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Characterization of Ethanolic Extract of *Streptomyces* sp. as a Pancreatic Lipase Inhibitors Produced by Endophytic *Streptomyces* sp. AEBg12

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Abstract

Endophytic *Streptomyces* sp. AEBg12 isolated from *Zingiber cassumunar* (Bangle) is known to produce pancreatic lipase inhibitory compound. However, the characteristics of this active compound has not been reported yet. This study aimed to determine the characteristics of pancreatics inhibitory compound produced by *Streptomyces* sp. AEBg12 and to assess the role of endophytic actinobacteria in producing pancreatic lipase inhibitor using endophytic-free bangle tissue culture, wild bangle and compared with the activity of *Streptomyces* sp. AEBg12 endophytes. Supernatant of *Streptomyces* sp. AEBg12 was extracted using ethanol, ethyl acetate, and n-hexane solvents. Toxicity test was performed using larvae of shrimp *Artemia salina*. The results showed that the best solvent to obtain pancreatic lipase inhibitor compounds was ethanol. Phytochemical analysis showed that ethanolic extract of endophytic *Streptomyces* sp. AEBg12 contained flavonoids. IC_{50} value of ethanol extract was 180.83 µg/ml. The result of TLC showed that ethanolic extract of *Streptomyces* AEBg12 had a blue luminescence band indicated that there were either flavone, flavanones, flavonols or isoflavones. Inhibitory activity of *Streptomyces* sp. AEBg12 was higher than wild bangle and bangle tissue culture. The information from this study can be be used as a basic data for further characterization of the active compound, which might be developed as an antiobesity agent through its pancreatic lipase inhibitory activity.

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INTRODUCTION

Obesity is an abnormal excess of gained weight that occurs due to an excessive fat accumulation. It is the result of energy balance disorders where calories that enter the body are more than calories needed. Among all the treatments for obesity, one of the most promising strategies for weight loss is by inhibiting fat absorption using pancreatic lipase. Fat is not directly absorbed by the intestine unless it has been degraded by pancreatic lipase. Many study of this mechanism were performed to determine the effectiveness of natural products as antiobesity agents. Pancreatic lipase is an enzyme secreted by pancreas and contribute in fat digestion (Shin et al., 2003).

Various types of medicinal plants were used to prevent obesity. This knowledge has been passed down from generation to generation based on their custom (Pradono et al., 2011). *Zingiber cassumunar* (Bangle) is one potential medicinal plants as a lipase inhibitor. Iswantini et al. (2011) reported that 100 ppm of ethanolic extract of bangle rhizomes had the highest inhibitory effect on pancreatic lipase activity (29.17%). The inhibitory effect was higher than 100 ppm inhibitory effects of Xenical ® / orlistat as a positive control, with inhibition percentage at 17.53%.

A part of plants, microbes are also able to produce secondary metabolites as a pancreatic lipase inhibitor, it can be used as an antiobesity drug. Endophytic microbes are microbes that can live in plant tissue and are able to form colonies without harming the host. Most of seed plants could associate with several endophytic microbes which are able to produce bioactive compounds. It was suspected that the bioactive compounds was associated with coevolution or genetic transfer from the host plant to endophytic microbes (Tan & Zou, 2001). From the preliminary study, a total of 9 isolates of endophytic actinobacteria from bangle which were capable of producing pancreatic lipase inhibitor was obtained. AEBg12 was the isolate which was able to produce the highest pancreatic lipase inhibitor. Based on morphological, microscopic and molecular observation, it was found that isolates of AEBg12 was *Streptomyces* sp. This study aimed to discover the content of the compound, toxicity values, inhibition values, TLC of ethanolic extract of *Streptomyces* sp. AEBg12 and the role of endophytic actinobacteria in producing pancreatic lipase inhibitor compared to plant tissue culture, and plant from the nature. However, the characterization of pancreatic lipase inhibitor compounds produced by *Streptomyces* sp. AEBg12 has not been studied. This study aimed to determine the characteristics of the ethanolic extract which contained pancreatics inhibitory compound produced by *Streptomyces* sp. AEBg12, and to assess the role of endophytic actinobacteria in producing pancreatic lipase inhibitor using endophytic- free bangle tissue culture plant, bangle plant from nature, and compared with the activity of endophytic *Streptomyces* sp. AEBg12 from bangle plant. It is expected that the output of the study can be used as a basic data for further molecular characterization and development of the active compound as an agent for antiobesity which is obtained from indigenous actinobacteria.

METHODS

Actinobacteria isolate was inoculated into Erlenmeyer flask containing 1000 ml yeast starch broth medium and incubated at 30°C for 10 days on a rotary shaker with 150 (Kekuda et al., 2011). The filtrate was collected by centrifugation at 4000 rpm at 4°C for 25 min to separate supernatant and biomass. Supernatant obtained from selected culture was extracted using various solvents to obtain the active compounds. The solvents tested were ethanol, n-hexane and ethyl acetate. Extraction was performed by adding solvent in to supernatant with a ratio of 1:1, then they were homogenized using magnetic stirrer for 2 hours to form water fraction and solvent fraction. Solvent fraction then separated and concentrated by rotary evaporator to obtain concentrated fraction. The fraction obtained then used to test the activity as an inhibitor of pancreatic lipase.

Inhibitor of pancreatic lipase activity test was carried out by using method of Etoundi et al. (2010) with some modifications. A total of 800µl of triolein mixture was added into a test tube containing 200µl swine lipase and 200µl sample. The solution was mixed and then the absorbance was measured using UV-Vis spectrophotometer at 450 nm wavelength. Then, the solution was incubated for 30 min at 37°C and the absorbance was measured as above. The percentage of pancreatic lipase inhibitory activity was calculated by using the formula:

Inhibition of pancreatic lipase = A - B / A x 100

where A = pancreatic lipase activity, B = pancreatic lipase activity after incubation

The toxicity test was performed using larvae of *Artemia salina*. Determination of IC_{50} values was performed by testing the inhibitory activity of the extracts on a wide range concentrations. After inhibition value of each concentration of the extract was obtained, then the equation

as a function of the extract concentration and the amount of inhibition produced was made.

Separation of pancreatic lipase inhibitor compounds was performed by using several solvents so that the right eluent could be obtained. The selected eluent then was dried and its predicted components were detected using reprostar 3 Camag integrated with WinCATS software. Detection was performed with UV at a lenght of 254 nm and 366 nm. Furthermore, the value Retention Factor (Rf) was determined by using WinCATS software with formula:
Rf = migration distance of the substance/migration distance of the solvent front

To determine the role of endophytic actinobacteria isolates in producing pancreatic lipase inhibitor, plant endophyte-free from tissue culture and bangle derived from nature wer eused. A total of 0.5 g samples plant was crushed aseptically then it was added by 0.5 ml of phosphate buffer 0.1 M (pH 8). Then, pancreatic lipase inhibitory activity of supernatant obtained was tested.

RESULTS AND DISCUSSION

Extraction of pancreatic lipase inhibitor of *Streptomyces* sp. AEBg12

Solvents which were used in this study consisted of ethanol, ethyl acetate and n-hexane. Extraction with various solvents are presented in Table 1. Extraction process with three solvent provided varies result for each solvent. The results showed that extraction with ethanol produced the highest yield (4.39 g) compared to other solvents, followed by ethyl acetate and n-hexane which were 0.48 and 0 g, respectively. The higher amount of pancreatic lipase inhibitor was produced by ethanol extract. It was probably because the ethanol had a high polarity. Ethanol had a low boiling point and it tend to be safe, non-toxic and harmless solvent. N-hexane could cause some negative effects such as disease and air pollution due to the characteristic of hexane which was toxic if it was consumed. In addition, it was a flammable liquid and had a low biodegradability (Azis et al., 2014).

The result showed that the content of polar compounds in supernatant of *Streptomyces* sp. AEBg12 were relatively larger than semi-polar and non-polar extract. The result is in line with the research done by Yuhernita (2011) that performed an extraction using a polar solvent (methanol), semi-polar (ethyl acetate) and non-polar (n-hexane) in which the content in polar compounds was higher than non-polar compounds. Ethanol extracts and ethyl acetate extracts were tested for their ability to inhibit the activity of pancreatic lipase. The content of the compound which would be extracted should be considered for selecting the solvents (Septiana & Asnani, 2012). Ethanol was a polar compound, so that another polar compound would be drag into the extract. The using of ethanol as a solvent in leaves extraction could dissolve alkaloid compounds, polyphenols, and flavonoids (Ayini et al., 2014).

Table 1. Yield of extraction of pancreatic lipase inhibitor of *Streptomyces* sp. AEBg12 with various solvents

Solvent	Yield (g)
n-hexane	0
Ethyl acetate	0.48
Ethanol	4.39

Phytochemical content of *Streptomyces* sp. AEBg12

Culture extracts of *Streptomyces* sp. AEBg12 were chemically analysed for its phytochemical content (Table 2). Major classes of active compounds contained in the extract could obtained through this analysis (Pujiyanto, 2012). Phytochemical analysis results showed that the water extract of *Streptomyces* sp. AEBg12 derived from bangle contained flavonoids, saponins and steroids, while ethanol and ethyl acetate extracts only contained flavonoids. Saponins and steroids in ethanol and ethyl acetate extract which were not detected probably because the compounds were small or they were not exist in the sample (Iswantini et al., 2011). Flavonoids, saponins and steroids allegedly able to act as pancreatic lipase inhibitor. These all compounds allegedly capable as an inhibitor of pancreatic lipase. The study of Iswantini et al. (2011) stated that the phytochemical analysis of bangle showed that they contained flavonoids, saponins, steroids and tannins. Flavonoids have been shown to inhibit the activity of lipase in vitro, including that contained in the rhizome of bangle. Saponins also proven capable to inhibit lipase activity both in vitro and in vivo. The study of Dzomba & Musekiwa (2014) showed that flavonoid extract from roots of *Dioscorea steriscus* could inhibit the activity of pancreatic lipase and α-amylase.

Toxicity test of ethanolic extract of *Streptomyces* sp. AEBg12

Toxicity test of ethanolic extract of *Streptomyces* sp. AEBg12 were conducted on larvae of shrimp *Artemia salina* (Table 3). The table showed that if the concentration is higher, the more sh-

rimp larvae were dead. The highest concentrations used in this study was 1000 µg/ml where the percentage of mortality reaches 100% and the lowest concentrations was 50 µg/ml where the percentage of mortality reaches 26.6 %. The values of LC_{50} of ethanol extract was 231.44 µg/ml,this means that at small concentrations, this extract was able to eliminate half of larvae *A. salina* population. According to Meyer et al. (1982), an extract was considered as a highly toxic when it had LC_{50} values below 30 µg/ml, considered as a toxic if it had LC_{50} values around 30 to 1000 µg/ml, and considered as a nontoxic if it had LC_{50} more than 1000 µg/ml. It showed that ethanolic extract of *Streptomyces* sp. AEBg12 were toxic and indicated that ethanolic extract of *Streptomyces* sp. AEBg12 contained a high bioactive compound.

Table 2. Phytochemical content of *Streptomyces* sp. AEBg12

Compounds	Solvent		
	Water	Ethanol	Ethyl acetate
Alkaloid	-	-	-
Flavonoid	+	++	+
Tannin	-	-	-
Saponin	+	-	-
Quinone	-	-	-
Steroid	+	-	-
Triterpenoid	-	-	-

This study used 48 hours old larvae of *Artemia salina*. Shrimp larvae at the age of 48 hours already had a complete limb so that the testing would be more certain (Muaja et al., 2013). Shrimp larvae toxicity test was performed as a preliminary study to observe the bioactivity potency and toxicity of each sample, so that the concentration of the extract which was safe for the test could be determined (Pradono et al., 2011).

Table 3. LC_{50} value of ethanolic extract of shrimp larvae

Concentration (µg/ml)	LC_{50} (%)
0	0
50	26.6
100	30
500	83.3
1000	100

Several researchers had performed the toxicity test using Brine Shrimp Lethality Test (BSLT). Kekuda et al. (2011) reported that isolate of *Streptomyces* origin from soil in Agumbe, Karnataka, India had LC_{50} values at 42.11 µg/ml with the highest concentration used was 1000 µg/ml with the percentage of mortality reached 100%. Tantithanagorngul et al. (2011) did initial screening of 459 isolates of *Streptomyces* origin from soil in Thailand. A total of 3 isolates were selected, namely 442, 449 and 145 (2010), they had a strong toxicity activity that is 10, 3.5 and 12.5 mg/ml respectively.

Pancreatic lipase inhibitory activity assay of extracts *Streptomyces* sp. AEBg12

Extract of *Streptomyces* sp. AEBg12 were tested for inhibitory activity against lipase. The result showed that ethanolic extract of *Streptomyces* sp. AEBg12 was able to produce the highest inhibition value compared to the ethyl acetate extract of *Streptomyces* sp. AEBg12 (Table 4). At a concentration of 1000 ppm, ethanolic extract of *Streptomyces* sp. AEBg12 was able to inhibit the activity of pancreatic lipase by 92.78%, while ethyl acetate extract of *Streptomyces* sp. AEBg12 inhibit by 65.28%. Ethanolic extract of *Streptomyces* sp. AEBg12 activity was higher compared with orlistat as a positive control that inhibit by 90.28%. This was because the number of secondary metabolites countained in ethanolic extracts of *Streptomyces* sp. AEBg12 were more compared to ethyl acetate extract of *Streptomyces* sp. AEBg12. It was probably due to the number of secondary metabolites of lipase inhibitor in ethanol extract was more compared to ethyl acetate extract. These results were in line with Pradono et al. (2010) which reported that ethanolic extract of tamarind leaves at a concentration of 150 ppm was able to inhibit the activity of pancreatic lipase enzymes to hydrolyze oleic acid by 49.0%. Ethanolic extract of tamarind leaves had the highest inhibitory activity than water extract of tamarind leaves and positive control that was act as an orlistat against human pancreatic lipase activity.

Table 4. Lipase inhibitory activity of various extracts of *Streptomyces* sp. AEBg12 and orlistat

Concentration µg/ml	Extract inhibitory (%)		Orlistat (%)
	Ethyl acetate	Ethanol	
100	18.89±1.73	38.33±3.63	40.56±1.92
250	29.44±1.27	57.5±1.67	47.22±1.27
500	33.33±1.67	66.67±1.67	71.11±2.68
750	52.5±0.83	82.78±2.68	75.56±1.27
1000	65.28±2.10	92.78±1.27	90.28±0.48

Mopuri & Meriga (2014) performed an extraction on *Terminalia paniculata* using various solvents. The results showed that ethanol extracts which was used to produce pancreatic lipase inhibitory activity had the highest content compared with extracts from another solvent with activity at 75%. Hadrich et al. (2014) also reported that ethanol and methanolic extracts of pomegranate skin were able to inhibit the activity of pancreatic lipase. The highest lipase inhibitor activity (100%) was obtained at a concentration of 1 mg/ml after 30 minutes of incubation. The study of Dzomba & Musekiwa (2014) showed that flavonoid produced from ethanolic extract of *Dioscorea steriscus* had a higher lipase inhibitor activity which was 95.88% compared to ethyl acetate and chloroform extract. Yuniarto et al. (2015) reported that ethanolic extract of *kumis kucing* leaves were able to inhibit pancreatic lipase up to 63.92% at 1000 µg/ml.

The results also showed that ethanolic extract of *Streptomyces* sp. AEBg12 at a concentration of 100 ppm could inhibit the pancreatic lipase up to 38.33%, while ethanolic extract of *Zingiber cassumunar* (bangle) at a concentration of 100 ppm could inhibit lipase inhibitor activity up to 29.17% (Iswantini et al., 2011). This result indicated that *Streptomyces* sp. AEBg12 had higher potency than the host plant, but further research was needed using the same substrate and enzyme.

The results also showed that the lower concentration of the extract, the lower its ability to inhibit pancreatic lipase enzyme activity and the higher concentration of the extract, the higher its ability to inhibit pancreatic lipase enzyme activity. These results were in line with other study which stated that ethanolic extract of *kumis kucing* leaves produced a higher inhibition percentage with the increases of concentration which were 38.55, 40, 44.26, 57.5 and 63.92% at concentrations of 0.1, 1, 10, 100 and 1000 ug/ml (Yuniarto et al., 2015). Based on these points, ethanolic extract of *Streptomyces* sp. AEBg12 had a greater effect on inhibition of pancreatic lipase activity, so it was possible to be used as an antiobesity drug.

In this study, IC_{50} values of each extract were determined. It indicated the concentrations of extract and orlistat. The results showed that the lowest IC_{50} values was obtained from ethanolic extract of *Streptomyces* sp. AEBg12 that was 180.83 µg/ml while IC_{50} values of ethyl acetate extract of *Streptomyces* sp. AEBg12 was 676.6µg/ml (Figure 1). IC_{50} value of ethanolic extract of *Streptomyces* sp. AEBg12 obtained was lower than orlistat (195.63µg/ml). The results showed that ethanolic extract of *Streptomyces*sp. AEBg12 had pancreatic lipase inhibitory activity which was higher compared to orlistat, so it was possible forfurther development as an antiobesity drug.

Broussonone A that were isolated from the stem barks of *Broussonetia kanzinoki* showed a noncompetitive inhibitory activity on pancreatic lipase with an IC_{50} of 28.4 µM (Ahn et al., 2012). Buthanol extract of *Streptomyces variabilis* strain of PO-178 produced pancreatic lipase inhibitor with IC_{50} of 44.32 mg/ml (Kekuda & Onkaroppa, 2014).The study of Adnyana et al. (2014) showed that ethanolic extract of pomegranate leaves inhibited pancreatic lipase with IC_{50} 20.64 ug/ml. *Syzygium aromaticum* extracts could inhibit pancreatic lipase with IC_{50} value of 0.015 mg/ml.

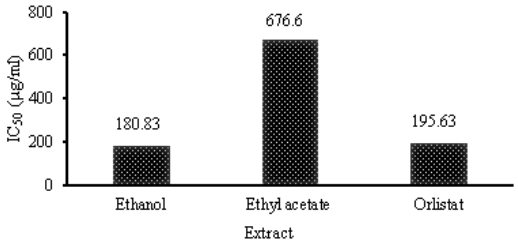

Figure 1. IC_{50} Values of extract ethanol, ethyl acetate of *Streptomyces* sp. AEBg12 and orlistat

Profile of TLC (Thin Layer Chromatography) of ethanolic extract *Streptomyces* sp. AEBg12

Ethanolic extract of *Streptomyces* sp. AEBg12 was further fractionated using thin layer chromatography (TLC). TLC was a method to analyze a mixture by separating the compounds which contained in the mixture. TLC method could be used to determine the number of components in a mixture, the identity and the purity of the compounds (Markham, 1988).

TLC testing on this study was also finding a mixed solvent that was able to separate active compounds contained in ethanol extract. From several experiments which has been conducted, eluent that wasable to separate the active components in the extract was indicated by separate bands formed from the result of chromatographic. The band produced from the elution of each eluent was examined under UV light at a wavelengths of 254 and 366 nm. The best eluent which wasable to separate active components of *Streptomyces* AEBg12 extract was a mixture of methanol:chloroform in the ratio of 9:1. This eluent could separate components contained in the extract into 4 bands with Retention factor (Rf) of 0.03; 0.65; 0.75; 0.76 (Table 5).

In this study, TLC of orlistat was also

performed using a mixture solvent containing methanol:chloroform in the ratio of 9:1. The result showed that *Streptomyces* AEBg12 and orlistat had a different color band. *Streptomyces* sp. AEBg12 had a blue luminescence band while orlistat has a green luminescence band, this indicated that *Streptomyces* sp. AEBg12 had different components compounds compared to orlistat (Figure 2). Detection of the components of ethanolic extract of isolate *Streptomyces* sp. AEBg12 was better observed at a wavelength of 366 nm than at 254 nm.

Table 5. The result of TLC to determine the best solvent

Solvent	Number of spot	Rf Value
Chloroform : n-hexane (9:1)	2	0.03; 0.60
n-hexane : ethyl acetate (2:8)	2	0,04; 0.65
n-hexane : ethyl acetate (3:7)	2	0.03; 0.65
Methanol : chloroform (9:1)	4	0.03; 0.65; 0.75; 0.76
Methanol : chloroform (8:2)	3	0.03; 0.60; 0.75
Methanol : chloroform (5:5)	2	0.04; 0.9
Methanol : chloroform (3:7)	3	0.07; 0,03; 0.9
Methanol : chloroform (2:8)	2	0.03; 0.65
Methanol : chloroform (1:9)	2	0.04; 0.65

According to Markham (1988), blue luminescence band showed on TLC plate when observed at a wavelength of 366 nm indicating several compounds: flavone, flavanones, flavonols and isoflavones. C-glycoside, a flavone found in leaves of *Eremochloa ophiuroides* could inhibit pancreatic lipase inhibitor with IC_{50} values range from 18.5 - 50.5 µM (Lee et al., 2010). Galangin, a flavonols found in *Alpinia galanga* was able to inhibit pancreatic lipase with IC_{50} 48.50 µM (Kumar & Alagawadi, 2013).

ImageJ program would convert the band showed on TLC plate into peaks showed in densitogram (Fereira & Rasband, 2012). A high peak indicating high luminescence color on the band on TLC plate. It could be seen that the peak of band 1 was higher than the other band.

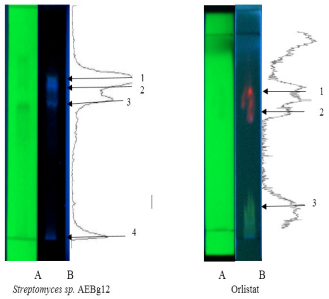

Figure 2. Visualization of thin layer chromatography of ethanolic extract of *Streptomyces* sp. AEBg12 and orlistat at a wavelength of A) 254 nm and B) 366 nm

Lipase inhibitor activity of tissue and host plant of *Streptomyces* sp. AEBg12

In this study, pancreatic lipase inhibitory activity produced by tissue culture endophyte-free bangle, bangle derived from the nature and endophytic actinobacteria were compared (Figure 2). The results were expected to give an overview of the role of the endophytic actinobacteria in producing lipase inhibitor.

The results showed that 2 months old plant tissue culture have a low capability to produce pancreatic lipase inhibitor compound compared to bangle derived from nature and *Streptomyces* sp. AEBg12. Inhibition value of plant tissue culture of bangle was 3.3%. Bangle derived from nature produced higher inhibition activity than the tissue culture bangle (23.9%), but it was lower compared to isolate AEBg12 (95.6%). The existence of endophytic actinobacteria in bangle which had the ability as a lipase inhibitor was in line with Tan & Zou (2001) which stated that endophytic microorganisms could produce certain phytochemicals similar to phytochemicals produced by the host plant, and it might be related to the evolution and gene transfer between the endophytic microorganisms with its host.

This results were in line with Pujiyanto (2012) which stated that endophyte-free plant which was obtained from 3 months old plant tissue culture have a low capability to produce inhibitor compound α-glucosidase (0.06%) compared with *Tinospora crispa* derived from nature (rod, 1.64; roots, leaves 3.39 and leaves, 4.52%). However, the ability of α-glucosidase inhibitor produced by endophytic actinobacteria BWA65 was higher over the host plant activity (10.98%).

Azadirachtin was a biopesticide produced by *Azadirachta indica* that were also found on endophytic fungi (Kusari et al., 2012). Azadirahtin were also detected in induced tissue culture from leaves explants (2.68% DM) at the age of 20 weeks and flower explant (2.48% DM) at the age of 12 weeks (Veeresham et al., 1998). The presence of pancreatic lipase inhibitor activity in plant tissue culture of bangle has not been reported.

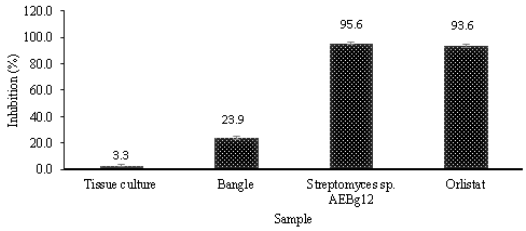

Figure 3. Activity of pancreatic lipase inhibitor produced by tissue culture of bangle, natural plants bangle, and *Streptomyces* sp. AEBg12.

Based on the data from this study, ethanolic extract of *Streptomyces* sp. AEBg12 is possibly a new compound that is able to be used as antiobesity drug through the approach to the pancreatic lipase inhibitor. The result also showed that endophytic actinobacteria from plants could produce the same secondary metabolite compounds as the host plants.

CONCLUSIONS

The results of this study concluded that the water extract of *Streptomyces* sp. AEBg12, endophytic actinobacteria in bangle contained flavonoids, saponins and steroids while ethanol and ethyl acetate extract contained flavonoids. IC_{50} value of ethanol and ethyl acetate extract were 180.9 µg/ml and 655.3 µg/ml respectively. IC_{50} value of ethanol extract was lower than orlistat that was 190 µg/ml. The result of TLC showed that ethanolic extract of *Streptomyces* AEBg12 had blue luminescence band indicated that there were either flavone, flavanones, flavonols or isoflavones. *Streptomyces* sp.AEBg12 produced inhibition value higher than bangle and plant tissue culture of bangle that was 95.6%.

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