Utilization of Anting-Anting (*Acalypha indica*) Leaves as Antibacterial

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**Abstract.** Anting-anting (*Acalypha indica*) plants is a species of plant having catkin type of inflorescence. This research aims to utilize anting-anting as antibacterial toward *Streptococcus mutans* and degradation of biofilm on teeth. Anting-anting leaves were extracted by maceration technique using methanol, chloroform, and *n*-hexane. Antibacterial and biofilm degradation assays were performed using microdilution technique with 96 well. *n*-Hexane extracts of anting-anting leaves gave the best antibacterial potency with minimum inhibitory concentration and minimum bactericidal concentration value of 500 µg/mL and exhibited good biofilm degradation activity. Fraction of F3 obtained from fractionation of *n*-hexane’s extract with column chromatography was a potential for degradation of biofilm with IC$_{50}$ value of 56.82 µg/mL. Alkaloid was suggested as antibacterial and degradation of biofilm in the active fraction.

1. **Introduction**

Anting-anting (*Acalypha indica*) plants is a species of plant having catkin type of inflorescence. This plants traditionally used to treat dysentery, diarrhea, malnutrition, and malaria$^1$. The activities of anting-anting is related to the chemical constituents such as saponins, tannis, flavonoid, and essential oil$^2$. The ethyl acetate extract of Anting-anting was reported as an anti-bacterial against *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Streptococcus faecalis, and Pseudomonas aeruginosa*.$^3$ However, this extract is not active as antibacterial against *Klebsiella pneumoniae, Escherichia coli*, and *Proteus vulgaris*.

Oral and dental disease is one of health problem which highly correlated to bacteria. On the tooth surface, bacteria makes solid biofilm called as plaque. Dental plaque adheres to the teeth and consists of many species microbe, salivary polymers and microbial extracellular products. The most common bacteria play a role in the formation of plaque is *Streptococcus mutans*.$^4$ This research aims to utilize anting-anting as antibacterial toward *S. mutans* and biofilm degradator on teeth. Antibacterial could decrease the number of *S. mutans* in mouth to less than normal levels. Eventually, the oral and dental would be healthy. On the otherhand, biofilms acts to protect and improve the nutrition of bacteria that live in it, the matrix of the biofilm protects the bacteria from antibacterial effect. That is why the product which has antibacterial and biofilm degradator activities is needed.
2. Materials and Methods

Materials used on this research are anting-anting leaves, solvents such as water, n-hexane, chloroform, methanol, dichloromethane, acetone, media for antibacterial activity test such as typtic soy broth (TSB), typtic soy agar (TSA), reagent for phytochemical analysis and Streptococcus mutans. The anting-anting leaves was collected from Biopharmacca Research Center Conservation and Cultivation unit, Darmaga Campus on December 2013 and determined by Biology Research Center of Indonesian Institute of Research, Cibinong, Jakarta. The Streptococcus mutans was obtained from Faculty of Medicine, University of Indonesia No. 63301.

The research steps including extraction, phytochemical test, fractionation, antibacterial test, degradation of biofilm test, as well as the identification of active component. The anting-anting leaves were extracted by maceration method using 3 different solvents. The first solvent was n-hexane, continue with chloroform and finally with ethanol to result 3 different types of extract. All extracts were determined the phytochemical content qualitatively and the antibacterial activities as well as the biofilm degradation activity. The most active extract then separated by column chromatography followed by preparative thin layer chromatography. The identification of compounds using the Fourier transform infrared spectrometry (FTIR) was performed to the most active fraction.

2.1. Antibacterial test

The antibacterial test against S. mutans was performed using micro-dilution method\(^6\). Extracts or fractions diluted in DMSO to obtain a concentration stock of 10000mg/mL. The stock samples were made into several concentrations (15.63-2000mg/mL). In each well of sterile 96 well plates, samples, TSB medium and bacterial inoculant were added. The mixture were incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) then determined. The Minimum bactericidal concentration (MBC) was determined after 24 hour incubation of MIC clear zone on new media.

2.2. Biofilm degradation activity test

The method used is micro-dilution method\(^6\). Biofilms are formed by synthetic saliva (Mc Dougall solution) put in a 96 well plate together with TSB medium, 3% glucose and bacterial inoculant. The mixture was incubated for 24 h at 37 °C. Once a biofilm is formed, the remaining medium is discarded. Extracts or fractions are added at a concentration of 16-2000 mg/mL and then incubated 24 hours at temperature of 37 °C. Biofilms attached to the wall of the wells is washed using phosphate buffer. Crystal violet 1% was added to the walls and left for 15 minutes. Well rinsed with sterile water three times and 95% ethanol was added. The suspension was incubated for 45 minutes and the solution was transferred to a new micro-plate. Suspension absorbance of each well was measured using a micro-plate reader at a wavelength of 595 nm to determine the % degradation. Chlorhexidine was used as positive control and 20% DMSO as a negative control.

3. Results and Discussion

3.1. Samples preparation

Anting-anting leaves were extracted by maseration technique using methanol, chloroform, and n-hexane. The yields of extraction on different solvents are varied. The highest yield found in methanol extract, while the lowest in n-hexane extract (Table 1). Based on qualitative phytochemical analysis, all extracts consisted of alkaloid. Flavonoid was only found at chloroform and methanol extract, while tannin was only found in methanol extract. The total phenolic contents of chloroform and methanol extracts were determined by spectrometric method. The results showed that the content of the two extracts were almost the same, about 1.3%. The tannin content on the methanol extract is about 2.21%. The tannin content was not found in the n-hexane and chloroform extracts. All extracts were used to determine its activity as antibacterial and biofilm degradation. The activities of each extract will be varied based on the phytochemical content in each extract.
The yield and phytochemical content of anting-anting leaves extracts

| Solvent of extraction | Phytochemical content | Yield (%w/w) | Phenolic content (%w/w) | Tannin content (%w/w) |
|-----------------------|-----------------------|--------------|--------------------------|-----------------------|
|                       | Tannin    | Saponin     | Flavonoid    | Alkaloid   |                      |                          |                          |                          |
| n-hexane              | -         | -           | +            | +          | 1.54                 | -                        | -                        |                          |
| Chloroform            | -         | -           | +            | +          | 2.44                 | 1.34                     | 2.21                     |                          |
| Methanol              | +         | -           | +            | +          | 4.67                 | 1.35                     |                          |                          |

Note: (+) present (-) absent

3.2. Screening and Fractionation the active extract

Antibacterial and degradation biofilm assay were carried out in order to screen the most active extract between the 3 extracts. The results showed in Table 2. n-Hexane extracts of anting-anting leaves gave the best antibacterial potency with minimum inhibitory concentration and minimum bactericidal concentration value of 500 µg/mL. This extract also exhibited a good biofilm degradation activity. The chloroform extract could not inhibit the bacterial growth till the highest concentration of 2000 µg/mL. The methanol extract inhibit the bacteria growth at concentration of 500 µg/mL, otherwise this extract could not kill the bacteria. The most active as biofilm degradation was found in chloroform extract.

| Sample name             | Antibacteria | Biofilm degradation |
|-------------------------|--------------|---------------------|
|                         | MIC(µg/mL)   | MBC(µg/mL) | IC50(µg/mL) |
| Methanol extract        | 500.0        | -          | 214.6       |
| Chloroform extract      | -            | -          | 149.8       |
| n-hexane extract        | 500.0        | 500.0      | 196.9       |
| F1                      | 250.0        | -          | 202.5       |
| F2                      | 250.0        | 2000.0     | 156.3       |
| F3                      | 250.0        | 2000.0     | 56.8        |
| F4                      | -            | -          | 76.7        |
| F5                      | -            | -          | 225.5       |
| F6                      | -            | -          | 141.5       |
| F7                      | -            | -          | -           |
| F8                      | -            | -          | -           |
| F9                      | -            | -          | -           |
| F10                     | -            | -          | -           |
| F11                     | -            | -          | -           |
| F12                     | -            | -          | -           |
| F13                     | -            | -          | -           |
| F3.1                    | 500.0        | 2000.0     | 139.2       |
| F3.2                    | 500.0        | 1000.0     | 180.7       |
| F3.3                    | 500.0        | 1000.0     | 138.7       |
| Tetracycline            | 15.6         | 15.6       | 2.58        |
| Chlorhexidine           |              |            |             |

Note: (-) concentration >2000.0 µg/mL
Based on the phytochemical content in the extracts, alkaloid would be the active component because only alkaloid is found in the n-hexane extract. Tannin in methanol extract would be active to inhibit the bacteria growth, but on this extract could not kill the bacteria. The active component for biofilm degradation would be from flavonoid on chloroform extract. To get the two activity from one prospective extract, n-hexane extract was selected.

n-hexane extracts was separated with silica gel column chromatography resulted 13 fractions. The fractionation was performed using column chromatography with silica gel as stationary phase and elution by dichloromethane:chloroform using step gradient method. All of fractions resulted from this fractionation was used to analyze the activity as antibacterial and biofilm degradation. Fraction 7 – 13 were not active as antibacterial and biofilm degradation, meanwhile fraction F3 was good biofilm degradator with IC₅₀ value of 56.82 µg/mL (Table 2).

Fraction F3 was further separated by preparative thin layer chromatography eluated with dichloromethane: chloroform (3:7). The fractionation of F3 resulted 3 subfractions, F3.1 – F3.3. All of the subfractions showed antibacterial activity but not as good as tetracycline as positive control. All subfractions also showed biofilm degradation activity but not as good as chlorhexidine as positive control. To determine the active ingredient as antibacterial and biofilm degradation, F3 was used.

FT-IR analysis was performed to F3 (Fig 1) in order to determine the active component in this fraction. The spectrum at wavenumber 3448 cm⁻¹ stalling indicated amine NH₂, 1512 cm⁻¹ bend for NH₂ (amine secondary), 1377 and 1242cm⁻¹ for CN (amine) and 1169 cm⁻¹ for ester. This information indicates the presence of alkaloid in F3. Based on phytochemical qualitative analysis and FT-IR analysis, F3 contain an alkaloid and was suggested as antibacterial and biofilm degradator in the active fraction.

4. Conclusion
n-Hexane extracts of anting-anting leaves is the most potent extract as antibacterial and good biofilm degradation. Fraction F3 obtained from n-hexane’s extract was good antibacterial and biofilm degradator. Alkaloid was suggested as antibacterial and biofilm degradator in the active fraction.

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