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Antibacterial activity against *Lactobacillus acidophilus* and *Streptococcus mutans* from endophytic mold of binahong leaf (*Anredera cordifolia* (Ten.) Steenis)

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Endophytic mold; Binahong leaf (*Anredera cordifolia* (Ten.) Steenis); Antibacterial activity; *Lactobacillus acidophilus*; *Streptococcus mutans*.

ABSTRACT

Endophytic microbe is usually used as a natural source to explore novel metabolites which has therapeutic activities. This research's aim is to determine the antibacterial activity of endophytic molds which grow in the tissue of binahong leaf (*Anredera cordifolia* (Ten.) Steenis) to resist *Lactobacillus acidophilus* and *Streptococcus mutans*. Four endophytic mold isolates were obtained, which were KTS1, KTS2, KTS3, and KTS4. The screening of antibacterial activities for *L. acidophilus* and *S. mutans* was carried out from the supernatant of each isolate, and the highest inhibition was obtained by KTS1 with the inhibition diameter of 2,82 mm for *L. acidophilus* and 2,89 mm for *S. mutans*. The grading extraction with n-hexane, ethyl acetate, and methanol was used to extract the supernatant. The microdilution method was used to determine the antibacterial activity by measuring the absorbances with a microplate reader at a 595 nm of wavelength. The respective of IC50 of KTS1 in the ethyl acetate (KTS1EA) and methanol (KTS1ME) against *L. acidophilus* were 9007,80 µg/ml and 10119,45 µg/ml. The relative potency of KTS1EA and KTS1ME to erythromycin against *L. acidophilus* were 0,0023 and 0,0020 times as respectively. The respective of relative potency of KTS1EA and KTS1ME to erythromycin against *S. mutans* were 0,0032 and 0,0021 times. However, the IC50 of KTS1EA and KTS1ME against *S. mutans* were 5878,03 µg/ml and 8928,75 µg/ml as respectively.

INTRODUCTION

Endophytic microbes (*endophytes*) are defined as microorganisms that living in the plant tissue by forming colonies without harming the host plant (Strobel & Daisy, 2003). These endophytes such as bacteria, mold, and yeast produce the similar bioactive compounds as produced by its host plant. This can occur due to the possibility of genetic transfer between endophytes and the host plant. These bioactive compounds can exhibit many important biological activities which potentially used as therapeutic agents. The endophytes usually receive nutrients from its host plant and can influence the development of the host plant (Kumala, 2014). The exploration of endophytes is usually performed to find novel metabolites which has biological activities.

The binahong plant (*Anredera cordifolia* (Ten.) Steenis) is considered as one of the famous medicinal plants because it has already widely been explored. It has the efficacy as an anticancer, antidiabetic, anticaries tooth, and antibacterial (Nurhartanti & Masduqi, 2020). It is also used to overcomes kidney failure and lower the cholesterol (Nurhartanti & Masduqi, 2020). The ethanolic extract of binahong leaf contains several active compounds, such as flavonoids, terpenoids, saponins and alkaloids (Samirana *et al.*, 2017). This flavonoid usually exhibits antimicrobial activity by forming complex bonds with bacterial cell walls (Indarto *et al.*, 2019). A study reported that binahong leaf (*Anredera cordifolia* (Ten.) Steenis) has an antibacterial activity against *Staphylococcus aureus* (Mengga *et al.*, 2022). The previous study also reported that binahong leaf extract has antibacterial activities against a dental caries causing bacteria which are *Lactobacillus acidophilus* and *Streptococcus mutans* (Permanasari *et al.*, 2024; Rimporok *et al.*, 2015; Soares, 2018).

Many dental health problems occur, one of which is dental caries. Untreated dental caries is found to be the most common dental case affecting to almost 2.5 billion people worldwide (The Global Oral Health Status Report, 2022). Untreated dental caries can lead to another serious health problems. Dental caries is a dental health problem caused by bacteria. Dental caries begins with the

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formation of dental plaque by microorganisms. The remaining food in the teeth causes the plaque which presence on the teeth surface. It is the initial mechanism for the occurrence of dental caries. Bacteria attach to the process which will cause demineralization (Michael *et al.*, 2008). *Lactobacillus acidophilus* and *Streptococcus mutans* are the main bacteria that cause dental caries (Anita *et al.*, 2015). To prevent dental caries, people must maintain the proper dental hygiene and health such as brushing the teeth, rinsing the mouth with an antiseptic, and reducing habit of eating sweet foods (Michael *et al.*, 2008). Dental caries infection can cause holes in the teeth, pain, and even tooth loss. However, the study of antibacterial activity of endophytes for a dental caries causing bacteria is yet very limited.

Here, we performed a study to explore the secondary metabolites from endophytes that exhibit antibacterial activity for a dental caries causing bacteria, which are *L. acidophilus* and *S. mutans*. In this study, the antibacterial testing was carried out for the endophytic mold of binahong leaf. This research began by the isolation of the endophytic molds from binahong leaves. The macroscopic and microscopic observations were also carried out. The cultivation of endophytic molds was carried out to obtain a supernatant containing secondary metabolites. The selected endophytic mold isolates were then extracted by a liquid-liquid extraction using the graded solvents. The antibacterial activity was tested for *L. acidophilus* and *S. mutans* using the microdilution method, as compared to the antibiotic of erythromycin. The measurements were conducted by reading the absorbance with a microplate reader at a 595 nm of wavelength. The antibacterial activities were measured as IC50 of inhibition percentage.

MATERIAL AND METHODS

Chemical and Reagents

Ethanol 75%, methanol, ethyl acetate, n-hexane, sodium hypochlorite 5.3% (NaOCl), and distilled water in analytical grade were from PT Smart-Lab Indonesia. Potato Dextrose Agar (PDA), Muller Hinton Agar (MHA), Potato Dextrose Yeast (PDY), Muller Hinton Broth (MHB), antibiotic erythromycin, chloramphenicol, DMSO 10%, Mayer's reagent, Dragendroff's reagent, Liebermann Bouchard reagent, Mg^{2+}, HCl 2N, and the culture of *Lactobacillus acidophilus* and *Streptococcus mutans* were obtained from CV. Eternal Space, Indonesia.

Isolation of Endophytic Molds

The fresh binahong leaves (*Anredera cordifolia* (Ten.) Steenis) were washed with clean running water, and was then sterilized in the 75% ethanol and the 5,3% sodium hypochlorite. After sterilizing, the samples were dried, then cut the bone of the leaf with a size of 0.5 x 0.5 cm. Then the leaf was planted directly into Potato Dextrose Agar (PDA) medium which was previously given 0.05% chloramphenicol (50 µg/ml). It was then incubated at a temperature of 27-29°C for 7 days. Endophytic mold isolates which showed different morphological characteristics were considered as distinct isolates. Four endophytic mold isolates were chosen and purified to obtain one type of isolate. Purification was carried out on PDA plate medium using the scratch plate method. Then the pure isolate obtained was transferred to a new PDA plate medium and incubated for 5-7 days at a temperature of 27-29°C (Kumala, 2014).

Macroscopic and Microscopic Morphological Observations

Macroscopic observation of endophytic mold morphology was done by observing colony characteristics, including color, elevation, edges, shape, consistency, colony odor and colony diameter (Nasichah *et al.*, 2016). Microscopic observations were carried out by making mold preparations using the slide culture method. This was done by dripping a glass object with PDA medium then leaving it for a while until it solidifies, then on top of the solid PDA medium, 1 tube of endophytic mold is placed which is taken using a tube needle, then covered carefully with a cover glass. The tissue was then finally incubated for 3 days at a room temperature. The preparations were observed under a microscope (Bahri *et al.*, 2021; Kumala, 2014).

Cultivation of Small Volumes of Binahong Leaf Endophytic Molds

The pure isolate of endophytic mold was then cultivated using a sterile PDY liquid medium. From each colony, the pure isolate of endophytic mold was put into a test tube containing 10 ml of PDY medium, then cultivated using a rotary shaker for 7 days. The cultivation results were then centrifuged. The supernatant will be used for antibacterial activity screening (Kumala, 2014).

The Screening for the Antibacterial Activity of Endophytic Molds

The screening for the potential antibacterial properties of endophytic molds in this study was carried out. The two test bacterial suspensions were prepared in a test tube containing Muller Hinton Broth (MHB) medium, then the suspensions were incubated for 24 hours at a temperature of 37°C. Next, the absorbance was measured using a UV- Vis at a 580 nm of the wavelength, until a transmittance of 25% was obtained, then the bacterial suspensions were put into an

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Erlenmeyer flask containing MHA media (ratio 1:10) then stirred until evenly distributed. Next, the medium solution was poured into a petri dish, then let it solidifies. The supernatant from each isolate was put on the paper disk.

The screening was done in the Laminar Air Flow (LAF) aseptically. The sterile disc was soaked in the supernatant, and wait until it is absorbed. After that, the sterile disc was placed on the medium and incubated for 2 days at a temperature, then the antibacterial potential was measured by observing whether there is an inhibitory zone around the disc. The diameter of the inhibition area was measured by the caliper. The isolate with the largest inhibition zone was continued for the extraction process (Kumala *et al.*, 2006).

Cultivation of Large Volumes of Binahong Leaf Endophytic Molds

The isolate with the largest inhibition zone was continued for cultivation. The cultivation was carried out with 300 ml of PDY liquid medium, and incubated for 7 days at a temperature of 27°C. The centrifugation was performed. As a result of centrifugation, the supernatant was obtained, then the liquid was extracted using graded solvents by n-hexane, ethyl acetate and methanol (1:1, v/v). The extraction was carried out by the grading solvents of n-hexane, ethyl acetate, and methanol. Each extract was concentrated using a vacuum rotary evaporator, then a viscous extract was obtained which was used to test antibacterial activity.

Phytochemical Screening

The phytochemical screening was performed to verify the bioactive compounds in the binahong leaf extracts. The identification was carried out for alkaloid, flavonoid, saponin, and terpenoid. The identification was conducted as described on the standard procedures (Julianto, 2019).

Antibacterial Activity Test Using Liquid Microdilution Method

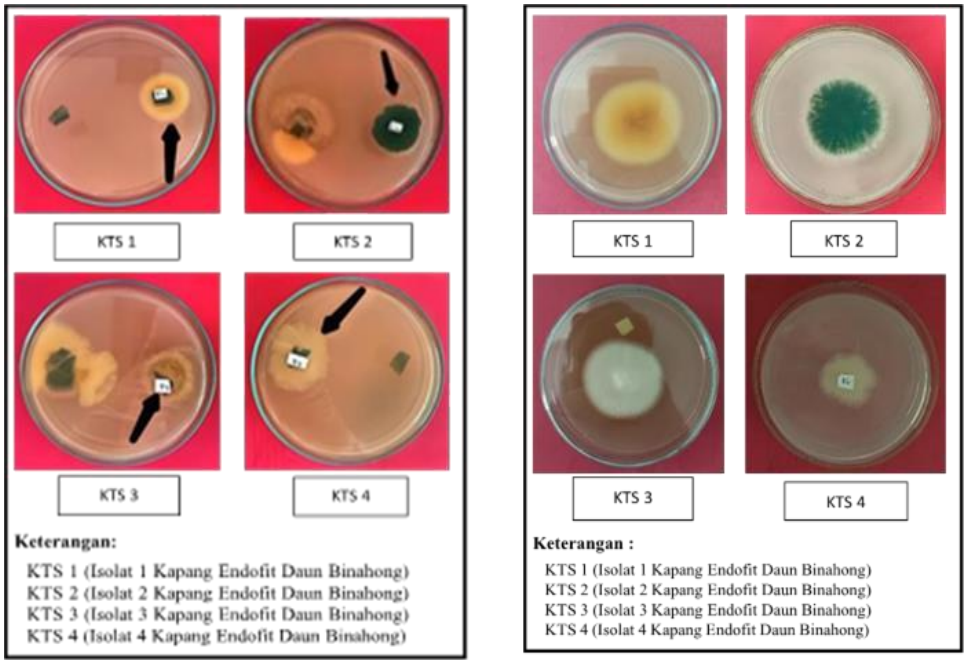
The antibacterial testing was performed with the microdilution method using a 96 well microplate under the LAF. The method was chosen as it is simple, easy, fast, sensitive, and does not require a lot of samples. The chemical used in the test was MHB medium, DMSO as a solvent control, and the antibiotic erythromycin as a positive control. The test solution was prepared by adding a 50 µl of MHB medium in microplate well 96, then adding a 100 µl of the samples, then adding a 50 µl of the test bacterial suspension which was equivalent to 25% transmittance. The control solution was carried out by adding a 100 µl of solution into microplate well 96, then a 50 µl of MHB medium and a 50 µl of DMSO. A positive control containing erythromycin was made by adding a 100 µl of erythromycin solution, adding a 50 µl of MHB medium and a 50 µl of the test bacterial suspension. The erythromycin control was made by adding a 100 µl of erythromycin solution, then a 50 µl of MHB medium and a 50 µl of DMSO. The bacterial control preparation was carried out by adding a 50 µl of MHB medium, a 50 µl of test bacterial suspension and a 100 µl of DMSO. The media control was made by adding a 100 µl of MHB medium and a 100 µl of DMSO. The total amount for each well was 200 µl. After completion of preparation, each was incubated at 37ºC for 24 hours and the absorbance was measured using a microplate reader with a wavelength of 595 nm.

RESULTS AND DISCUSSION

Isolation of Binahong Leaf Endophytic Molds

The isolation of endophytic molds from binahong leaves was the initial and critical stage of the research. The isolation must be done under the aseptic condition to eliminate all forms of contaminant microorganisms. The surface of sterile leaves was cut lengthwise in several sections of 0.5 cm x 0.5 cm of the measurements. This process was carried out to make it easier for endophytic mold that live in binahong tissue to grow on the PDA medium. The isolation process was carried out by planting directly the leaves on the PDA medium. A chloramphenicol of 0.05% (50 mg/ 1000 ml) was given to inhibit the bacterial contamination (Kumala, 2014).

The colonies that grow on PDA medium of binahong leaves were considered as endophytic mold isolates. Based on the results, four isolates of endophytic molds were obtained as shown in **Figure 1a**. They were coded as KTS1, KTS2, KTS3 and KTS4. Each isolate was inoculated back into another petri dish containing PDA medium to be stored as a pure culture. The aim of this purification is to obtain the types of endophytic mold isolates with the same morphological characteristics in one petri dish (Kumala, 2014). The results of the purified isolates can be seen in **Figure 1b.**



(a) **(b)**

Figure 1. (a) Four Isolates of Endophytic Molds from Binahong Leaf, (b) Purification of Four Endophytic Mold Isolates from Binahong Leaf

Macroscopic and microscopic characterization

The purified KTS1, KTS2, KTS3, and KTS4 were observed for their macroscopic and microscopic characteristics, as described in the **Table 1** and **Figure 2**. The macroscopic and microscopic characterizations were carried out on endophytic molds aged 5-7 days. The aim is to determine the morphology of the endophytic mold isolate. The microscopic characterizations were made by looking at the morphological form of the mold in the form of septate hyphae, colored or dark pigmented hyphae, the shape of the hyphae (spiral, nodule, or have rhizoids), branching of the hyphae tips, and whether or not conidia were present (Kumala, 2014).

Based on the results of the macroscopic characterization of four isolates as shown in **Table 1**, it can be concluded that the morphology of all isolates has different characteristics on the surface of the isolate and the shape of the hyphae. With the color differences as shown in the KTS1 isolate, there is a white and yellow color, the edges of the mold colonies also have radial lines which are marked by the presence of transparent white hyphae. KTS2 shows that the mold has a very striking color, namely greenish white around which is transparent at the edge of the colony, and has thick colonies. KTS3 has a pure white color and has a cotton-like texture, the edges of the colonies have radial lines. KTS4 has a white color, there are no thick and smooth radial lines at the edges of the colony.

Table1. Macroscopic Characterization of Endophytic Mold Isolates from Binahong Leaf

Isolate codes	Parameter			
	Color	Diameter	Radial Line	Colony Form
KTS1	White yellow	5,6 cm	Yes	Velvet
KTS2	Green in the middle and white in the edge	5,3 cm	None	Thick stringy cotton
KTS3	White	5,2 cm	Yes	Hyphae resemble cotton
KTS4	White	3,5 cm	None	Granular thick and slippery

The results of microscopic characterization of four isolates as shown in the **Figure 2** found that the morphology of KTS1 is the velvety hyphae (thin hairs and separate edges) which can be described as yellow, septate hyphae, and do not have conidia. The KTS2 has the form of thick and greenish filamentous hyphae that are septate and have conidia at the tip. Then, KTS3 is a long and non-septate white colored hypha. The KTS4 isolate has a thick and smooth granular hypha with the combination colors of white and yellow septate and do not have conidia. All four isolates differ in their macros- and microscopic characterizations.

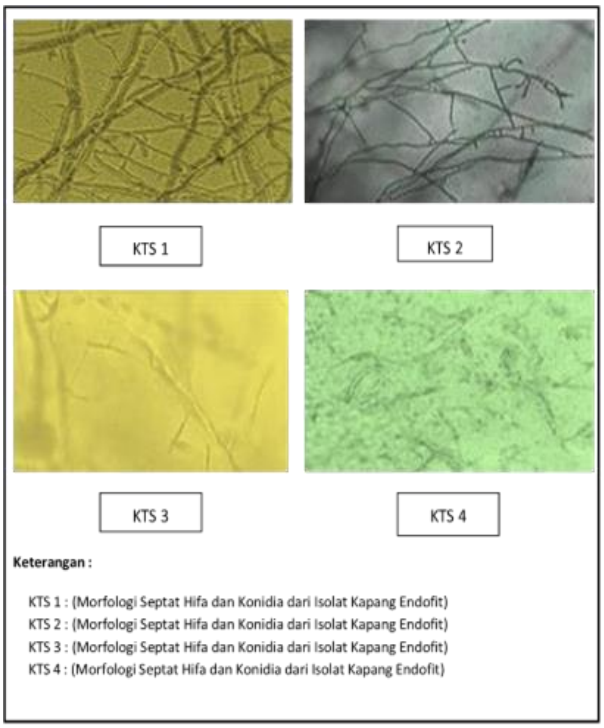


Figure 2. Microscopic Characterizations of Endophytic Mold Isolates from Binahong Leaf with 40x Magnification

The Small Volumes Cultivation

The cultivation process is carried out to produce secondary metabolite compounds from the four isolates of binahong leaves. The PDY medium was used for cultivation as it has a good composition for the growth of endophytic mold. Endophytic molds will produce secondary metabolites at the stage where the number of dead microorganism cells is equivalent to the number of growing cells, which is called the stationary phase (Pratiwi, 2008). At this stage a nutritional crisis occurs which causes microorganisms to release secondary metabolites for self-defense. PDY medium has a composition consisting of (Potato Dextrose Broth) which functions as a carbon source, yeast extract functions as a mineral source, and $CaCO_3$ functions as a pH balancer. Small volume cultivation was carried out by inoculating each colony in 10 ml PDY medium. The secondary metabolites were supposedly presence in the supernatant.

The Results of the Antibacterial Activity Screening

The supernatant produced from the small volume cultivation process was screened for antibacterial activity. The aim was to determine the isolate that has the largest inhibition zone against the bacteria. The screening results of the antibacterial activity from KTS1, KTS2, KTS3, and KTS4 were shown in **Table 2** and **Figure 3**.

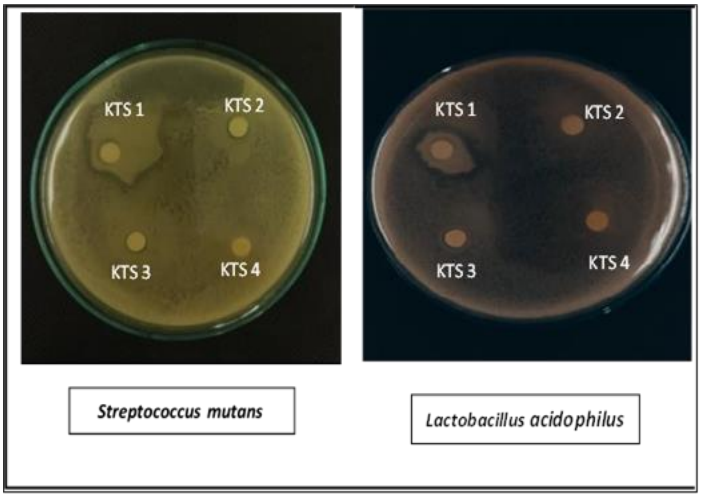


Figure 3. The Screening Results of Antibacterial Properties of Four Isolates from Binahong Leaf Against *L. acidophilus* and *S. mutans*

The results shown in the **Table 2** found that the largest of inhibition diameter is the KTS1 isolate with the inhibition diameter of 2,82 mm against *L. acidophilus* and 2,89 mm against *S. mutans.* The KTS1 were then chosen for large volumes of cultivation.

Table 2. The Screening Results for Antibacterial Activity of Endophytic Mold Isolates of Binahong Leaf Against *S. mutans* and *L. acidophilus*

No	Isolate codes	Inhibition Diameter (mm) for ***S. mutans***	Inhibition Diameter (mm) for ***L. acidophilus***
1	KTS1	2,89	2,82
2	KTS2	1,51	1,25
3	KTS3	1,36	1,35
4	KTS4	1,76	1,82

The Secondary Metabolite Extractions

The cultivation supernatant obtained from the KTS1 isolate will then be subjected to a liquid-liquid extraction. The extraction aims to extract the metabolite compounds. The liquid-liquid extraction process uses solvents with different polarities to find out whether the three solvents could completely extract the secondary metabolite compounds. From the extraction process, three extracts were obtained which were methanol, ethyl, and n-hexane extract. Each extract of KTS1 was then continued for rotary evaporator resulting in only two condensed extracts, which were ethyl acetate (KTS1EA) and methanol extract (KTS1ME) as shown in **Table 3**. These two extracts were tested for antibacterial properties for *S. mutans* and *L. acidophilus.*

Table 3. The Extraction Results of Endophytic Mold Isolates from Binahong Leaf

Solvent	Liquid Extract (ml)	Viscous Extract (g)	Rendement (%)
Methanol	346	5,4089	1,5708
Ethyl Acetate	335	3,5342	5,4089
n-Hexane	314	NA	NA

The Results of Phytochemical Screening

The phytochemical screening was the preliminary process to overview the bioactive compounds contained in the sample. The positive results of KTS1AE and KTS1ME were obtained in the testing of alkaloids, flavonoids, saponins, and terpenoids. The screening results can be seen in **Table 4**. This finding showed that the KTS1AE and KTS1ME have broad bioactive compounds. In the previous research, binahong leaves have secondary metabolites which exhibit antibacterial properties (Sianipar *et al.*, 2020).

Table 4. Metabolite Compound Identification Results

Isolates	Screening test	Results
KTS1EA	Alkaloids	+
	Flavonoids	+
	Saponins	+
	Terpenoids	+
KTS1ME	Alkaloids	+
	Flavonoids	+
	Saponins	+
	Terpenoids	+

Note: (+) is a positive phytochemical screening. (-) is a negative phytochemical screening

From the previous research, each of those bioactive compounds could exhibit antibacterial properties with their mechanisms. For example, alkaloids have antibacterial and inhibitory effects by affecting the peptidoglycan component of bacterial cells, weakening cell wall layer formation and causing the death. The flavonoid acts as an antibacterial with several actions, inhibiting membrane function so that it can damage bacterial cell membranes. The saponin acts as an antibacterial agent because it acts as a toxin in the protoplasm, which causes bacterial cells to die due to the layer of their cell walls. The terpenoid exhibited antibacterial mechanism by damaging bacterial cell membranes and releasing of intracellular compounds (Indarto *et al.*, 2019).

The results of antibacterial activities

The antibacterial testing was carried out by the microdilution method using a 96-well microplate. This method was chosen because it requires a small number of samples and the obtained results are more accurate. In this procedure, the erythromycin was used as an antibiotic which usually inhibits *S. mutans* and *L. acidophilus*. The variation of the sample solution was prepared by 14960 µg/ml, 7480 µg/ml, 3780 µg/ml, and 1870 µg/ml. While the erythromycin was prepared by 48 µg/ml, 24 µg/ml, 12 µg/ml, and 6 µg/ml. The percentage of inhibition was described in **Table 5**.

Table 5. The Percentage of Inhibition of KTS1EA and KTS1ME of Binahong Leaf to *Lactobacillus Acidophilus* and *Streptococcus Mutans*

Concentration (µg/ml)	KTS1EA to *S. mutans*		KTS1ME to *S. mutans*		KTS1EA to *L. acidophilus*		KTS1ME to *L. acidophilus*	
	% inhibition	IC50	% inhibition	IC50	% inhibition	IC50	% inhibition	IC50
14960	76,78	5878,03	68,84	8928,75	72,94	9007,80	68,49	10119,45
7480	60,80		46,72		49,07		40,66	
3740	35,61		36,11		27,28		24,70	
1870	23,80		24,59		18,98		15,96	

The IC50 of KTS1EA and KTS1ME to *S. mutans* were 5878,03 µg/ml and 8928,75 µg/ml as respectively shown in **Table 5**. Meanwhile, the IC50 value of KTS1EA and KTS1ME were respectively 9007,80 µg/ml and 10119,45 µg/ml. The IC50 of erythromycin to *S. mutans* and *L. acidophilus* were calculated to be 19,092 µg/ml and 20,9061 µg/ml respectively as shown in **Table 6**.

Table 6. The IC50 of Erythromycin to *S. Mutans* and *L. Acidophilus*

Concentration (µg/ml)	*S. mutans*		*L. acidophilus*	
	% Inhibition	IC50 (µg/ml)	% Inhibition	IC50 (µg/ml)
48	82,04	19,0920	84,35	20,9061
24	60,11		56,63	
12	35,50		30,95	
6	22,71		15,96	

Therefore, the relative potency of KTS1EA and KTS1ME to erythromycin against *S. mutans* were 0,0032 and 0,0021 times as respectively. While, the relative potency of KTS1EA and KTS1ME to erythromycin against *L. acidophilus* were 0,0023 and 0,0020 times as respectively. Hence, the KTS1EA and KTS1ME of binahong leaf have an antibacterial activity for *Streptococcus mutans* and *Lactobacillus acidophilus*. The level of antibacterial activity of KTS1EA and KTS1ME were weak as the IC50 value of those isolates were considered as lower than erythromycin against for *L. acidophilus* and *S. mutans*. The weak antibacterial activity was probably coming from the lower content of bioactive compounds. Although, those two isolates performed a broad variation of bioactive compounds in the phytochemical screening results, the composition of those bioactive compounds may influence the total antibacterial activities of the samples.

CONCLUSIONS

In this study, it can be concluded that the endophytic molds from the binahong leaf (*Anredera cordifolia* (Ten.) Steenis) were successfully exhibit antibacterial activity against *L. acidophilus* and *S. mutans* as respectively. Against *Lactobacillus acidophilus*, the respective IC50 of KTS1EA and KTS1ME were 9007,80 µg/ml and 10119,45 µg/ml with the relative potency of 0,0023- and 0,0020-times to erythromycin. However, the respective IC50 value of KTS1EA and KTS1ME were 5878,03 µg/ml and 8929,75 µg/ml with a relative potency of 0,0032- and 0,0021-times to erythromycin against *Streptococcus mutans*.

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