

Original Article

METABOLITES ACTIVITY OF ENDOPHYTIC *STREPTOMYCES* SP. IPBCC. B.15.1539 FROM *TINOSPORA CRISPA* L. MIERS: α -GLUCOSIDASE INHIBITOR AND ANTI-HYPERGLYCEMIC IN MICE

YULIN LESTARI^{a,d}, YESSY VELINA^b, MIN RAHMINI WATI^{c,d}

^aDepartment of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Campus IPB Dramaga, Bogor 16680, Indonesia, ^bDepartment of Biology, Faculty of Education, Raden Intan University, Lampung 35131, Indonesia, ^cDepartment of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University, Campus IPB Dramaga, Bogor, 16680, Indonesia, ^dBiopharmaca Research Center, Bogor Agricultural University, Campus IPB Taman Kencana, Bogor 16151, Indonesia
Email: yulinlestari@gmail.com

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ABSTRACT

Objective: This research work aimed to assess the capability of endophytic *Streptomyces* sp. IPBCC. b.15.1539 isolated from *Tinospora crispa* in producing α -glucosidase inhibitor compound and examined the effect of its ethyl acetate extract containing α -glucosidase inhibitor in lowering blood glucose in streptozotocin mice.

Methods: *Streptomyces* sp. IPBCC. b.15.1539 was grown in a bioreactor filled with International *Streptomyces* Project 2 medium, for 5, 10, 15, and 20 days, and assayed for its *in vitro* α -glucosidase inhibitory activity. The ethyl acetate extract was examined for its IC₅₀ value. An *in vivo* experiment was set up using thirty mice, which were divided into five treatment groups: (a) acarbose (0.03 mg/30 g body weight) used as a positive control, (b) placebo used as a negative control, (c-e) treatment groups were treated with ethyl acetate extract at 0.036 mg/30 g body weight (P1), 0.36 mg/30 g body weight (P2), 0.036 mg/30 g body weight (P3).

Results: The crude extract produced 98.5% α -glucosidase inhibitory activity, with 15,6 biomass after 10 days of production. The ethyl acetate extract at a concentration of 1000 μ g/ml produced 96.08% α -glucosidase inhibitory activity, while acarbose at the same concentration gave 97.46% inhibition. The IC₅₀ for the ethyl acetate extract was 0.047 μ g/ml, while for acarbose was 0.003 μ g/ml. The ethyl acetate extract applied as the P1 treatment group lowered blood glucose levels in streptozotocin mice by 26%.

Conclusions: The α -glucosidase inhibitor of *Streptomyces* sp. IPBCC. b.15.1539 of *T. crispa* has the potency as an antidiabetic agent for type 2 DM therapy.

Keywords: *Streptomyces* sp., *Tinospora crispa*, Inhibitor α -glucosidase, Anti-hyperglycemic, mice.

INTRODUCTION

Global diabetes mellitus (DM) prevalence data shows serious increases. The total number of diabetics worldwide is projected to rise from 171 million in 2000 to 366 million in 2030 [1] and become the growing global problem of overweight, obesity and physical inactivity. Both modern and traditional antidiabetic therapies have been used to treat people with type 2 DM, which is the most common form of DM. Type 2 DM comprises 90% of people with diabetes around the world [2]. One of the modern antidiabetic drug treatment mechanisms is based on α -glucosidase inhibitor activity, which inhibits the absorption of glucose from the intestine to the blood. Acarbose is a pseudo-oligosaccharides which acts as a competitor for α -glucosidase, non digestible and non-toxic. The α -glucosidase inhibitor of acarbose is used in the therapy of type 2 DM (non-insulin-dependent) [3, 4]. On the other hand, various medicinal plants have been traditionally used to treat diabetics. Recent scientific evidence supports the use of the medicinal plants in DM therapy, e. g. *Terminalia arjuna*; *Tinospora crispa*, *T. cordifolia*, *Lagerstroemia speciosa*; *Andrographis paniculata*, *Phaleria macrocarpa*, *Curcuma aeruginosa*, *C. xanthoriza*, *Centella asiatica*, *Xoncus arvensis*, *Caesalpinia sappan*, *Alloe vera*, *Parcia speciosa*, *Gynura procumbens*, *Physalis peruviana*, *Hibiscus sabdariffa*, *Tribulus terrestris*, and *Berberis aristata* [5-12].

Tinospora crispa has been studied for its potential antidiabetic treatment mechanism. Water extract of *T. crispa* significantly lowered blood glucose levels and increased plasma insulin levels in diabetic rats [13]. This effect may be due to the modulation of Ca²⁺ concentration in pancreatic beta cells [14]. Other data showed that *T. crispa* treatment reduced plasma glucose levels as much as 7.45% for 40 days in rats induced by streptozotocin [15]. These

evidences clearly show that *T. crispa* has potency as a source of antidiabetic compounds. However, *T. crispa*, a herbaceous climbing plant, is considered to be slow growing. A huge amount of biomass and consistent supply is required to produce active compounds at a large scale. Therefore, although there is no doubt that *T. crispa* has an antidiabetic potency, its slow growth is a major constraint to its widespread application. For that reason, new approaches should be thought, e. g. exploring the capability of endophytic microbes especially actinomycetes which reside in *T. crispa* plant tissue and can function as antidiabetic compound producers.

Actinomycetes are known to produce bioactive compounds with various biological function including α -glucosidase inhibition. Published data on actinomycetes which function as an α -glucosidase inhibitor, have been mainly on non-endophytic actinomycetes. Supporting evidence is available for *Actinoplanes* sp. SE50/110 [16, 17], *Actinoplanes* sp. CKD485-16 [18], *Streptomyces glaucescens* [19], *Micromonospora* sp. VITSDK3 (EU55138) [20], and *Actinoplanes* sp. A56 [21]. Nowadays, acarbose which was originally isolated from *Actinoplanes* sp. from Africa has been successfully commercialized as an antidiabetic drug [22].

Our previous work demonstrated that α -glucosidase inhibitor, an antidiabetic compound, associated with *T. crispa* was also produced by its endophytic actinomycetes. Several endophytic actinomycetes are found to reside in *T. crispa*, and they are capable of producing the α -glucosidase inhibitor similar to that of their host plant. Production of the α -glucosidase inhibitor by *T. crispa* seems to be influenced by the contribution of its endophytic actinomycetes [23]. Amongst the isolated endophytic actinomycetes of *T. crispa*, *Streptomyces* sp. IPBCC. b.15.1539 has been selected, due to its high potency as a source of α -glucosidase inhibitor. Here, we describe our

further work on the effect of ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 in lowering blood glucose of streptozotocin diabetic mice. The described findings are the first report in elucidating the role of endophytic actinomycetes from *T. crisper* which can lower blood glucose in streptozotocin mice. Data from the *in vivo* experiments clearly support the *in vitro* assessment for the capability of *Streptomyces* sp. IPBCC. b.15.1539 as antidiabetic agent through its α -glucosidase inhibitory activity. The microbial based approach may be more efficient for producing antidiabetic compounds than the host plant especially for very slow growing *T. crisper* plant.

MATERIALS AND METHODS

Growth and production of metabolites

Streptomyces sp. IPBCC. b.15.1539 (1% volume) was grown in a bioreactor filled with ISP 2 medium, for 5, 10, 15, and 20 days, and assayed for its α -glucosidase inhibition. The optimum time for the production of α -glucosidase inhibitor was determined based on the *in vitro* α -glucosidase inhibitory activity produced by crude extract and ethyl acetate extract containing the active compounds.

α -glucosidase inhibitory activity

The stock enzyme solution was prepared by adding 1 mg of α -glucosidase (Sigma) into 100 ml phosphate buffer pH 7 containing 200 mg Bovin Serum Albumin. A total of 1 ml of the stock enzyme solution was 25 times diluted with phosphate buffer pH 7. The substrate solution consisted of 50 μ l of 20 mM p-nitrophenyl α -D-glucopyranoside, 50 μ l of phosphate buffer pH 7 and 10 μ l of dimethyl sulfoxide solution (DMSO). The mixture was incubated for 5 min at 37 °C, then 50 μ l of phosphate buffer solution and the enzyme was added, and incubated for further 15 min. The reaction was stopped by adding 800 μ l of 200 mM sodium carbonate. The released P-nitrophenol absorbance was measured using a spectrophotometer (Thermo Spectronic Genesys 20) at 400 nm wavelength. Acarbose (Glucobay; Bayer), the commercial α -glucosidase inhibitor, was used as a comparison. A concentration of 50 mg acarbose was diluted until 1% (w/v) concentration. Inhibition activity of crude extracts containing α -glucosidase inhibitor was calculated as percentage of inhibition by the following formula: $[(C-S)/C \times 100\%]$, where C is the control absorbance and S is the sample absorbance [24].

IC₅₀ of ethyl acetate extract containing α -glucosidase inhibitor

The IC₅₀ value was obtained by testing the inhibitory activity of the extract at different concentrations, i.e. 1000 μ g/ml, 500 μ g/ml, 250 μ g/ml, 125 μ g/ml, and 62.5 μ g/ml [25]. The line graph equation was made as a function of the extract concentration (x) and an inhibition activity produced (y).

Acclimatization

Mice were acclimatized in cages for 7 days in order to adjust to the new environment. The *in vivo* experiment was conducted by considering the animal ethics conduct. Cages were placed in the room with the dark and light arrangement each for 12 hours, at room temperature with humidity around 49-64%. Feed containing 25% protein, 3% fat, 5% crude fiber, 10% ash, and 12% moisture content and distilled water, were given ad libitum.

Anti-hyperglycemic activity based on oral glucose tolerant test (OGTT)

Mice were divided into 6 groups which each group consisted of 5 mice. The mice were fasted for 6 hours and still be given a drink, then the blood was taken for determination of initial glucose levels. Group 1 was given a 10% sucrose solution (90 mg/30 g bw), group 2 as a negative control was given distilled water, and group 3 as a positive control was given acarbose (0.03 mg/30 g bw), groups of 4 to 6 were given treatment group of ethyl acetate extract each at 0.036 mg/30 g bw (P1), 0.36 mg/30 g bw (P2), 3.6 mg/30 g bw (P3). After 30 minutes, all groups were orally given 10% sucrose solution (90 mg/30 g bw). Then the blood sample was taken at 30, 60, 120 and 180 minutes after administration. Blood glucose levels were calculated by glucometer (GlucoDr) and then the percentage of blood glucose levels was calculated [26].

Anti-hyperglycemic assay using streptozotocin induction in mice

Mice were fasted for 6 hours and intravenously injected with streptozotocin which previously dissolved in 50 mM sodium citrate pH 4.5 at a dose of 40 mg/kg. After 15 days of treatment, the mice which experienced an increase in glucose levels above 150 mg/dl were classified as diabetic mice [27].

The diabetic mice were divided into 5 groups, each group consisting of 6 mice which were given treatment for 15 days. Group 1 was given distilled water as a negative control, group 2 as a positive control was given acarbose (0.03 mg/30 g bw), group 3, 4, and 5 were given treatment of ethyl acetate extract each at 0.036 mg/30 g bw (P1), 0.36 mg/30 g bw (P2), 3.6 mg/30 g bw (P3). At 5, 10, and 15 days after treatment, the mice tails were cut at the tip, then the blood glucose levels were measured using glucometer (GlucoDr), and calculated the percentage of changing blood glucose levels [26].

Data analysis

The experiment was set up using a completely randomized design (CRD). All data were shown as mean values \pm standard deviation (mean \pm SD). Data were analyzed using the Statistical Analysis System (SAS) program version 9.1.3 based on analysis of variance (ANOVA) and followed by Duncan test 5% significance level.

RESULTS

α -glucosidase inhibitory activity

The data showed that α -glucosidase inhibitory activity of the crude extracts and the average weight of biomass increased at 5-10 days of the production time and decreased in 15-20 days (fig.1). The crude extract containing α -glucosidase inhibitor showed 98.5% of α -glucosidase inhibitory activity after 10-days of production, with 15.6 mg weight of produced biomass.

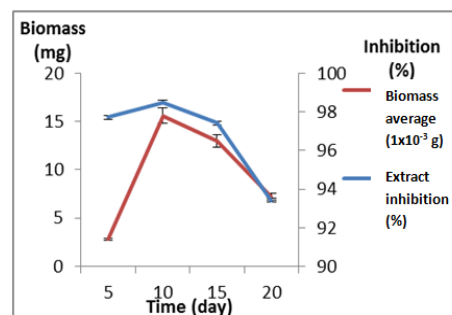


Fig. 1: The α -glucosidase inhibitory activity and biomass production of *Streptomyces* sp. IPBCC. b.15.1539 grown at ISP2-medium for 5-20 days at room temperature.

The inhibitory activity of ethyl acetate extract containing α -glucosidase inhibitor at a concentration of 1000 μ g/ml was 96.08%, while acarbose at the same concentration gave 97.46% inhibition (table 1). The higher concentration of α -glucosidase inhibitor increased the percentage value of α -glucosidase inhibition.

Table 1: Activity of ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539

Concentration (μ g/ml)	Inhibition (%)	
	Acarbose	Ethyl acetate extract
62.5	88.13	83.33
125	88.89	86.60
250	91.53	90.85
500	96.33	93.46
1000	97.46	96.08

Results of logarithmic equation showed that $y = 4.6686 \ln(x) + 64.287$ with $R^2 = 0.9908$ (fig. 2a). The value of $x = 0.047 \mu\text{g/ml}$. The results were then compared with acarbose which showed $y = 3.7654 \ln(x) + 71.677$ with $R^2 = 0.9417$ and $x = 0.003 \mu\text{g/ml}$. The IC_{50} of acarbose was lower than the IC_{50} value of α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 (fig. 2b).

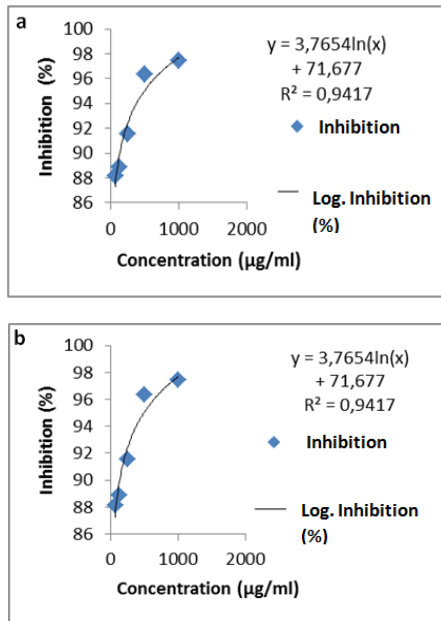


Fig. 2: The IC_{50} value of α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 (a) and acarbose (b) Anti-hyperglycemic activity of OGTT

The anti-hyperglycemic effect of ethyl acetate extracts containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 is shown in fig. 3. In *in vivo* data showed that giving of 10% sucrose

(90 mg/30 g bw) sharply increased blood glucose levels of mice and reached the highest value after 60 min. Blood glucose levels decreased toward normal in 2 to 3 hours of observation. Treatment with ethyl acetate extracts containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 could inhibited the increased in blood glucose levels of mice given with sucrose.

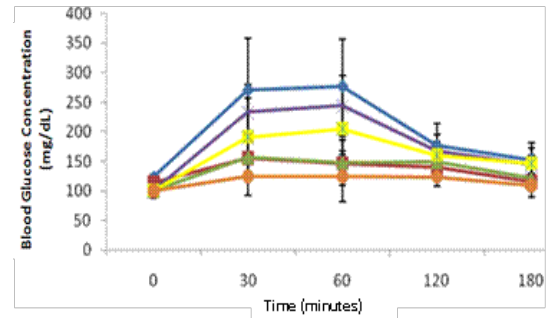


Fig. 3: Normal and hyperglycemic blood glucose level and acarbose for mice treated with ethyl acetate extract of *Streptomyces* sp. IPBCC. b.15.1539 (1, 10 dan 100 times sequentially). P1 extract, P2 extract, P3 extract, sucrose, positive control, and negative control

Data analysis of variance at 95% level of confidence indicated that the administration of P3 treatment (3.6 mg/30 g bw) of ethyl acetate extract was significantly different from 10% sucrose treatment (90 mg/30 g BW) (table 2). Further, the P3 treatment was significantly different to P1 and P2. The area under the curve (AUC) between blood glucose levels over time showed 626.5 mg. hour/dL AUC values after administration of the 10% sucrose (table 2). Giving acarbose to the treated mice caused 34 % decreased in AUC values which became 413.3 mg. hour/dL. Meanwhile, the ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 at a concentration of P1, P2 and P3 were able to reduce the AUC value by 10%, 18.9% and 24.7%, respectively.

Table 2: The effect of ethyl acetate extracts containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 in lowering blood glucose level of mice after 180 minutes treatment

Mice	AUC of blood glucose level (mg. hour/dl) (N=5)					
	Sucrosa	Control-	Control+	P1	P2	P3
X±SE	626.5±137.4	413.3±46.1	422.75±61.5	546.15±81.9	508.±103.1	471.7±72.1
AUC ^(*)	100 % ^a	65.96% ^c	67.47% ^c	90.04% ^{ba}	81.09% ^{ba}	75.29% ^{bc}
Reduced AUC		34.04%	32.53%	9.96%	18.91%	24.71%

(*) Number followed by similar letter at similar row does not indicate the differences amongst treatments based on Duncan test at 5% level of significant.

Anti-hyperglycemic activity in streptozotocin mice

Testing the antihyperglycemic activities with the low-dose of streptozotocin induction (Multiple low-dose Streptozotocin (MLDSTZ)) showed that the ethyl acetate extract capable of lowering blood glucose level from day 5 to day 15 (fig. 4).

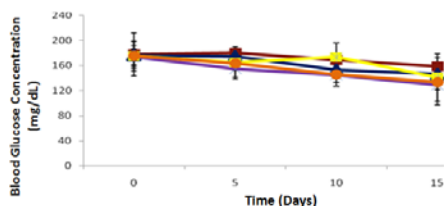


Fig. 4: Changing of blood glucose level in diabetic mice induced by streptozotocin for 15 days treatment. P1, P2, P3, Positive Control, Negative Control

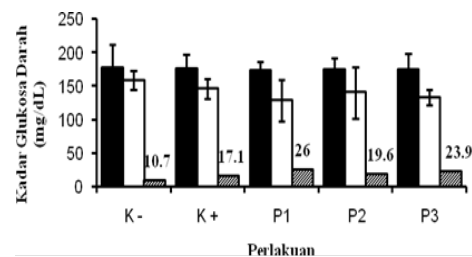


Fig. 5: Changing of blood glucose level in diabetic mice induced by streptozotocin after 15 days treatment. Day 0, Day 15, % of reduced blood glucose. K-: negative control; K+: positive control; P1, P2, P3: treatment

Kolmogorov-Smirnov test indicated that the data was normally spreaded. The analysis of variance at the 95% level of confidence showed that there was a significant effect amongst the treatments. The P1 difference from to P2, P3, negative control and positive

control. These indicated that the ethyl acetate extract of *Streptomyces* sp. IPBCC. b.15.1539 capable of lowering blood glucose levels of diabetic streptozotocin mice. The highest decreased in blood glucose levels was by 26% and it was found at day 15 for mice treated with P1, whereas the positive control gave 17% reduction (fig. 5).

DISCUSSION

The highest α -glucosidase inhibitory activity is correlated with the average of biomass produced by *Streptomyces* sp. IPBCC. b.15.1539 grown on ISP-2 medium for 10 days. Production of secondary metabolites and biomass are influenced by the growth conditions such as nutritional composition. The ISP-2 medium contains malt extract and yeast extract as a source of nutrients and glucose as a carbon source. The high of α -glucosidase inhibitory activity probably due to relatively rapid cell growth for 10 days in a bioreactor, which then slowing down the activity and growth on the day 15 to 20. The old cells is associated with the process of sporulation. At the final phase of their life cycle, microbial cells need to adapt to unfavorable environmental conditions, which may cause the production of secondary metabolites [28]. Microbes need water, energy sources, carbon, nitrogen, minerals, and vitamins for nutrients and oxygen for aerobic processes [29]. The production of secondary metabolites requires media that can stimulate cell growth. The production of α -glucosidase inhibitor by *Streptomyces* sp. IPBCC. b.15.1539 may be stimulated by nutrient limitations that exist in a bioreactor used during the production period.

Microbial natural products are considered as important sources of various lead compounds used in pharmaceutical industry. Actinomycetes is known as source of various important commercially microbial natural products used in the medical field. Acarbose as an α -glucosidase inhibitor is produced by member of Actinomycetes, e. g. strains of the genera *Actinoplanes* and *Streptomyces*. Acarbose is produced industrially using developed strains of *Actinoplanes* sp. SE50/100 [30], and it is known as the most potential inhibitor of the sucrose metabolism through inhibition of the activity of α -glucosidase [31] used in the treatment of type 2 diabetes disease. Acarbose works as a competitive inhibitor of α -glucosidase enzyme that catalyzes the break glycosidic bond in the release of glucose, which causes inhibition of glucose absorption, resulting in lower glucose levels after eating.

The α -glucosidase is an enzyme that can hydrolyze the substrate likes p-nitrophenyl- α -D-glucopyranose into product, e. g. p-nitrophenol and glucose [24]. Diabetics treated with α -glucosidase inhibitor like acarbose will enabling them to better utilize starch-or sucrose-containing diets by slowing down the intestinal release of α -D-glucose. Acarbose able to inhibit the activity of sucrase, maltase, dextrinase, and glucoamylase [30]. The acarbose, as α -glucosidase inhibitor works as the competitor for the enzyme, thus blocking the active site of the enzyme. The α -glucosidase inhibitor binds to the substrate in the form of enzyme substrate complex and produces the p-nitrophenol and glucose. The enzyme activity was measured by the absorbance of p-nitrophenol which gave the yellow colour. The difference in absorbance value of the sample with or without addition of the enzyme represents the percentage of α -glucosidase inhibitory activity.

In this work, we found that endophytic *Streptomyces* sp. IPBCC. b.15.1539 isolated from an antidiabetic medicinal plant namely *T. crispa* proved to have capability as the source of α -glucosidase inhibitor. The IC_{50} value is the concentration of the extract value which gave 50% of the α -glucosidase inhibitory activity. The IC_{50} for the ethyl acetate extract was 0.047 μ g/ml, while for acarbose was 0.003 μ g/ml. The data indicate that IC_{50} value of ethyl acetate extract containing α -glucosidase inhibitor was much higher than acarbose. This condition may be related to the degree of purity of the extract. Acarbose is a commercial product in the form of pure preparations potent inhibitor of the activity of sucrose metabolism [31]. The lower the IC_{50} value, the higher the activity of the active compounds in inhibiting the activity of α -glucosidase. The intensity of inhibition in the sample are categorized as very active if it is able to produce α -glucosidase inhibition at concentrations of less than 50

μ g/ml [32], which is also showed by the sample used in this research work.

For the antihyperglycemic activity of oral glucose tolerance test (OGTT), it clearly shows that the ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 has the potency in lowering postprandial blood glucose levels of mice after the administration of 10% sucrose solution. The ethyl acetate extract containing α -glucosidase inhibitor is capable to lowering blood glucose levels in hyperglycemic diabetic mice. This phenomenon indicates that the ethyl acetate extract containing α -glucosidase inhibitor has promising to be further developed as antidiabetic agent. In addition, the α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 may have other different active metabolites produced. Acarbose belongs to the class of pseudooligosaccharide [30], while the ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 has been assayed for the present of flavonoid [25]. It has been reported that the flavonoid component has the ability to suppress postprandial hyperglycemic blood glucose levels [33]. Flavonoids given orally to diabetic mice, capable to lower plasma blood glucose levels by increasing glucose uptake in peripheral tissues and regulate the activity of the expression of enzymes involved in carbohydrate metabolism pathway [34]. Active compounds from plants *Cynanchum acutum* L. i.e. quersetin, tamarixtin and kemferol had antidiabetic activity by lowering blood glucose levels [35]. Flavonoids such as quersetin can stimulate cell division pancreatic beta cells that produce insulin secretion [36]. The data reinforces the notion that the ethyl acetate extract containing α -glucosidase inhibitor produced by *Streptomyces* sp. IPBCC. b.15.1539 has potential as α -glucosidase inhibitor as well as antihyperglycemic in diabetic mice.

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CONFLICT OF INTERESTS

Declared None

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