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Short communication: Endophytic actinobacteria isolated from ginger (*Zingiber officinale*) and its potential as a pancreatic lipase inhibitor and its toxicity

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Abstract. *Rahayu S, Fitri L, Ismail YS. 2019. Short communication: Endophytic actinobacteria isolated from ginger (*Zingiber officinale*) and its potential as a pancreatic lipase inhibitor and its toxicity. Biodiversitas 20: 1312-1317.* Endophytic actinobacteria from ginger (*Zingiber officinale* Rosc.) is a bacterium that is capable of producing secondary metabolites that are the same as their hosts. This study aims to look at the potential of endophytic actinobacteria from ginger as a pancreatic lipase inhibitor and its toxicity. Endophytic actinobacteria were isolated, purified, then tested for pancreatic lipase inhibitors and their toxicity using the BSLT method (*Brine Shrimp Lethality Test*) and phytochemical tested on ethanol extract of selected isolates. Seven endophytic actinobacterial isolates were isolated from the ginger rhizome. The isolates had different morphological diversity based on colony and microscopic observations and 5 isolates had pancreatic lipase inhibitor activity. The highest inhibitors were found in AJ4 isolates (89.9%), compared with pancreatic lipase inhibitors crude extracts of ginger (68.9%) and orlistat (88.1%) as positive controls. The LC_{50} value of AJ4 isolates was 653,381 ppm and the value of LT_{50} was 17,569 hours. AJ4 isolates contain terpenoids, phenols, tannins, flavonoids, alkaloids, and saponins. This research data is considered as new information about the potential of endophytic actinobacteria from ginger as pancreatic lipase inhibitors and their toxicity.

Keywords: endophytic actinobacteria, lipase inhibitors, ginger, toxicity, *Zingiber officinale*

INTRODUCTION

Obesity is a disease caused by increased release of fatty acids, hormones and pro-inflammatory molecules that alter the metabolic and endocrine functions of adipose tissue (Weisberg et al. 2003). Obesity causes diabetes mellitus, hypertension, dyslipidemia, heart, lung, neurological disorders, and cancer (Mohamed et al. 2014). Some of them are taking drugs that can inhibit appetites such as sibutramine, hydroxycitric acid, oleoyl-estrone, and epigallocatechin gallate. Then using pancreatic lipase inhibitors such as lipstatin, panclicins, valilactone and hesperidin (Sukhdev and Singh 2013). However, these treatments have side effects for the body (Vasudeva et al. 2012). As a result of the many side effects of the drug, the community returns to traditional herbal medicine, namely using medicinal plants.

Ginger (*Zingiber officinale*) is a medicinal plant from the family Zingiberaceae and widely available in Indonesia. Ginger has irregular, branched rhizome characteristics, rough fibrous, radiating and the inside is pale yellow (Kurniasari et al. 2008). Ginger water extract can inhibit pancreatic lipase absorption (Han et al. 2005). This shows that ginger has an effect as antiobesity. Bioactive compounds taken directly from plants will require very much biomass or parts of the plants. An efficient way to obtain these bioactive compounds is to use endophytic microbes obtained from the inside of the plant. Endophytic microbes are expected to be able to produce a number of bioactive that are the same as their hosts without having to extract from plants (Simarta et al. 2007).

Actinobacteria can produce secondary metabolites similar to their host plants (Tan and Zou 2001). Actinobacteria has the potential to produce different bioactive metabolites including antimicrobial, anticancer, antidiabetic and other pharmaceutical compounds (Golinska et al. 2015). Therefore, it is necessary to do research on actinobacteria from ginger which has the potential as a pancreatic lipase inhibitor.

MATERIALS AND METHODS

Isolation of endophytic actinobacteria

The part of the medicinal plant used is the ginger rhizome. 1 g of ginger rhizome was washed and carried out surface sterilization by soaking 70% alcohol for 5 minutes, followed by 1% hypochlorite solution for 5 minutes, then rinsed with sterile distilled water and sterilized with 70% alcohol for 5 minutes. The ginger rhizomes were then crushed and taken 0.1 mL of liquid sample and inoculated on agar HV media, then incubated for 14-30 days at room temperature. Actinobacterial colonies that grow was purified using ISP 2 media (Taechowisan et al. 2003). The results of isolation were identified based on morphological criteria, namely the characteristics of the colonies above

the plates, the morphology of the substrate and hyphae, the pigments produced and Gram staining (Cao et al. 2004). Then the pancreatic lipase inhibition activity test was carried out and its toxicity.

Pancreatic lipase inhibition activity test.

Isolates were inoculated into a 200 mL Erlenmeyer flask containing 50 mL ISP 2 media actinobacterial colonization. Then incubated for 10 days in a shaker incubator at a speed of 100 rpm and a temperature of 30ºC. The isolates were then transferred into a microtube to be centrifuged at 6000 rpm for 30 minutes at 4ºC.

Activity testing uses the Etoundi et al (2010) method modified by Fitri et al (2017a). Substrate was made by adding 1% olive oil and 1% tween 80 into phosphate buffer at pH 8. The enzyme was made by weighing lipase enzyme (L3127 Sigma) as much as 0.1 g, then added PBS to 3 mL then homogenized with vortex. Wavelength measurements using a Microplate Spectrophotometer. The available microplate is included as many as 20 µL then added 20 µL enzyme, awaited 10 minutes later plus 80 µL of the substrate made three replications. Then the wavelength is measured at 450 nm. Then the solution was incubated for 30 minutes at 37ºC then measured the absorbance again. The absorbance obtained is converted to inhibition power.

The percentage of pancreatic lipase inhibition activity was calculated according to Muharni et al. (2013) using the following formula:

$$\% \text{ inhibition} = \frac{Ak - As}{Ak} \times 100$$

Where:
Ak = control absorbance
As = sample absorbance

Commercial slimming drugs, orlistat, were used as positive controls in this study. The orlistat solution was made with one orlistat capsule weighing 120 mg plus 120 mL Phosphate Buffer Saline (PBS), and then the solution was homogenized using 20 µL of vortex to be tested. Standard controls use PBS and negative controls use crude ginger extract. Crude ginger extract is made by washing the ginger rhizome and drying it. Then crushed and filtered to get the filtrate. A total of 20 µL of filtrate was tested for inhibition of pancreatic lipase activity as above.

Toxicity test using the BSLT method (Brine Shrimp Lethality Test)

The selected isolates actinobacterial samples were made concentrated by calculating concentrations of 10, 50, 100, 500 and 1000 ppm. The test sample was taken 1 mL according to the calculation of concentration and put in a test tube and then added 1 mL of seawater. Each concentration is made of three tubes (triplo). 10 Artemia larvae were put into the test sample then added seawater to a volume of 5 mL. The same treatment is carried out for the solvent of the test sample (buffer) which functions as a negative control. The number of dead and living larvae was observed and calculated and the LC_{50} and LT_{50} values were determined using probit analysis after 24 hours (Ningdyah et al. 2015).

Phytochemical test of actinobacterial bacteria

The phytochemical test was carried out to determine the secondary metabolite compounds possessed by selected isolates of ginger endophytic actinobacteria which were used as characteristic compounds of pancreatic lipase inhibitors. Among them are tests of flavonoids, alkaloids, saponins, steroids/terpenoids, and phenols/tannins (Harborne 1987).

Data analysis

Data from the research results were analyzed descriptively and displayed in the form of images and tables. Pancreatic lipase test results were seen based on the calculation of pancreatic lipase inhibition. The results of the toxicity test were calculated based on probit analysis LC_{50} and LT_{50}.

RESULTS AND DISCUSSION

Isolation of endophytic actinobacteria from ginger

A total of 7 actinobacterial isolates were successfully isolated from the ginger rhizome. Characterization of actinobacteria was carried out based on morphology, color of aerial mycelium, color of substrate mycelium, color of dissolved mycelium, and surface of colonies. Endophytic actinobacterial isolates obtained from ginger have diverse morphology. The AJ1 isolate has the color of aberrant mycelium and the substrate, which is cream, has the color of orange dissolved pigment and the surface of the convex colonies. The AJ2 isolate has the color of aerial celium and substrate, namely brown, does not have soluble pigments and is convex. The AJ3 isolate has the color of gray aerial mycelium, the color of the brown substance mycelium, does not have soluble pigments and is convex. Isolates AJ4, AJ5 and AJ6 have the color of white aerial mycelium, the color of the yellow substrate mycelium, do not have soluble pigments and are convex. The AJ7 isolate has the color of gray aerial mycelium, the color of the green substrate mycelium, has no soluble pigments and the surface of the colonies is convex (Table 1).

Table 1. Morphological characteristics of endophytic ginger actinobacteria on ISP 2 media

Isolate code	Color of aerial mycelium	Color of substrate mycelium	The color of the dissolved pigment	Surface of the colony
AJ1	Beige	Beige	Orange	Convex
AJ2	Chocolate	Chocolate	-	Convex
AJ3	Gray	Chocolate	-	Convex
AJ4	White	Yellow	-	Convex
AJ5	White	Yellow	-	Convex
AJ6	White	Yellow	-	Convex
AJ7	Gray	Green	-	Convex

Note: AJ1 = Endophytic Actinobacteria Ginger isolate 1; AJ2 = Endophytic Actinobacteria Ginger isolate 2; AJ3 = Endophytic Actinobacteria Ginger isolate 3; AJ4 = Endophytic Actinobacteria Ginger isolate 4; AJ5 = Endophytic Actinobacteria Ginger isolate 5; AJ6 = Endophytic Actinobacteria Ginger isolate 6; AJ7 = Endophytic Actinobacteria Ginger isolates 7

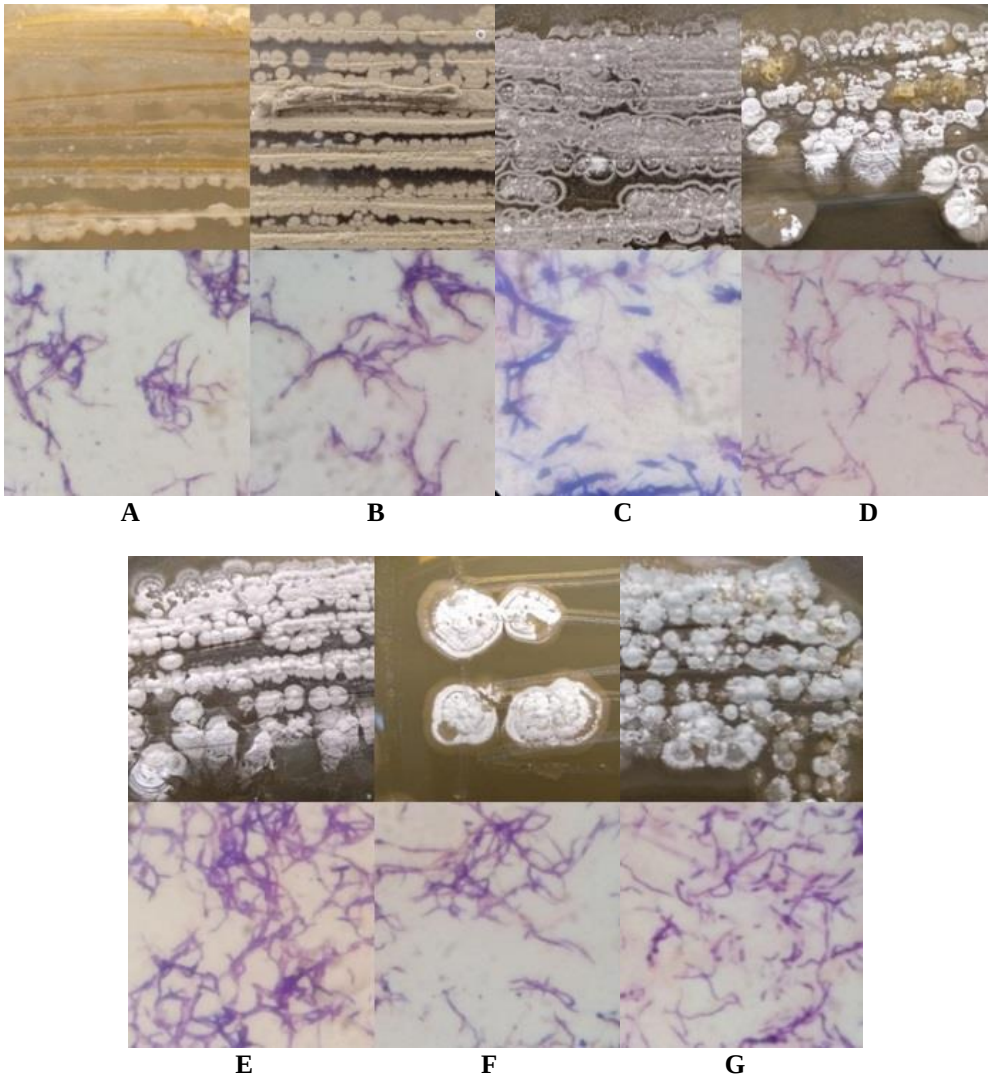

Figure 1. Colony and endophytic actinobacterial morphology of 1000x ginger enlargement. Type of spores: A. AJ1, biverticillate with spirals; B. AJ2, flexuous; C. AJ3, monoverticillate; D. AJ4, flexuous; E. AJ5, flexuous; F. AJ6, flexuous; G. AJ7, fascicled)

Endophytic actinobacterial isolates of ginger have four different types of spore chains, namely the type of biverticilliate with spiral, flexuous, monoverticillate without spiral and fascicled (Anandan et al. 2016). Isolate AJ1 has the form of a chain of biverticillate spores with spirals, isolates AJ2, AJ4, AJ5, and AJ6 have the same spore form, flexuous, AJ3 has monoverticillate spores and AJ7 has fascicled spores (Figure 1). Based on observations of morphology and characteristics of spores, the seven isolates were thought to be a group of the genus *Streptomyces.*

The *Streptomyces* genus dominates 50% of endophytic actinobacterial strains (Dinessh et al. 2017). The results of Fitri et al. (2017a) which isolated endophytic actinobacteria from medicinal plants, namely *A. galanga, K. Galanga, Z. Cassumunar, K. Rotunda* and *M. paniculata* which produced 35 isolates with different morphologies. The 5 selected isolates were genus from *Streptomyces*. The research conducted by Kim et al. (2012) who isolated actinobacteria from root samples in local herbal plants of Korean society. There are several genera found that dominate the roots of the herbal plants, namely *Streptomyces* (45.9%), *Micromonospora* (18.8%), *Rhodococcus* (6.6%), *Microbispora* (4.9%) and *Micrococcus* (4.9%). Other parts are found in the genus *Microbacterium*, *Streptacidiphilus*, *Arthrobacter*, *Dietzia*, *Kitasatospora*, *Herbiconiux*, *Mycobacterium*, *Nacardia*, *Rathayibacter*, and *Tsukamurella*. The isolation results obtained 49 isolates consisting of 4 genera namely *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardiodes* spp. Sheng et al. (2009) isolated endophytic actinobacteria from medicinal plants collected from tropical rainforests in Xishuangbanna, China. There are 2,174 endophytic actinobacterial isolates from isolation using different selective media. A total of 46 isolates were selected based on morphology in the media obtained by 32 genera.

Activity of pancreatic lipase inhibitors

Testing of pancreatic lipase inhibitor activity from 7 endophytic ginger actinobacterial isolates produced 5 isolates that had pancreatic lipase activity. The 5 isolates that had the ability to inhibit pancreatic lipase were isolated AJ1, AJ2, AJ4, AJ6 and AJ7 with each inhibition of (50.8%), (77.4%), (89.8%), (71.8 %) and (42%) (Figure 2). Isolates that do not have the ability as pancreatic lipase inhibitors are isolated AJ3 and AJ5.

The inability of these isolates to produce pancreas lipase inhibitors is thought to be because these isolates do not have secondary metabolites to be able to become pancreatic lipase inhibitors. The highest pancreatic lipase inhibitors were found in AJ4 isolates, which amounted to 89.8%. This high ability in pancreatic lipase inhibitors is suspected because these isolates have secondary metabolite compounds with high levels of compounds that can inhibit pancreatic lipases in large quantities.

This test uses the ginger rhizome as a comparison and orlistat as a positive control. In the crude extract of ginger rhizome, pancreatic lipase inhibitor activity was 68.5%. Three isolates produced higher pancreatic lipase inhibitor activity than crude ginger extract. The isolates were AJ2, AJ4, and AJ6 isolates. Whereas AJ1 and AJ7 isolates produced lower inhibitor values than crude ginger extract. This shows that some of the ginger endophytic actinobacterial isolates have a higher ability to produce inhibitory values compared to their host plants (Figure 2). This result is in accordance with the study of pancreatic lipase inhibitors conducted by Fitri et al. (2017b), where AEBg12 isolates from the bangle plant have higher pancreatic lipase inhibitors than their host plants. The resistance value of the AEBg12 isolate obtained was 95.6%. Whereas with bangle plants produce pancreatic lipase inhibitors of 23.9%.

In this study, positive controls used orlistat (commercial slimming drugs commonly consumed by the public). The activity value of pancreatic lipase in orlistat solution was 88.1% with standard deviation was 36,37. The value of activity in orlistat was lower than the activity value of AJ4 isolates (89.8%). Based on the highest value of pancreatic lipase activity in this test, the AJ4 isolate is considered the best isolate and has high potential as a pancreatic lipase inhibitor. The results of Weibel et al. (1987) stated that the isolation of *Streptomyces toxytricini* produced Lipstatin which acts as an inhibitor of pancreatic lipase. The resistance produced by Lipstatin is quite strong and is considered a new source of pancreatic lipase inhibitors.

Orlistat is a pharmacological agent for weight loss through inhibition of gastric and pancreatic lipases (Shalaby et al. 2014). In 1988 it was approved and considered as the only drug available for long-term control of obesity (Hadvary et al. 1988). Although orlistat has been shown to be able to induce significant weight loss, it has cardiostimulant and gastrointestinal side effects (Chaput et al. 2007). That's why it's very important to find new inhibitors that are sourced from nature (Seyedan et al. 2015).

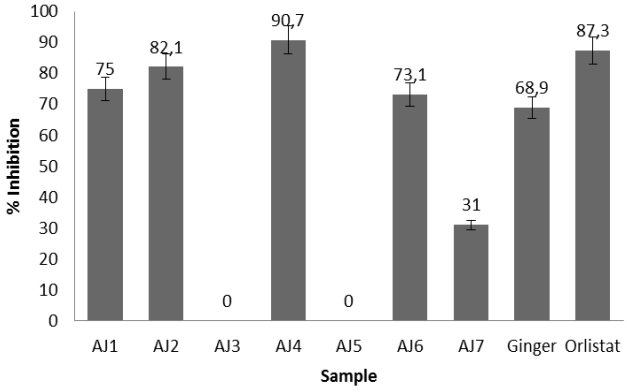

Figure 2. Graph of mean pancreatic lipase activity of endophytic ginger actinobacterial isolates, crude ginger and orlistat extract

Toxicity

The toxicity test of the AJ4 isolate ethanol extract was carried out based on the BSLT method. Based on table 2 shows that the AJ4 isolate had an LC_{50} value of 653,381 ppm. This shows that the AJ4 isolate at extract concentration of 653,381 ppm was able to kill half the population of *Artemia salina* shrimp larvae. The results of Iswantini et al. (2010) on the extracts of gelugur acid (*Garcinia atroviridis),* galangal (*Alpinia galanga)* and kencur (*Kaempferia galanga)* as pancreatic lipase inhibitors showed LC_{50} values of each extract of 103.4 ppm, 1445.5 ppm, and 47.974 ppm. According to Meyer et al. (1982) the higher the level of toxicity of secondary metabolites of a compound, the more potential the compound is represented by the smaller LC_{50} value.

If further testing is carried out to create a new drug, especially at the stages of being safely absorbed in the human body. Then the test must be below the concentration of the LC_{50} value. The results of the toxicity test will be used to determine the concentration limits of extracts in the test of its activity as pancreatic lipase inhibitors (Iswantini et al. 2010). AJ4 isolate is toxic. However, the range of toxic numbers in isolates is classified as moderate. This shows that both isolates have the potential to be used as anti-obesity drugs. According to Meyer et al. (1982), an extract is considered very toxic if it has an LC_{50} value below 30 µg/mL, is considered toxic if it has an LC_{50} value of 30 to 1000 ppm, and is considered not toxic if it has an LC_{50} value exceeding 1000 ppm. LT_{50} is the Median Lethal Time, which is the time needed to cause the death of 50% of the population of test animals. The LT_{50} value of the AJ4 isolate was 17,569. This shows that the AJ4 isolate was able to kill 50% of the test larvae well at that concentration.

Phytochemical AJ4 isolate

AJ4 isolates were selected isolates from this pancreatic test which were then tested for phytochemical tests. The results of phytochemical tests (Table 3) of AJ4 isolates showed results of terpenoid compounds, phenols, tannins, flavonoids, alkaloids and saponins.

Table 2. Tables of LC_{50} and LT_{50} values

Isolate code	LC_{50} (ppm)	LT_{50} (hour)
AJ4	653,381	17,569

Table 3. Phytochemical results of endophytic ginger actinobacterial isolates and ginger rhizomes

Completely agree	Isolate AJ4	Ginger rhizomes (Agustina et al. 2016)
Steroid	-	+
Terpenoid	+	+
Fenol	+++	+
Tanin	+++	-
Flavonoid	++	+
Alkaloid	+	+
Saponin	++	+

Note: -: Not detected; +: Detected; ++: Moderately detected; +++: Detected a lot

AJ4 isolates contain flavonoids, and this composition is also found in host plants, ginger. Flavonoid compounds inhibit compounds that can inhibit pancreatic lipase inhibitors. According to Iswantini et al. (2010), flavonoids are secondary metabolites which are thought to inhibit pancreatic lipase inhibitors. This is based on his research which contains pancreatic lipase inhibitors in gelugur acid (*Garcinia atroviridis*), galangal (*Alpinia galanga*) and kencur (*Kaempferia galanga*) which produce research on flavonoids. The results of the study by Fitri et al. (2017b) also showed *Streptomyces* sp. AEBg12 from Bangle (*Zingiber cassumunar*) which produces pancreatic lipase inhibitors, produces a flavonoid composition in the three extracts distributed. The results of a study conducted by Han et al. (2005) show how ginger extract can inhibit fat in the pancreatic in vitro test using ginger water extract. Although there are differences in AJ4, flavonoids have a role in inhibiting pancreatic lipase inhibitors.

Dinessh et al. (2017) state that endophytic actinobacteria can produce secondary metabolites by inducing physiological and biochemical changes directly in the host plant. Endophytic actinobacteria also benefit host plants. The mechanism occurs after actinobacteria infect plant tissues, then form a relationship where plants and actinobacteria are mutually beneficial. Plants provide nutrients, and protection from other bacteria. Then actinobacteria provide certain functional metabolites that can encourage plant growth and provide plant protection against pathogens that can interfere with plant growth.

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