2013; Shamli and Chandra, 2015; Malakar and Choudhury, 2015) but slightly different to study by Sivaraman *et al.* (2010) which stated that water spinach leaves gave a negative result for steroid/triterpenoid and saponin compound. The slightly different result could be caused by the different growth locations of water spinach.

Density determination for all three extracts was crucial to be determined because all extracts should be similar to avoid fake antibacterial activity due to too concentrated/diluted extract. The density of water spinach herbs ethanol, ethyl acetate, and n-hexane extract were 0.780; 0.795; 0.787 g/ml, respectively, and these results were similar density in three extracts. Hence the antibacterial activity of three extracts could be compared to each other.

Total plate count test should be performed to determine the number of bacterial colonies used in the test because the number of bacteria greatly determined the outcome of antibacterial activity testing. Total plate count was performed to a turbidity of 0.5 McFarland by the CLSI method (CLSI, 2012). Temperature 37ºC was selected as an incubation temperature because this temperature was the optimum temperature *P. acnes* (Achermann *et al.*, 2014). This present research concluded that the turbidity of 0.5 McFarland of *P. acnes* was equal to 2.87 x 10^8cfu/ml.

Antibacterial activity testing was started with a sensitivity test to Gram-positive and negative bacteria. For this research, *Staphylococcus aureus* bacteria was selected as representative of Gram-positive bacteria, while *Escherichia coli* bacteria were selected as representative of Gram-negative bacteria. Both bacteria were chosen due to their commonness for antibacterial testing, and it could grow quickly (Harris *et al.*, 2002; Bachir and Abouni, 2015). Sensitivity test were done by using the agar disc-diffusion method due to its simplicity, low cost, and could be done for many microbial strains (Balouiri *et al.*, 2016). All solvents that had been used to reconstitute extract also were tested because some organic solvent also had an antibacterial activity such as ethanol, dimethyl sulfoxide, and methanol (Wadhani *et al.*, 2008). This present research demonstrated that all solvents dimethyl sulfoxide 2.5% v/v, ethyl acetate, and n-hexane didn't have any antibacterial activity due to the absence of an inhibition zone on

the disc diffusion method. It was similar to previous research by Wadhani *et al*. (2008), which stated that dimethyl sulfoxide with concentration under 3% v/v didn't have any antimicrobial activity.

The present study revealed that ethanol and ethyl acetate extracts had antibacterial activity against *S. aureus,* while only ethanol extract had the antibacterial activity against *E. coli*. From this result, it could be concluded that water spinach herbs had better antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. It was similar to the previous study by Sivaraman *et al.* (2010) which demonstrated that 1 mg/ml methanolic extract of water spinach leaves had a better activity to *Staphylococcus aureus* (25 mm) and *Bacillus subtilis* (20 mm) than *Pseudomonas aeruginosa* (18 mm) and *Escherichia coli* (15 mm). The previous study also stated that aqueous extract 1 mg/ml also had better activity against Gram-positive (18-19 mm) than Gram-negative bacteria (8-11 mm). A study by Shamli and Chandra (2015) gave a different result, which demonstrated that both acetone and petroleum ether extract of water spinach had a bigger inhibition zone to Gram-negative bacteria than Gram-positive bacteria. From these all studies, it could be concluded that water spinach extract had antibacterial activity. Antibacterial activity of water spinach herbs extract could be caused by many secondary metabolites that were contained, such as flavonoid, alkaloid, tannin, saponin, phenolic compound, and steroid/triterpenoid based on phytochemical screening analysis. A study by (Omojate *et al.*, 2014) demonstrated that flavone had an antimicrobial mechanism by forming a complex with extracellular protein. Tannin had antimicrobial activity by inactivating microbial adhesins, enzymes, and envelope transport proteins, meanwhile saponin by altering the permeability of cell walls.

Antibacterial activities of water spinach herbs extracts were tested to target bacteria *P. acnes* by using the agar disc diffusion method. The present study demonstrated that all three extracts didn't produce 100% clear inhibition zone, so it could be concluded that all extract had bacteriostatic activity. Ethyl acetate and ethanol of water spinach herbs extracts had a stronger antibacterial activity to *P. acnes* (25.00-27.67 mm) than n-hexane extract (13.00 mm). Ethanol extract gave the clearest inhibition zone, so it could be denoted as the best antibacterial activity among all extracts. This result might be caused by ethanol extract contained more secondary metabolite such as flavonoid, saponin, tannin, phenolic compound, and steroid/triterpenoid. This result was also supported by Chusnie and Lamb's study, which demonstrated that flavonoid compounds like quercetin had antibacterial activity through inhibition of DNA gyrase (Cushnie and Lamb, 2005). The previous study by (Su *et al.*, 2014) also demonstrated that luteolin and apigenin, which were also contained in water spinach (Manvar and Desai, 2013) had antibacterial activity against *S. aureus*.

MIC and MBC determination were performed only for ethanol extract that was concluded as the best antibacterial extract. MIC determination was done by the microdilution method by (CLSI, 2012). There was no research for water spinach extract's MIC value to *P. acnes*. The agar disc diffusion method wasn't used for determining because it wasn't possible to determine the exact amount of antimicrobial compounds diffusing through the medium (Balouiri *et al.*, 2016). MIC was defined as the lowest concentration of antimicrobial agent that can inhibit microbial growth after 18-24 h of incubation. Meanwhile, MBC was defined as the lowest concentration of antimicrobial agent that can kill 99.9% of tested microbe after 24 h of incubation (Brunton *et al.*, 2011). The MIC value of ethanol extract to *P. acnes* was 1280 μg/ml, while the MBC value was >5120 μg/ml. This result was quite much different to MIC and MBC values of tetracycline hydrochloride 2 μg/ml and 128 μg/ml, respectively. This difference might be caused tetracycline hydrochloride was an isolate while extract still had a lot compound. Study by (Aligiannis *et al.*, 2001) demonstrated that extract had strong antimicrobial activity if MIC $\leq$ 0.5 mg/ml, moderate antimicrobial activity if MIC 0.6-1.5 mg/ml, and weak activity when MIC value > 1.6 mg/ml. Based on this present result, it could be concluded that ethanol extract had moderate activity as antibacterial agents against *P. acnes*.

Equivalency of antibacterial activity of ethanol-water spinach herbs extract to tetracycline hydrochloride was conducted by the agar disc-diffusion method with few changes from the sensitivity test. Seven concentrations of tetracycline hydrochloride were used to make a standard curve equation. Tetracycline hydrochloride was chosen as reference antibiotic due to its wide spectrum activity (Katzung, 2003) and used as one of the first-line antibiotics for acne vulgaris therapy (Zaenglein *et al.*, 2016). The equivalency of antibacterial activity of ethanol-water spinach herbs extract was reported in terms of tetracycline hydrochloride equal using the standard curve equation y = 5.6793x+6.0829, R^2=0.9975. This equation had met the requirements for a good linear equation for microbiology testing R^2> 0.95 (Shabir, 2006). Based on the equation, 1163.87 μg ethanol-water

spinach herbs extract (density 1% extract was 0.780) was equal to 1 μg tetracycline hydrochloride.

CONCLUSION

All of three water spinach herbs extracts had a better antibacterial activity to Gram-positive than Gram-negative bacteria. All extracts had antibacterial activity against *P. acnes*. Ethanol extract had the best antibacterial activity against *P. acnes* with MIC value 1280 μg/ml, MBC >5120 μg/ml, and 1163.87 μg ethanol extract (density 1% extract was 0.780) was equal to 1 μg tetracycline hydrochloride. Ethanol water spinach herbs extract could be exploited as a natural medicinal alternative therapy for acne vulgaris.

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