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Pancreatic Lipase Inhibitory Activity of Endophytic Actinobacteria from *Rhododendron* spp.

Witri Winanda[1], Irmanida Batubara[2,4], Yulin Lestari[3,4]*

[1]Graduate School, IPB University, Indonesia
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Indonesia
[4]Tropical Biopharmaca Research Center, IPB University, Indonesia
*Corresponding Author: yulinl@apps.ipb.ac.id

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Abstract. Antiobesity medication is available as therapeutic compounds that can reduce fat digestion by the inhibition of pancreatic lipase. Actinobacteria have the potency as source of bioactive compounds with various biological function including as pancreatic lipase inhibitor. However, the potency of endophytic actinobacteria from *Rhododendron* spp. as source of pancreatic lipase inhibitor producer has not been reported yet. The aim of this study was to examine the potential of pancreatic lipase inhibitory activity of 23 endophytic actinobacteria from *Rhododendron* spp.; to characterize their colony based on morphology and molecular analysis. Screening test of pancreatic lipase inhibitor was conducted using the supernatant of endophytic actinobacteria, lipase pancreatic porcine (L3126) and p-nitrophenyl butyrate. The supernatant of selected isolates was extracted using ethyl acetate. The result showed that various inhibitory activities ranging between 0.00 until 91.69%. There were 11 out of 23 isolates that have potential as pancreatic inhibitor. Amongst them, the extract of four selected isolates, i.e. RZP 1.3, RSSB 3.2, RSS 2.1, and RJB F3.2 demonstrated inhibitory percentage of more than 80%. The RJB F3.2 extract showed to have IC_{50} value by 431.48 µg mL^{-1} compared to control, i.e. Xenical (89.07 µg mL^{-1}). Phytochemical analysis exhibited that the extract of the selected isolates contained alkaloid which may function as pancreatic lipase inhibitor. Based on the morphological character, the selected isolates have various morphological colonies and 16S rRNA gene sequence revealed the sequence homology to *Streptomyces* spp. The data clearly indicate that endophytic actinobacteria from *Rhododendron* spp. have potency as pancreatic lipase inhibitor producer and further studies could be explored for the development of antiobesity agent.

Key words: actinobacteria; IC_{50}; pancreatic lipase inhibitor; 16S rRNA; *Rhododendron* spp.

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INTRODUCTION

Fat plays an important role as source of energy. In this case, dietary fats represent the major source of unwanted calories. However, excessive fat intake in absence of physical activities may induce obesity due to fat accumulation in the body as a result of imbalance of energy intake and consumption (Hofbauer, 2002). Obesity is an imbalance condition between energy intake and expenditure. Obesity constitutes one of the main health concerns causing deleterious problems such as type 2 diabetes, cardiovascular disease, and cancer (Sukhdev & Singh, 2013). Fat from food is hydrolyzed by lipase (triacylglycerol acyl hydrolase EC 3.1.1.3), enabling to convert long chain fatty acid into fatty acids and glycerols (Mukerjee, 2003). Pancreatic lipase is a primary enzyme in fat degradation since its hydrolytic activity reaches 50-70%. This provokes an idea to manage obesity through the involvement of pancreatic lipase inhibitor (Sukhdev & Singh, 2013). The principle of this inhibitor is to reduce fat absorption by suppressing activity of pancreatic lipase (Weigle, 2003).

Some of medicinal plants have the opportunity to be used as antiobesity agents. Ethanol extract of *Guazuma ulmifolia*, *Zingiber cassumunar* and *Murraya paniculata* were reported to contain flavonoid, steroid, triterpenoid, saponin, and tannin that used as inhibitor of pancreatic lipase (Iswantini et al., 2011). Meanwhile, bioactive compounds of *Rhododendron* were found to be flavonoid, tannin, and saponin which previously used as anti-inflammatory agent (Verma et al., 2010). In addition, ethanol extract of *Rhododendron arboreum* flowers was reported to show antihiperlipidemic activity through decreasing level of glycerol and triglyceride (Verma et al., 2012).

Secretion of bioactive compounds from plant closely relates to existence of endophytic microorganisms. They colonize plant tissues without having negative impact on the host. Endophytic microorganisms such as bacteria and fungi are known capable of producing the same metabolites compounds as their host plants (Strobel & Daisy, 2003; Tan & Zou, 2001). Bacteria specifically from endophytic actinobacteria are source of bioactive compounds. Pujiyanto et al. (2012) reported that

BWA65, endophytic actinobacteria of *Tinospora crispa* was potential as the source for α-glucosidase inhibitor, where the ethyl acetate extract of BWA65 demonstrated the highest inhibition by 77.8%. Meanwhile, the supernatant of other endophytic actinobacteria, i.e. AEBg12 from *Zingiber cassumunar* exhibited high inhibitory pancreatic lipase, reaching up to 95.3% (Fitri et al., 2017).

Bioactive compounds from endophytic actinobacteria have been showed to have antibacterial activity (Dehnad et al., 2010). Recently, endophytic actinobacteria from *Rhododendron* spp. were reported as antibacterial agent (Fitriandini et al., 2017). However, scientific reports on endophytic actinobacteria of *Rhododendron* spp. as pancreatic lipase inhibitor has not been explored. Therefore, this work aimed to perform *in vitro* experiment in order to evaluate pancreatic lipase inhibitory activities, characterization of macroscopic and microscopic morphological colony as well as molecular identification of endophytic actinobacteria from *Rhododendron* spp. The capability of endophytic actinobacteria *Rhododendron* spp. reported here can be considered as a new information. These may also contribute to the further development of finding antiobesity agent using microbial-based product.

METHODS

Endophytic actinobacteria samples

A total of 23 isolates of endophytic actinobacteria were isolated from 6 species of *Rhododendron* (*R. sessilifolium, R. javanicum* from Jambi, *R. javanicum* from Java, *Rhododendron* sp., *R. jaka*, and *R. zoelleri*) available as author's isolate collection. The plant samples were originally obtained from Cibodas Botanical Garden, West Java, in collaboration with Indonesian Institute of Sciences. Actinobacteria were sub-cultured on International *Streptomyces* Project 4 (ISP4).

Screening of pancreatic lipase inhibitor activities

All of endophytic actinobacterial isolates were inoculated into 30 mL of liquid ISP2 medium. Isolate was incubated in a rotary shaker (120 rpm) at room temperature for 10 days. Cell biomass was removed using centrifuge (HERMLE Labortechnik GmbH Z 326 K) at 2500 ×g for 20 minute to obtain supernatant for further test.

Pancreatic lipase of porcine L3126 (6 mg) was dissolved in 10 mL of buffer phosphate saline (0.1 M buffer phosphate saline at pH 7.2, 0.15 M NaCl and 0.5% Triton-X-100). Substrate p-nitrophenyl butyrate P-NPB (8.40 µL) was dissolved in 10 mL of acetonitrile. The reaction mixture with three replications consisted of P-NPB (25 µL), lipase (50µL), supernatant (25 µL), and buffer phosphate saline (100 µL) (Chedda, et al., 2016). Xenical was applied as positive control, while blank solution (in absence of supernatant) was used as negative control. The mixture was homogenized and incubated at 37°C for 30 min. Absorbance was determined using ELISA reader at 400 nm. Inhibitory activity of pancreatic lipase was calculated as follows:

$$\text{Inhibition (\%)} = \frac{[(AB-ABC)-(AS-ASC)]}{AB-ABC} X100\%$$

Note: AB = absorbance of blank, ABC = absorbance of blank control, AS = absorbance of sample, ASC = absorbance of sample control

Extraction and determination of IC_{50} from ethyl acetate extracts of endophytic bacteria from *Rhododendron* spp.

Extraction was performed at ratio of 1:1 (supernatant:ethyl acetate), and concentrated using rotary evaporator at 45°C. IC_{50} value was determined to know the concentration of ethyl acetate extract responsible for 50% of inhibition against pancreatic lipase activity. The extract was dissolved in dimethyl sulfoxide (DMSO) and tested at extract concentration of 62.5, 125, 250, 500, 1000 µg mL^{-1}. Based on correlation between percentage inhibition and concentration of samples, the IC_{50} was determined.

Phytochemical test from ethyl acetate extracts of endophytic bacteria from *Rhododendron* spp.

Phytochemical test was conducted based on Harbone (1987) to obtain types of bioactive compounds generated by endophytic bacteria from *Rhododendron* spp. As in the flavonoid test by adding extract with 0.1 g of ethyl acetate dissolved in water and chloroform (1:1), 1 mL HCl, and 1 mL amyl alcohol, then homogenized. If the amyl alcohol layer has an orange color, the extract indicates that it contains flavonoid. For saponin test, the bottom layer was filtered then followed by adding aquadest and heated for 5 min, then homogenized. The foam formation indicates saponin positive. Tannins test was done by diluting 0.1 g of ethyl acetate extract in 10 mL aquadest, then heated; Iron (III) Chloride was added to 1 mL of the filtrate, then blue color indicates that it contains tannins. For steroid and terpenoid test, the bottom layer was filtered and dried. The Liebermann-Burchard reagen was added to the filtrate. For steroid test, the solution has green or blue color, while if red or purple color indicates that it contains terpenoid.

Morphological characterization

The colony morphology of endophytic actinobacteria were observed by growing them in ISP2, ISP3, ISP4, and Yeast Starch Agar (YSA)

media for 14 days incubation at room temperature (25-28^{o}C). Macroscopic features were observed, i.e. the color of aerial and substrate mycelia and diffusible pigment; while arrangement of spores chain was observed under light microscope at magnification of 400×.

Molecular identification based on 16S RNA

DNA genome of 16S rRNA gene of endophytic actinobacteria was isolated using protocol of Genomic DNA Mini Kit (Blood/Cultured cell) (Geneaid; Taiwan). DNA purity after isolation was evaluated using Nanodrop. The quantified DNA was then amplified using PCR technique with GoTaq Green Mastermix (Promega). Specific primer for 16S rRNA gene of actinobacteria 27F used 27F (5"AGAGTTTGATCCTGGCTCAG -3") and 16Sact 1114R (5"GAGTTGACCCCGGCRGT-3") (Martina, et al., 2008). Amplification product of 16S rRNA gene was sequenced according to standard protocol of DNA sequencer service company. Base sequence was corrected using Sequence Alignment Editor in MEGA 7.0. All sequences of 16S rRNA gene were compared to database EzTaxon (https:www.ezbiocloud.net/). Topology of phylogenetic tree was evaluated using neighbour-joining method (p-distance analysis) with bootstrap at 1000 replications.

Data analysis

A linear regression analysis to determine the IC_{50} value was calculated using Microsoft Excel 2010. All data of inhibition percentage and IC_{50} value were analyzed using SPSS 22.0 software, ANOVA test One-Way with level of significance α=0.01.

RESULTS AND DISCUSSION

The pancreatic lipase inhibitor activity of endophytic actinobacteria

The supernatants of endophytic actinobacteria were analyzed for inhibitory activity of pancreatic lipase. As presented in table 1, there was variance at inhibition percentage, ranging from 0% until 91.69%. Additionally, 11 out of 23 isolates were potentially recorded as pancreatic lipase inhibitor, while the remaining isolates did not exhibit inhibitory activities. From 11 isolates, there were four isolates that demonstrated high inhibitory percentage, i.e. RZP 1.3, RSSB 3.2, RSS 2.1 and RJB F3.2.

Table 1. Screening of 23 endophytic actinobacteria from *Rhododendron* spp. as pancreatic lipase inhibitors

Isolate	Inhibition (%)	Isolate	Inhibition (%)
RJB F1.2	27.02± 2.40^{d}	RZP 2.2	0.00^{a}
RJB F1.10	0.00^{a}	RZPB 1.1	0.00^{a}
RJB F3.2	85.15 ± 4.40^{f}	RZPB 4.1	0.00^{a}
RJJB 1.2	0.00^{a}	RZPB 7.1	0.00a
RJJB 2.2	19.94 ± 3.60^{c}	RSS 2.1	88.57 ± 4.90^{fg}
RJJB 3.1	0.00^{a}	RSS 4.3	0.00^{a}
RJJB 3.2	12.67 ± 4.30^{b}	RSS 5.3	0.00^{a}
RJJM B1.3	22.30 ± 2.40^{c}	RSPB 6.1	0.00^{a}
RJJMB3.3	28.42 ± 5.80^{d}	RSSB 3.2	90.49 ± 0.26^{g}
RJJM B6.1	35.24 ± 6.50^{e}	RBJBB 5.1	36.04 ±1.51^{e}
RZP1.1	0.00^{a}	RBJBB 1.1	0.00^{a}
RZP 1.3	91.69 ± 0.36^{g}		

Description: Different superscript letters indicate significant different values (One-Way ANOVA, p<0.01)

Pancreatic lipase is involved in fat metabolism. Thus, inhibition of pancreatic lipase was used in obesity management, enabling to retard fat hydrolysis. In this work, the inhibition was based on absorbance of p-nitrophenol (P-NPB), in which the color changed into yellow after its degradation (Gupta et al., 2014). The reduction of enzymatic activity could be represented by a reduced product concentration, as indicated by changes in color and absorbance detected at wavelength of 400 nm. The secretion of bioactive compounds by each type of actinobacteria may differ in its bioactivity. At initial screening using the supernatant, RZP 1.3 demonstrated the highest inhibition up to 91.69%. Bioactive compounds flavonoid, alkaloid (Ono et al., 2006), saponin (Karu et al., 2007), and triterpenoid (Li et al., 2007) capable of inhibiting pancreatic lipase are included as secondary metabolite.

Ethyl acetate extract inhibition of potential endophytic actinobacteria and its phytochemical compounds

Yields of four isolates are presented in table 2. The yield could represent the abundance of bioactive compounds successfully extracted by solvent. Ethyl acetate extract of RJB F3.2 was found to have the highest yield percentage of 0.014%, indicating that 0.014% of isolate was extractable. The extract was

then analyzed for IC_{50} value. RJB F3.2 showed inhibition percentage towards pancreatic lipase by 50% at the lowest concentration (431.48 µg mL^{-1}) compared to other isolates. Meanwhile, IC_{50} of Xenical demonstrated the 50% inhibition at concentration of 89.07µg mL^{-1}.

Table 2. Yield and IC_{50} value of ethyl acetate extracts of endophytic actinobacteria from *Rhododendron* spp.

Isolate	Yield (%)	IC_{50} (µg mL^{-1})
Xenical (control)	-	89.07 ± 7.00^{a}
RJB F3.2	0.014	431.48 ± 24.24^{b}
RZP 1.3	0.005	616.57 ± 33.95^{c}
RSS 2.1	0.002	607.76 ± 14.09^{c}
RSSB 3.2	0.011	880.73 ± 24.89^{d}

Description: Different superscript letters indicate significant different values (One-Way ANOVA, $p<0.01$)

The IC_{50} of positive control was much lower in comparison with *Rhododendron* spp. ethyl acetate extracts. This is caused to the difference in purity of bioactive compounds, in which Xenical possessed better purity than crude extracts. *Streptomyces* was reported to exert significant contribution to pancreatic lipase inhibition. Singh et al. (2017) reported that *S. tendae* was found to be active as pancreatic lipase inhibitor with IC_{50} value of 147.58 µg mL^{-1}, which was higher than Orlistat as positive control (IC_{50} value of 0.65 µg mL^{-1}). Other cases showed that pancreatic lipase inhibitor from *Streptomyces* soil isolate which showed 61.67% with 50 mg mL^{-1} butanol extract concentration (Kekuda et al., 2011).

In this experiment, alkaloid was found in the four ethyl acetate extracts examined which might indicated its dominancy compared to flavonoid, saponin, steroid, and terpenoid (Table 3). Dechakhampu and Wongchum (2015) reported that flavonoid and alkaloid compounds from medicinal plants (*Memecylon edule* Roxb., *Garcinia vilersiana* Pierre., *Cryptolepis elegans* Wall., and *Phyllanthus chamaepeuce* Ridl.) demonstrated a strong inhibition against pancreatic lipase. On the other hand, from endophytic actinobacteria of *Zingiber cassumunar*, flavonoid compounds were found and could be a promising candidate as inhibitor of pancreatic lipase (Fitri et al., 2017).

Table 3. Bioactive compounds from ethyl acetate extracts of endophytic actinobacteria from *Rhododendron* spp.

Bioactive compounds	Isolate			
	RJB F3.2	RZP 1.3	RSS 2.1	RSSB 3.2
Alkaloid	+	+	+	+
Flavonoid	+	-	+	+
Saponin	+	-	-	+
Steroid	-	-	-	+
Terpenoid	+	-	+	-
Tannin	-	-	-	-

Description : (+) detected, (-) not detected

Morphological diversity of endophytic actinobacteria

The four selected isolates showed different morphological appearances. The aerial mycelia existed as white to gray and substrate mycelia was displayed as yellow to brown in some medium (Figure 1). All of isolates did not produce the pigment dissolved in four types of test medium.

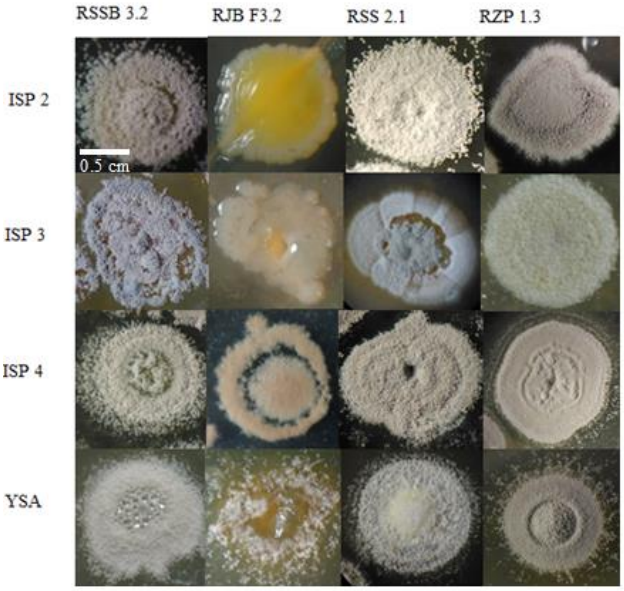

Figure 1. Macroscopic morphological colony of endophytic actinobacteria *Rhododendron* spp. grown in ISP2, ISP3, ISP4, and YSA for 14 days

Actinobacteria produce a variety of pigments responsible for the color of substrate and aerial mycelia (Flärdh & Buttner, 2009). The dissimilar appearance of aerial and substrate mycelia may result from biotic and abiotic conditions, including age of actinobacteria, temperature, pH, and nutrition (Whitman et.al., 2012). Microscopic identification was used to observe spore diversity of endophytic actinobacteria *Rhododendron* spp. The three isolates (RSSB 3.2, RZP 1.3, and RSS 2.1) demonstrated spiral spore chain, while RJB F3.2 showed

retinaculum-apertum spores (open loops) (Madigan et al., 2015). Based on their morphological data, all the isolates have the *Streptomyces* character.

Molecular identity of potential endophytic actinobacteria based on 16S rRNA gene

Sequence data of 16S rRNA gene showed that RJB F3.2 was similar to *Streptomyces fradiae* strain DSM 40063 (100% similarity), *S. coeruleoprunus* NBRC 15400 (99.22% similarity), and *S. somaliensis* DSM 40738 (98.83% similarity). On the other hand, three other isolates RZP 1.3, RSS 2.1, and RSSB 3.2 showed similarity to *S. tendae* ATCC 19812 (99.90%), *S. rubrogriseus* LMG 20318 (99.90%), and *S. tritolerans* DAS 165 (99.81%) (Table 4). These findings were confirmed with the phylogenetic tree construction for each isolate that existed in the same clade with their homologue (Figure 2).

Table 4. The similarity of sequences on 16S rRNA gene from endophytic actinobacteria *Rhododendron* spp.

Isolate code	Homology	Similarity (%)	Accession number
RJB F3.2	*S. fradiae* DSM 40063	100	MIFZ01000280
	S. coeruleoprunus NBRC 15400	99.22	AB184651
	S. somaliensis DSM 40738	98.83	AJ0077403
RZP 1.3, RSS 2.1 and	*S. tendae* ATCC 19812	99.90	D63873
RSSB 3.2	*S. rubrogriseus* LMG 20318	99.90	AJ781374
	S. tritolerans DAS 165	99.81	DQ345779

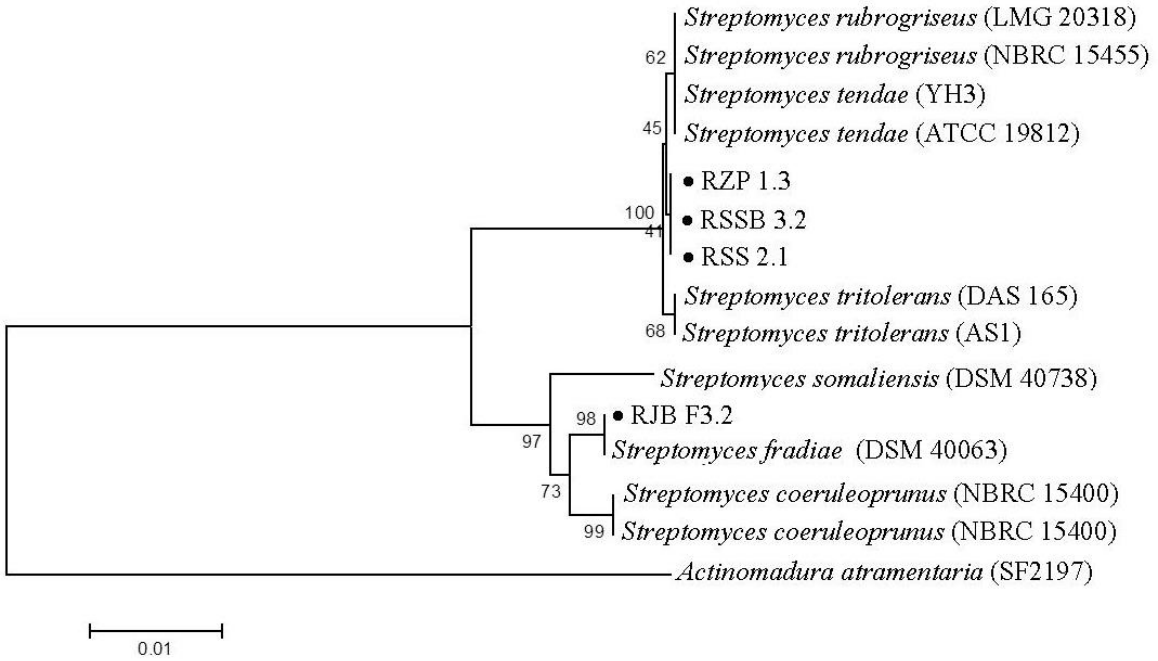

Figure 2. Neighbour-joining (p-distance analysis) of 16S rRNA gene of endophytic actinobacteria *Rhododendron* spp. (bootstrap 1000 replications)

Based on the of phylogenetic tree with considering p-distance analysis, RJB F3.2 was closely related to *S. fradiae* DSM 40063 (100%). Bioactive compounds from *S. fradiae* was reported as antibiotics. Tylosin, a macrolides antibiotic can be produced by *S. fradiae* (Bate, et al., 2000). Meanwhile, three other isolates RZP 1.3, RSS 2.1, and RSSB 3.2 were closely related to *S. tendae* and *S. rubrogriseus* (99.90%). Recently, *S. tendae* have been reported to have potential as pancreatic lipase inhibitors (Singh et al., 2017). Species that have similarity of > 99.0% to *S. tendae* showed to have antimicrobial activity (Laidi et al., 2006).

Based on morphological character and molecular identity, the four actinobacterial isolates belong to *Streptomyces* spp. However, phenotypic and molecular identification of 16S rRNA gene may be insufficient to prove that actinobacterial isolates are identical to homologue species. Hence, more specific approach is required, such as polyphasic taxonomy (Ramasamy et al., 2014). Sequence homology of 16S rRNA gene that was performed similarity of < 97% could be a novel species (Stackebrandt et al., 2002). Meanwhile, the other taxonomists stated that performed similarity of > 97% could be a different species (Prakash, et al., 2007).

Streptomyces spp. have been studied as pancreatic lipase inhibitor. Butanol extract from *S. variabilis* strains PO-178 could be used as pancreatic lipase inhibitor with IC_{50} value of 44.31 mg mL^{-1} (Kekuda et al., 2014). *Streptomyces tendae* is also recognized as of pancreatic lipase inhibitor with IC_{50} value of 147.58 µg mL^{-1} (Singh et al., 2017). Ethyl acetate extract of endophytic actinobacteria from *Rhododendron* spp. have the potency to be used as antiobesity agent. The data obtained from this work clearly indicate that endophytic actinobacteria from *Rhododendron* spp. have the potency to be developed as antiobesity agent. This new information, can be used as a reference for developing pancreatic lipase inhibitor compounds in the future.

CONCLUSION

The activity of pancreatic lipase inhibitor from 23 supernatant of endophytic actinobacteria from *Rhododendron* spp. showed to have percent inhibition varied between 0% until 91.69%. The ethyl acetate extract from the four selected isolates, i.e. RZP 1.3, RSSB 3.2, RSS 2.1 and RJB F3.2 have the pancreatic lipase inhibitory activity of > 80%. The ethyl acetate extract of those isolates qualitatively contained alkaloid. Ethyl acetate extract of RJB F3.2 has the lowest IC_{50} values of 431.48 µg mL^{-1} compared to three other isolates examined. Based on colony morphology and sequence data of 16S rRNA gene, the four isolates have similarity to *Streptomyces* spp.

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