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Antibacterial Activity Tests of N-hexane, Ethyl Acetate, and Methanol Leaves (Vitex) Extract (pinnata) against *Streptococcus mutans*

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Abstract

BACKGROUND: *Vitex pinnata* is known as Laban, which is a medicinal plant used traditionally for generations to generations. Laban leaf extract with various concentrations has antibacterial activity. Laban leaf extract is known to inhibit the formation of Streptococcus mutant in human teeth.

AIM: To exam, the minimum inhibitory concentration (MIC) and minimum kill concentration (KBM) extract Laban leaves (*V. pinnata*) as an antibacterial against *Streptococcus mutants*.

METHODS: This research was purely experimental research with design randomized pretest-posttest and control group. The study was conducted at the Laboratory Microbiology Faculty of Animal Health, Syiah Kuala University, Banda Aceh, from March to June 2019. The sample of this study was the Laban leaf from the Aceh Besar geothermal area. This research included preparation raw materials, chemical characterization of raw materials, determination of minimum and maximum components mixture, determining the optimum formula based on the best inhibitory potential, inhibitory testing antibacterial, standardized test, and test (one-way ANOVA).

RESULTS: MIC of n-hexane extract showed the results of calculations; the percentage of bacterial inhibition was at a MIC of 1.56% on average colony −1.45 CFU/ml. In ethyl acetate extract, the MIC was 0.20% on the average colony −0.17 CFU/ml. The methanol extract can inhibit bacteria at the smallest concentration of 0.05% average colony −1.48 CFU/ml. Methanol extract inhibits bacteria more quickly. Concentration results minimum kill was 1.56%, 0.78%, 0.39%, 0.20%, 0.10%, and 0.05%. The smallest concentration of extract (n-hexane, ethyl acetate, and methanol) can kill *Streptococcus mutans* bacteria that are marked in the absence of bacterial colonies on microbiological growth media.

CONCLUSION: Extracts of n-hexane, ethyl acetate, and methanol from Laban leaves have inhibitory activity on the growth of *S. mutans* bacteria. The smallest concentration of extract (n-hexane, ethyl acetate, and methanol) is able to kill *S. mutans* bacteria.

Introduction

Dental health and mouth of Indonesians still need to get serious attention, especially the hard layer of plaque that sticks to teeth and contains a lot of bacteria. Bacteria that play a role in plaque formation and caries development are *Streptococcus mutans* [1]. *S. mutans* is the bacterium that most contributes to the incidence of dental caries due to its ability to produce acids that can reduce the pH of the oral cavity and cause it tooth demineralization [2], [3]. *S. mutans* change polysaccharide extracellular to lactic acid through a process of homofermentative so as to form a colony on the tooth surface, is acidogenic, and as a trigger for tooth decay [4]. Bacteria *S. mutans* is resistant to acidic conditions, so it can invade biofilms and trigger the occurrence of dental caries [5]. Dental caries can be inhibited by inhibiting growth.

S. mutans bacteria and inhibits the work of the enzyme glucosyltransferase (GTF). GTF enzyme used by *S. mutans* to form biofilms in teeth by converting glucose into glucan [6]. Dental plaque is caused by the formation of biofilms by mouth microbes [7]. Dental caries is a dental disease that affects the majority of the population in Indonesia, with a prevalence of 72.1% [8]. Eating factor is one of the factors that can trigger dental caries (Samaranayake 2002). Prevention of dental caries can be done by blocking *S. mutans* attachment in salivary pellicles [9]. Laban plant (*Acalypha indica* L.) is known as a wrong medicinal plant that grows in the mountains. Laban leaf extract with various concentrations has antibacterial activity. Laban leaf extract is known to inhibit formation *S. mutans* in teeth. Laban plants contain flavonoid compounds, saponins, and tannins [10]. All parts of Laban plants can be used for the treatment of diseases, leaves to treat fever,

hypertension, and bark to treat wounds, stomachaches, and dysentery, while the root as a tea drink that can eliminate fatigue, back pain, and body ache [10]. Phytochemical test results of the Great Aceh Agam Seulawah Geothermal region, Laban extract containing alkaloids, flavonoids, saponins, terpenoids, tannins, and bark containing, alkaloids, saponin, terpenoid, and tannin [11]. While extract compounds from Laban have antimicrobial, anti-inflammatory, antidiabetic, antioxidant, antitumor, antifungal, and antibacterial [12]. Based on the description above and in order optimizing the potential utilization of Laban leaves as an antibacterial, the purpose of the study it tests the minimum inhibitory concentration (MIC) and the minimum kill concentration (KBM) Laban leaf extract (*Vitex pinnata*) as an antibacterial against *S. mutans*.

Methods

This research was purely experimental research with a randomized research design pretest-posttest controlled group design. The study was conducted at the Faculty of Microbiology Laboratory Animal Health Syiah Kuala University, Banda Aceh, from March to June 2019. Samples were leaves geothermal laboratory of Aceh Besar. Wet Laban leaf sample collection of 10 kg, dried in the open air without direct sunlight. Laban leaf samples dried until blended until smooth and weighed as much as 3 kg, macerated with n-hexane as a non-polar solvent for 3 × 24 h and continuously filtered. Inhibition testing Laban leaf extract antibacterial is done by looking at the MIC and KBM against *S. mutans*. Bacterial suspension test standardized with a McFarland 0.5 solution, so the amount of bacterial density used the same 1.5 × 108 CFU/ml, and coloring is done. MIC of Laban extracts determined by the microdilution method using a microplate consisting of 96 wells. Extract solution with an initial concentration of 3.13% was mixed with 100 ml dimethyl sulfoxide.

Next sterile microplate provided with 96 wells (8 rows and 12 columns) [13], each well filled with 150 µl brain heart infusion liquid as a medium. Then, the extract of Laban leaves is taken with a concentration of 3.13% as much as 150 µl and put into the first well on rows 1, 2, 5, and 6 and series dilutions were carried out, the results of dilution were 3.13%, 1.56%, 0.78%, 0.39%, 0.20%, 0.10%, and 0.05% [14]. Then, in all rows, wells 5, 6, 7, and 8 put a suspension *S. mutans* bacteria, each as much as 10 µ [13]. Lines 1 and 2 (Group 1) contain media and samples (M + S), rows 3 and 4 (Group II) contain only media (M), rows 5 and 6 (Group 3) contain media and samples, and bacteria (M + S + B) that function as a test group to determine inhibitory concentrations minimum, and lines 7 and 8 (Group 4) contain media and bacteria (M + B) function as positive controls. Then, microplate dreaded facultative anaerobes in estimator at 37°C for 48 h. The determination of MICs was based on turbidity and reading results incubation with a microplate reader. The absorbance value was used to determine the concentration value minimum inhibition using the formula: {(M + S + B) - (M + B)/(M + B) – (M)} × 100%. The KBM is the smallest concentration of the Laban leaf extract still kills *S. mutans* which is characterized by the absence of bacterial colonies on the media Mueller–Hinton (MHA) after contact with Laban leaf extract. To determine KBM of each microplate well on rows 5 and 6 that are not turbid or clear, *S. mutans* was taken and planted on sterile MHA media. Then, put in an indicator with a temperature of 37°C for 48 h until you see a bacterial colony showed bacterial growth from MHA media which remained sterile or showed no bacteria. The KBM is determined by concentration the smallest which shows no growth of *S. mutans* [13].

Results

Determination of MIC value

Bacterial growth in the microdilution method using indicator solutions and observations turbidity. Turbidity was observed in n-hexane, ethyl acetate, and leaf methanol extract Laban visually against the microplate after incubation for 48 h, at a temperature of 370°C. Values the MIC were determined by reading the results of turbidity on the microplate with a wavelength plate reader of 620 nm. Then, the absorbance value of n-hexane extract, triacetate, and methanol is used to determine the value of MIC using the formula: {M + S + B) - (M + B)/(M + B) - (M)} × 100%. Inhibitory concentration values the minimum is the smallest concentration that gives the first negative result.

The analysis results in Table 1 show the n-hexane extract at a concentration of 1.56%; there is the first negative value. The first negative value indicates starting to occur inhibition of bacteria with a MIC value of 1.56% with the number of colonies (−1.45 CFU/ml).

Table 1: Bacterial MIC value of n-hexane extract

Concentrations (%)	Amount colony (CFU/ml)			Amount colony average (CFU/ml)
	P1	P2	P3	
3.13	5,256	6,190	9,309	5.85
1.56	13,979	2,645	1,381	−1.45
0.78	7,473	0,869	1,381	1.37
0.39	2,194	2,119	0,731	1.12
0.20	37,710	0,579	−1,663	1.19
0.10	1,120	0,608	0,249	0.37
0.05	11,363	2,674	0,484	1.50

P1 = 1 repetition; P2 = repeat 2; P3 = repetition 3. CFU = Per milliliter colony forming unit. MIC: Minimum inhibitory concentration.

The results of the analysis in Table 2 show that the ethyl acetate extract concentration is 0.20% first negative. There is a first negative value that indicates

the occurrence of the inhibition began bacteria with a MIC value of 0.20% with the number of colonies (−0.17 CFU/ml).

Table 2: Bacterial MIC values of ethyl acetate extract

Concentrations (%)	Amount colony (CFU/ml)		
	P1	P2	P3
3.13	0.204	3.435	0.979
1.56	1.242	0.510	0.230
0.78	0.307	2.549	2.191
0.39	0.209	20.364	0.821
0.20	−0.041	−0.704	0.222
0.10	1.686	0.061	0.113
0.05	0.778	−0.861	0.087

P; 1 = repetition 1; P2 = repeat 2; P3 = repeat 3 CFU = Per milliliter colony forming unit. MIC: Minimum inhibitory concentration.

The results of the analysis in Table 3 show the methanol extract at a concentration of 0.05%; there is a negative value. The negative value indicates bacterial inhibition with inhibitory concentration values minimum of 0.05% on the average colony of −1.48 CFU/ml.

Table 3: Bacterial MIC values of methanol extract

Concentration (%)	Amount colony (CFU/ml)		
	P1	P2	P3
3.13	−15.477	1.537	−5.741
1.56	1.856	2.465	−3.139
0.78	0.739	0.581	−2.482
0.39	2.095	0.362	−4.345
0.20	0.255	0.427	−6.311
0.10	1.423	0.361	−6.376
0.05	0.415	0.122	−4.982

P1 = 1 repetition; P2 = repeat 2; P3 = repeat 3 CFU = Per milliliter colony forming unit. MIC: Minimum inhibitory concentration.

The results of the microdilution test conducted on the third extract (hexane, ethyl acetate, and methanol); it was found that the extract can inhibit bacteria faster which is the methanol extract on the concentration of 0.05%. The results of the analysis using the SPSS program obtained homogeneity test values significant of 0.909> α (0.05).

The third variant of the extract (hexane, ethyl acetate, and methanol) compared is the same or homogeneous. Following the ANOVA test at a significant level >0.05, all three extracts are “significantly different.” ANOVA test output results generated sig. 0.017 < 0.05, meaning that the average of the three extracts was significantly different (Table 4). Since the results are different, then do further tests with the Duncan method. Further test results using the Duncan test note that the average value of methanol has the smallest value of −0.2650. Meaning with using methanol can inhibit bacteria at the lowest concentration of 0.05% on average colony −1.48 CFU/ml.

Table 4: Analysis of bacteria minimum inhibitory concentration (MIC)

Concentrate test (%)	Average colony (CFU/ml)	
	Hexane	Ethyl acetate
3.13	5.85	1.54
1.56	−1.45	0.66
0.78	1.37	1.68
0.39	1.12	7.13
0.20	1.19	−0.17
0.10	0.37	0.62
0.05	1.50	0.00

MIC: Minimum inhibitory concentration.

Determination of KBM value

KBM is obtained by subculturing 100 µL, then incubating for 48 h at 370°C, starting from n-hexane, ethyl acetate, and methanol extracts of Laban leaves. Result the KBM is 1.56%, 0.78%, 0.39%, 0.20%, 0.10%, and 0.05%. The smallest concentration extract (n-hexane, ethyl acetate, and methanol) is able to kill *S. mutans* bacteria characterized by the absence of bacterial colonies on microbiological growth media. Subculture results in the third stack extract do not have the KBM as in the following table.

Based on the results of the KBM test at a concentration of 3.13%.1.56%, 0.78%, 0.39%, 0.20%, 0.10%, and 0.05%, the Laban leaves methanol extract did not show its ability in kill bacteria (Table 5).

Table 5: Analysis minimum kill concentration value

Concentration test (%)	Average amount of colony (CFU/ml)	
	Hexane	Ethyl acetate
3.13	+	+
1.56	+	+
0.78	+	+
0.39	+	+
0.20	+	+
0.10	+	+
0.05	+	+

CFU = Colony forming per milliliter unit, (−): No bacterial growth, (+): Yes bacterial growth, KBM: Minimum kill concentration.

Discussion

The antibacterial test results of Laban leaf extract against *S. mutans* pillow showed that Laban leaf extract has antibacterial activity and Laban leaf extract has the ability inhibits the bacteria *S. mutans*. The MIC value is concentration the smallest that gives negative results. MIC of n-hexane extract shows the results of the calculation of the percentage of bacterial inhibition found at the inhibitory concentration minimum of 1.56% on average colony −1.45 CFU/ml. In ethyl-acetate extract inhibitory concentration minimum 0.20% on the average colony of −0.17 CFU/ml. The methanol extract can inhibit bacteria at the smallest concentration of 0.05% at an average colony of −1.48 CFU/ml. Methanol extract is faster to inhibit bacteria. The results of the analysis using the SPSS program obtained homogeneity test values significant of 0.909> α (0.05). The third variant of the extract (hexane, ethyl acetate, and methanol) compared is the same or homogeneous. Following the ANOVA test at a significant level> 0.05, all three extracts are “significantly different.” ANOVA test output results generated sig. 0.017< 0.05, meaning that the average of the three extracts was significantly different. Because it is different, then do further tests with the Duncan method. Duncan test results note that the average value of methanol the smallest is −0.2650. This means that using methanol can inhibit bacteria on the smallest concentration of 0.05% at an average colony of −1.48 CFU/ml. Inhibition of bacterial growth *S. mutans* is thought to be due to compounds in the methanol extract of the leaves of Laban such as compounds, alkaloids, flavonoids, tannins, saponins, and polyphenols that cause bacterial dead.

Leaf Laban contains flavonoids, saponins, tannins, triterpenoids/steroids, and glycosides [11]. While extract compounds from Laban have activity antimicrobial, anti-inflammatory, antidiabetic, antioxidant, antitumor, antifungal, and antibacterial [12]. Laban also contains other components such as lignans, phenylpropanoids, sesquiterpenoids, and iridoid glycosides [15]. Alkaloid compounds containing antibacterial which can interfere with the constituent components of peptidoglycan in bacterial cells, thus biological activity becomes damaged so that the growth of *S. mutans* is stopped [16]. Laban leaves contain flavonoid compounds that function as anti-bacterial agents form complex compounds to extracellular proteins that cause damage to the composition and changes in the mechanism of cell membranes. A higher concentration will result in a thicker solution wherein more solution thick diffusion will be difficult to diffuse compared to a thinner solution, consequently, the inhibitory power will decrease [17] so that in this study, the KBM from Laban leaf extract against *S. mutans* bacteria at the smallest concentration of the extract (n-hexane, ethyl acetate, and methanol) can kill and inhibit the bacteria *S. mutans*.

Conclusion

Extracts of n-hexane, ethyl acetate, and methanol from Laban leaves have inhibitory activity *S. mutans* bacterial growth. MIC of n-hexane extract shows that the calculation of the percentage of bacterial inhibition is at the inhibitory concentration minimum 1.56% (−1.45 CFU/ml), at minimum ethyl acetate extract inhibitory concentration 0.20% (−0.17) CFU/ml) and methanol extract can inhibit bacteria at the smallest concentration of 0.05% on average colony −1.48 CFU/ml. The smallest concentration of the extract (n-hexane, ethyl acetate, and methanol) is capable of kill *S. mutans* bacteria which is characterized by the absence of bacterial colonies on the media microbiological growth.

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