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Research Article

ALPHA-GLUCOSIDASE INHIBITOR ACTIVITY AND CHARACTERIZATION OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM SOME INDONESIAN DIABETIC MEDICINAL PLANTS

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ABSTRACT

An alpha glucosidase inhibitor is one of the compounds for the treatment of diabetes. This inhibitor can retard the liberation of glucose from dietary complex carbohydrates and delay glucose absorption, resulting in reduced postprandial plasma glucose levels and suppress postprandial hyperglycaemia. The purpose of this study was to isolate and select alpha glucosidase inhibitor-producing endophytic actinomycetes from various diabetic medicinal plants. Endophytic actinomycetes were isolated from the roots, leaves and stems of diabetic medicinal plants: *Alloe vera, Tinospora crispa, Phaleria macrocarpa, Curcuma aeruginosa, Centela asiatica, Xoncus arvensis, Andrographis paniculata, Caesalpinia sappan, Curcuma xanthoriza, Parcia speciosa, Gynura procumbens, Physalis peruviana* and *Hibiscus sabdariffa*. Sterilized plant sample were inoculated on the HV Agar medium containing 50 ppm cycloheximide and 30 ppm nalidixic acid and were incubated for 2-3 weeks at room temperature. Sixty-five isolates were obtained and tested for their ability to inhibit the alpha-glucosidase. Identification for the selected isolates was based on 16S rDNA sequences. The inhibitor activity to alpha glucosidase was determined spectrophotometrically at 400 nm using p-Nitrophenyl-alpha-D-glucopyranoside as a substrate, and acarbose as a positive control. The results showed that endophytic actinomycetes isolated from selected antidiabetic plants produced various inhibition activities. The highest inhibition activity to alpha-glucosidase was shown by BWA65 found from *Tinospora crispa*. Production of alpha-glucosidase inhibitor compounds in this plant largely related with the contribution of its actinomycetes endophytes. The molecular identification based on 16S rDNA sequence revealed that the potential BWA65 isolate showed 92% similarity to *Streptomyces olivochromogenes*.

Keywords: Alpha-glucosidase inhibitor, Endophytic actinomycete, Diabetes mellitus, Indonesian medicinal plants.

INTRODUCTION

Diabetes mellitus (DM) is the highest cause of death among other chronic diseases. This disease can cause complications such as cardiovascular disease, kidney failure, blindness, impotence and gangrene. More than 95% of diabetes is type 2 diabetes or often called non-insulin dependent diabetes[1]. DM cannot be cured, but can be controlled. Treatment of DM in principle is to maintain blood glucose levels in normal conditions (80-120 mg/dl). Both modern and traditional antidiabetic drugs have commonly use by Indonesian community experiencing DM. One of antidiabetic drugs mechanism is by inhibiting digestion of complex carbohydrates (starch) into glucose in the small intestine, resulting of reducing the intake of glucose from the intestine into the blood. One of active compounds that have this activity is an alpha glucosidase inhibitor. The alpha glucosidase inhibitor can be produced by some organisms, including microbes. An example is acarbose, a commercial alpha glucosidase inhibitor produced by *Actinoplanes* sp., an actinomycetes isolated from Kenya[2].

Traditionally, DM treatments utilize various types of medicinal plants which contain active ingredients that can decrease blood sugar levels. Empirically, some medicinal plants are known to have capability to cure diabetes. The plant active ingredients are commonly used to decrease blood sugar levels. Various medicinal plants have been reported as anti hyperglicaemic e.g. *Terminalia arjuna*, *Tinospora crispa*, *Phaleria macrocarpa*, *Andrographis paniculata*, *Momordica charantia, Tribulus terestris, and Berberis aristata* [3-10].

Exploration of endophytic microbes is expected to produce important secondary metabolites that have properties similar to that produced by the host plant metabolites. Endophytic microbes that live in plants can produce secondary metabolites similar to those produced by its host as a result of genetic exchange and evolution of a long relationship[11,12]. Medicinal plants for diabetes are a potential source of microbial producers of alpha glucosidase inhibitors. With potential isolates obtained from medicinal plants, we will be able to produce an alpha glucosidase inhibitor compounds for diabetes drug microbiologically, with greater numbers and better quality. The purpose of this study was to obtain isolates of endophytic actinomycetes from some Indonesian medicinal plants that have been known to have antidiabetic properties, potentially as an alpha glucosidase inhibitor producer.

MATERIALS AND METHODS

Medicinal plants samples

Thirteen medicinal plants were collected from the Collection of Medicinal Plants Garden of Biopharmaca Research Center, Bogor Agricultural University, Bogor, Indonesia. The sample of medicinal plants namely: *Alloe vera*, *Tinospora crispa*, *Phaleria macrocarpa*, *Curcuma aeruginosa, Centela asiatica*, *Xoncus arvensis*, *Andrographis paniculata*, *Curcuma xanthoriza*, *Physalis peruviana*, *Gynura procumbens, Hibiscus sabdariffa, Caesalpinia sappan and Parcia speciosa*.

Isolation of endophytic actinomycetes

Isolation of endophytic actinomycetes was base on previous researcher[13]. Surface sterilization of the plant samples were done by soaking in alcohol 70% (1 minute), sodium hypochlorite 1% (5 minutes), alcohol 70% (1 minute) and finally rinsed with sterile distilled water. The sterilized samples were then aseptically grounded and added by 4 ml of 12.5 mM sterile phosphate buffer. Amount of 100 µl of sample suspension was platted on Humic Acid Vitamin (HV) agar medium contain 50 ppm of cycloheximide and 30 ppm of nalidixic acid, and incubated for 2-3 weeks at room temperature (25-28^{0}C). Actinomycetes colonies that grow from agar medium were purified on Yeast Malt Extract Agar (YMA) medium and stored in the refrigerator for further examination.

Selection of an alpha glucosidase inhibitor-producer

All isolates obtained were grown in a liquid medium containing 0.1% soluble starch, 0.5% peptone, and 0.1% yeast extract (pH 7) for 14 days with agitation (120 rpm) at room temperature. The cell biomass were separated by centrifugation at 1432 x g for 20 minutes and the supernatant were tested for alpha glucosidase inhibitor activity according to previous researcher[14]. Isolates with the greatest inhibitory activity was selected for further investigation.

Assay of alpha glucosidase inhibition

Inhibition of alpha glucosidase activity was examined according to previous researcher[14]. Enzyme inhibition assay was measured based on solving the substrate to produce colored products. Enzyme alpha-glucosidase (Sigma) with a concentration 0.75 units / ml was dissolved in 0.1 M phosphate buffer pH 7. As a substrate, we used p-nitrophenyl-alpha-D-gluco pyranoside 20 mM dissolved in 0.1 M phosphate buffer pH 7. The mixture of reaction contains 125 µl substrate, 240 µl 0,1 M phospate buffer pH 7 and 10 µl sample. After the reaction mixture was incubated at 37^0C for 5 minutes, amount of 125 µl of enzyme were added and incubated for 15 minutes at 37^0C. The reaction was stopped by adding 500 µl sodium carbonate and p-nitrofenol produced was measured its absorbance at 400 nm. As a comparison, we used 1 mg/ml solution of acarbose (Sigma). Inhibition of alpha glucosidase activity was determined by the formula:

$$\text{Inhibition (\%)} = (Ac-(As-Ab) / Ac \times 100\ \%$$

(Ac: absorbance of control, Ab: absorbance of background, As: absorbance of sample)

Role of endophytic actinomycetes in the production of alpha-glucosidase inhibitor

To determine the role of endophytic actinomycetes in producing alpha-glucosidase inhibitors, we used free endophytic of selected medicinal plant tissue culture, *Tinospora crispa*. The 0.5 g of samples plant tissue culture was grounded and added 0.5 ml phosphate buffer, and then centrifuged 1432 x g for 5 minutes. The supernatant obtained was tested against the alpha glucosidase inhibitory activity. In the same way, the inhibition activity of the naturally grown plant was also examined. The ability of plant inhibitors were compared with the inhibitor from endophytic actinomycete culture

Molecular identification and morphological characterization

DNA extraction and amplification of 16S rDNA gene

DNA extraction of BWA65 isolate was carried out using GES methods[15] followed 16S rDNA gene amplification using Primer 20F (5'-GATTTTGATCCTGGCTCAG-3') and 1500R (5-GTTACCTTGTTACGACTT-3'). PCR reactions were done using Thermalcycler (Trootsa Shuzo Co, Ltd., Shiga, Japan) for 30 cycles. Amplification results was visualized by electrophoresis using Mupid Mini Cell in 1% agarose gel in TAE buffer (Tris-EDTA Acetate) for 25 minutes at 100 V. Purification of PCR products was done with PEG precipitation method. 16s rDNA pure samples was stored at -20^0C.

Cycle sequencing

The cycle sequencing was carried out by using primers 520 F (5'-GTGCCAGCAGCCGCGG-3'), 920R (5'-CCGTCAATTCATTTGAGTTT-3'), 520R (5'-ACCGCGGCTGCTGGC-3'), 920F (5'-AAACTCAAATGAATTGACGG-3'), 20 F (5'-GATTTTGATCCTGGCTCAG-3') and 1500 R (5-GTTACCTTGTTACGACTT-3'). The composition used for each tube was 0.5 ml 10 pmol primer, 1 µl purified DNA, 0.5 ml of Big Dye Terminator sequence premix kit (Applied Biosystems Inc., Warington, UK), 1.5 ml 5x sequence buffer and deionized water until a volume of 10 ml. Subsequently the mixture reaction was performed by PCR amplification of 40 cycles.

Preparation and sequencing

Preparation was performed by mixing 10 ml of the product cycle sequencing with 1 ml of 3M Na-acetate, 1 ml of 125 mM EDTA (pH 8) and 25 ml absolute ethanol. The next stage was centrifugation at 8586 x g for 25 minutes at 4^0C. Supernatant discarded and the pellet was washed with 70% ethanol and then re-centrifuged 8586 x g for 10 minutes. The supernatant was discarded followed by drying pellets for 10 minutes. Dried DNA pellets were added to 10 ml HiDi-formamide (Applied Biosystems Inc., Warington, UK). Samples were then heated at 95°C for 2 minutes and immediately cooled in ice. The next step, sample was injected to ABI 3130 sequencer (Applied Biosystems Inc., Foster, Calif.).

Analysis of molecular data

DNA sequences were analysed using the BioEdit program and data blasting at the NCBI Gene Bank data library. Phylogenetic analysis was conducted using multiple alignment programs Clustal X version 1.83. Construction of phylogenetic trees as based on genetic distance with the Neighbor Joining method. Construction of evolutionary distance in degrees of confidence was done using the bootstrap values in NJ plot program.

Observation of morphology

Morphological observations were carried out by microscopic observation with light microscope at magnification of 100x, 400x and scanning electron microscope (JSM-5310LV) with magnification of 10,000x.

RESULTS AND DISCUSSION

Isolation of endophytic Actinomycetes

Endophytic microbes are microbes that live inside plant tissues at specific periods and are able to live by forming colonies in plant tissue without harming their host.

In this study, 13 samples of medicinal plants used as sources of endophytic actinomycetes isolates, namely: *Alloe vera, Tinospora crispa, Phaleria macrocarpa, Curcuma aeruginosa, Centela asiatica, Xoncus arvensis, Andrographis paniculata, Caesalpinia sappan, Curcuma xanthoriza, Parcia speciosa, Gynura procumbens, Physalis peruviana* and *Hibiscus sabdariffa*.

In this study, 65 endophytic actinomycetes isolates were successfully isolated from different species of medicinal plants using HV agar medium. All of the isolated endophytic actinomycetes obtained can be regarded as the culturable isolates, although they may not represent all the endophytic microbial populations living on these plants. This is due largely that microbes cannot all grow on synthetic medium.

The result of endophytic actinomycetes isolates from each medicinal plant examined was shown in Figure 1 and Table 1. Around 69.2% of tested plants contained endophytic actinomycetes. *Tinospora crispa* contained the highest number of endophytic actinomycetes (32 isolates), *Curcuma aeruginosa* (9 isolates), *Gynura procumbens* (6 isolates), *Curcuma xanthoryza* (5 isolates) and other plants (1-4 isolates). The results of investigation showed that most endophytic actinomycetes obtained from the roots (45 isolates), followed by the rhizome (14 isolates), the stems (3 isolates) and the leaves (3 isolates).

Each plant generally contains several endophytic actinomycetes that live in the plants. Each higher plants may contain some endophytic actinomycetes that can produce biological compounds or secondary metabolites that allegedly as a result co-evolution or genetic transfer (genetic recombination) from the host plant into endophytic microbes[11].

The results indicate that several endophytic actinomycetes capable of producing active compounds similar to their host. This phenomena support the findings from other workers. Strobell and Daisy[16] reported that endophytic *Taxomyces andreanae* produced paclitaxel in *Taxus* plants. Anticancer compound paclitaxel was also produced by the plant *Taxus brevivolia*. Taechowisan *et al*[17] found that *Streptomyces aureofaciens,* endophytic in ginger plant produced arylcoumarin compound which has antitumor activity, where the ginger plant also has anti-tumor compounds such as reported by previous researcher[18]. Meanwhile Castillo *et al*[19] showed that *Streptomyces* NRRL 30562, the endophytic *in Kennedia nigriscans* plant capable of producing broad-spectrum of antibiotics. This plant is traditionally used to prevent infection of microbes in the wound by Aboriginal tribes.

Screening of endophytic actinomycetes producing α-glucosidase inhibitor

Examination for all endophytic actinomycetes revealed that 12 isolates which comprised of 10 isolates from *Tinospora crispa*, 1 isolate from *Caesalpinia sappans* and 1 isolate from *Curcuma aeruginosa* respectively, generate positive alpha-glucosidase inhibitors, whereas 53 other isolates were having negative results (Table 1).

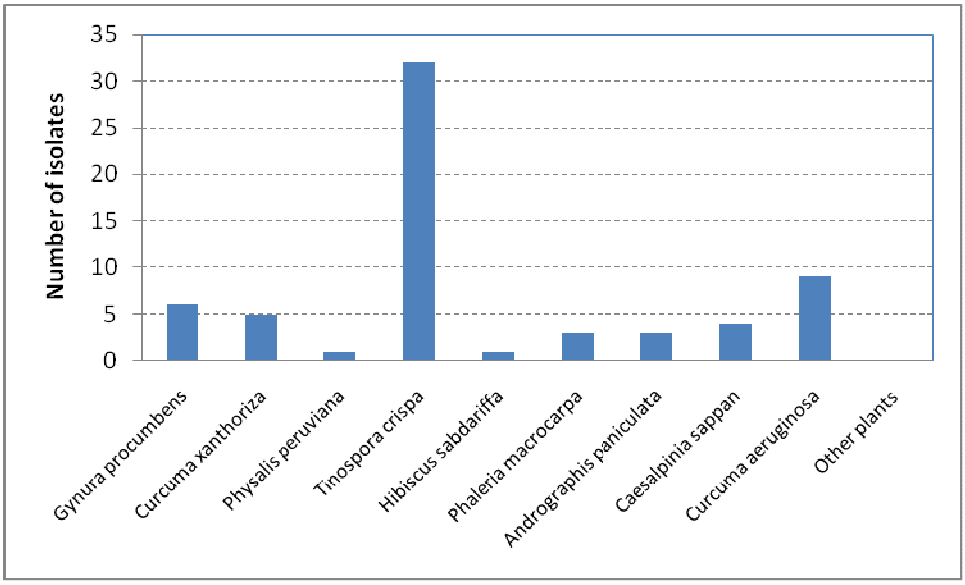

Fig. 1: The number of endophytic actinomycetes isolates from each plant

Table 1: The endophytic actinomycetes isolates from various diabetic medicinal plants and their properties of alpha glucosidase inhibitor

Medicinalplants	Part of plants	Number of isolates	Code of isolates	Characteristic onYMA medium	Inhibitory activity (%)
Gynura procumbens	roots	4	SNA 11	no spores, pink colony	-
			SNA 12	no spores, red colony	-
			SNA 2	no spores, brown colony	-
			SNA 21	no spores, dark brown colony	-
	Stems	2	SNB 1	no spores, brown colony	-
			SNB 1A	no spores, pink colony	-
	Leaves	1	SND 22	no spores, red colony	-
Alloe vera	Roots	-			
	Leaves	-			
Curcuma xanthoriza	Roots	-			
	Stems	-			
	Leaves	-			
	Rhizome	5	TLR 1	brown spores	-
			TLR 2	white spores	-
			TLR 21	gray spores	-
			TLR 3	white spores	-
			TLR 4	gray spores	-
Centela asiatica	Roots	-			
	Stems	-			
	Leaves	-			
Physalis peruviana	Roots	-			
	Stems	-			
	Leaves	1	CP1	white spores	-
Tinospora crispa	Roots	32	BWA 14	no spores, brown colonies, producing a brown pigment	-
			BWA14A	no spores, brown colonies	-
			BWA 15	brownish white spores	0.684
			BWA 15A	gray spores	-
			BWA 16	gray spores	-
			BWA 2	white-gray spores	-
			BWA 3	white spores, produce reddish pigments	-
			BWA 33	white spores	-
			BWA 34	white spores, black colony	-
			BWA 35	no spores, brown colony	2.66
			BWA 36	no spores, brown colony	4.853
			BWA 3A	white spores	0.478
			BWA 4	white spores, brown colony	1.162
			BWA 4A	gray spores	0.273
			BWA 51	white spores	-

			BWA 54	no spores, dark brown colonies, dark brown pigment	1.162
			BWA 61	no spores, dark brown colonies, brown pigment	-
			BWA 62	no spores, copper brown colonies	-
			BWA 63	white-brown spores	-
			BWA 64	white spores	-
			BWA 65	no spores, dark brown colony	4.511
			BWA 66	white spores	-
			BWA 71	no spore, brown colony	0.752
			BWA 72	no spores, yellow-brown colony	-
			BWA 73	white spores, brown colony	0.889
			BWA 74	no spores, brown colony	-
			BWA 75	white spores	-
			BWA 76	white spores, brown colony, black pigmen	-
			BWA 82	no spores, brown colony	-
			BWA 84	no spores, yellow colony	-
			BWA 85	white spores	-
			BWA 86	no spores, brown colony	-
			BWA 93	no spores, black colony	-
	Stems	-			
	Leaves	-			
Hibiscus sabdariffa	Roots	-			
	Stems	1	ROB 12	no spore, pink colony	-
	Leaves	-			
	Flower	-			
Phaleria macrocarpa	Roots	3	MDA 2	brown spores	-
			MDA 22	brown spores	-
			MDA 52	no spore, orange colony	-
	Stems	-			
	Leaves	-			
	Fruits	-			
Andrographis paniculata	Roots	2	SBL A1	no spore, black colony	-
			SBL A2	white spores, no pigment	-
	Stems	-			
	Leaves	1	SBLD 3	no spore, brown colony	-
Xoncus arvensis	Roots	-			
	Stems	-			
	Leaves	-			
Caesalpinia sappan	Roots	4	SC A 13	white spores	0.547
			SC A 11	white-brown spore	-
			SC A 14	white spores	-
			SCA 1	white spores	-
	Stems	-			
	Leaves	-			
Parcia speciosa	Roots	-			
	Stems	-			
	Leaves	-			
Curcuma aeruginosa	Roots	-			
	Stems	-			
	Leaves	-			
	Rhizomes	9	TIR 11	white spores, brown colony	-
			TIR 12	white spores - brown	-
			TIR 13	gold spores, brown colony	3.623
			TIR 14	no spore	-
			TIR 1A	white spores, brown colony	-
			TIR 1B	white spores	-
			TIR 1B2	white spores, brown colony	-
			TIR 2	gray spores, dark brown colony	-
			TIR 3	gray-white spores	-
Number of isolates		65			

On further testing quantitatively assay using nitrophenyl-alpha-D-gluco pyranoside as a substrate showed that the crude extract (supernatant) from four isolates of endophytic actinomycetes of *Tinospora crispa* had alpha glucosidase inhibition activity. Crude extract of BWA65 isolates from *Tinospora crispa* produce the highest inhibition (11.01%) to alpha glucosidase, that was equal to 80% if compared to 1mg/ml of acarbose (13.61%) as control (Figure 2).

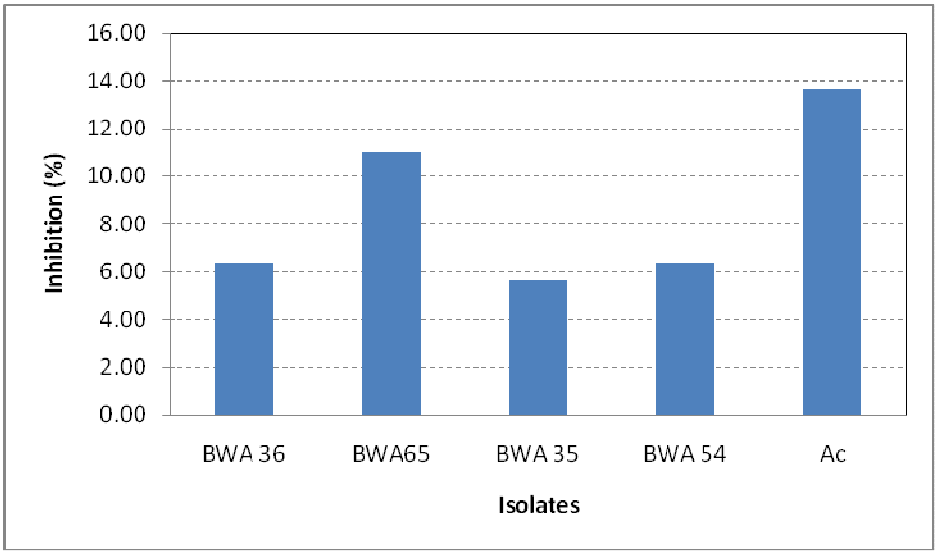

Fig. 2: Inhibitory activity of the alpha glucosidase by endophytic actinomycetes isolated from *Tinospora crispa*

The discovery of endophytic actinomycetes isolates from *Tinospora crispa* which produce the alpha glucosidase inhibitor in this research, strengthen the opinions that any plant can contain several endophytic microbes that can produce biological compounds or secondary metabolites that allegedly as a result genetic transfer (genetic recombination) from the host plant into endophytic microbes[11]. Information about the presence of hypoglycemic agents in *Tinospora* had been reported by previous researchers[20-23]. There were reported that the daily administration of alcoholic or aqueous extract of *Tinospora cordifolia* decreased the blood sugar in alloxan-induced hyperglycemia in rats and rabbits in the dose of 400 mg/kg.

In this study alpha glucosidase inhibitor activity was assayed by comparing the activity produced by host plants *Tinospora crispa*, free endophytic *Tinospora crispa* plant tissue culture and endophytic actinomycetes isolates. The result can illustrate the role of endophytic actinomycetes in contributing to the production of the inhibitor compounds. The results showed that free of endophytes plant tissue culture has only a very low capability to produce inhibitor compounds.

Moreover, the native plants part of *Tinospora crispa* capable of producing much larger inhibitor compounds (Figure 3). However, the capability of endophytic actinomycetes BWA65 was more than twice the activity of the host plants. The data clearly indicates that the endophytic actinomycetes in this plant contribute significantly to the production of alpha-glucosidase inhibitor compounds.

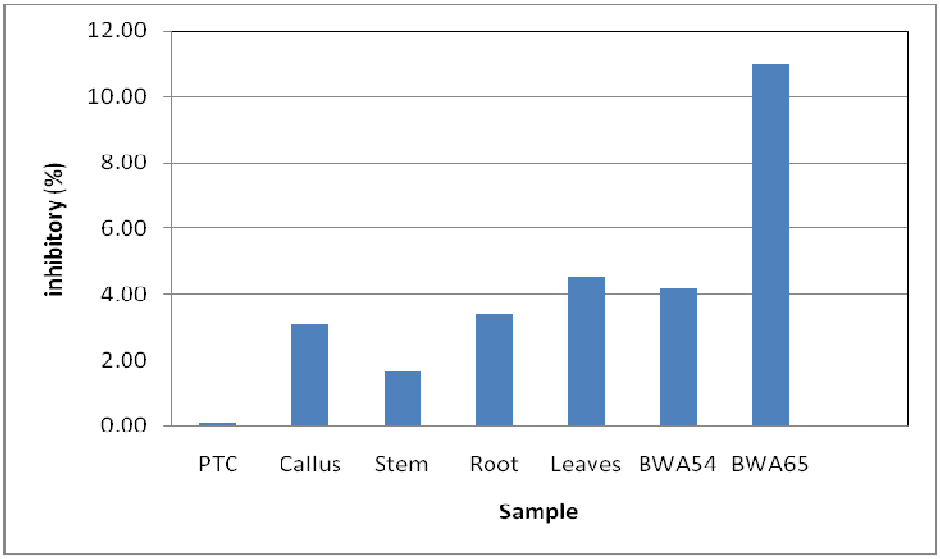

Fig. 3: The activity of alpha-glucosidase inhibitor produced by plant tissue culture (PTC, callus), native plants parts (stem, root, leaves) and endophytic actinomycetes isolates (BWA54, BWA65)

Morphologycal and molecular identification of isolate BWA65

In this study we used three types of media to see the culture characteristics of isolates BWA65 ie: Yeast Extract Malt Extract Agar (YMA), Yeast Extract Soluble Starch Agar (YSA) and Oatmeal Agar (OA). The results of investigation showed that BWA65 has good growth on YSA and OA media, and moderate growth on YMA media. In all media, the BWA65 isolate produced white aerial mycelium. The substrate mycelium of isolate BWA65 was brown on OA media and dark brown on both YSA and YMA media.

This isolate also produce soluble pigment dark brown on YSA media and pink pigment on OA media. The culture characteristics of BWA65 isolate on various media are listed in Table 2.

Tabel 2: Cultural characterizatics of potential isolate BWA65 on various media (7 days incubation at room temperature)

Cultural characteristic	Media		
	YMA	YSA	OA
Growth	moderate	good	good
Aerial mycelium	white	white	white
Substrate mycelium	dark brown	dark brown	brown
Soluble pigment	no pigment	brown	pink

The morphological observation under a light microscope with 400x magnification showed the spiral spore chains. The spiral spore chains are typical characteristics for *Streptomyces*. Further testing by SEM showed that BWA65 isolates has unbranched aerial hyphae and spores cylindrical spiral chains with smooth surface (Figure 4).

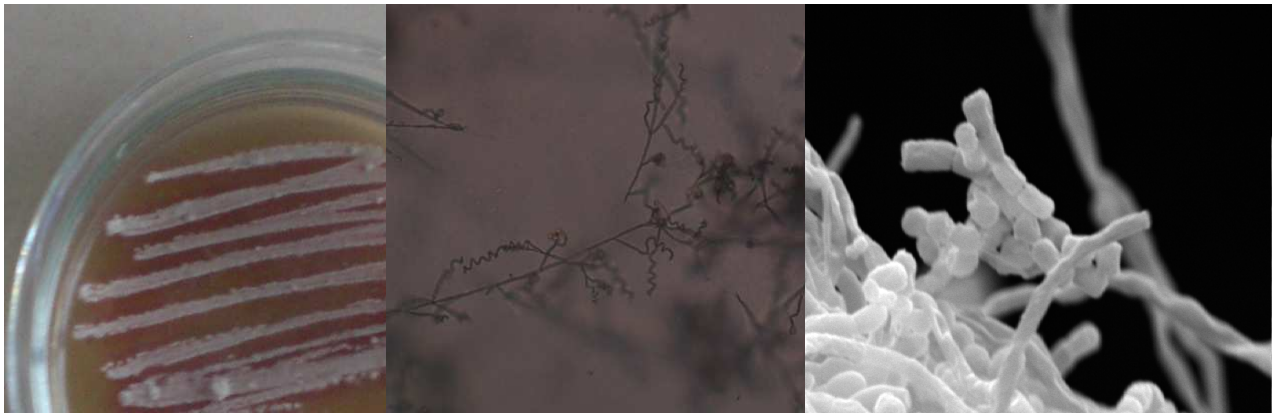

Fig. 4: The morphology of the endophytic actinomycetes BWA65 observed on Oatmeal Agar, ligh microscope (400x) and SEM (10,000x)

Phylogenetic tree of endophytic actinomycete of isolate BWA65 based on the 16s rDNA sequences is showed at Figure 5. The results of molecular identification using partial sequences of 16S rDNA showed that BWA65 has a 92% similarity with *Streptomyces olivochromogenes.* Base on other previous studies, *Steptomyces olivochromogenes* was known to produce many active compounds such as: glucose isomerase, xilose isomerase and phospolipase[24-25]. Until now, there has been no report of alpha-glucosidase inhibitor produced by *Steptomyces olivochromogenes*. Accordingly, we believe that the isolate BWA65 was a new type of alpha-glucosidase inhibitors producer. The results of this investigation also indicate that the BWA65 may be a novel species.

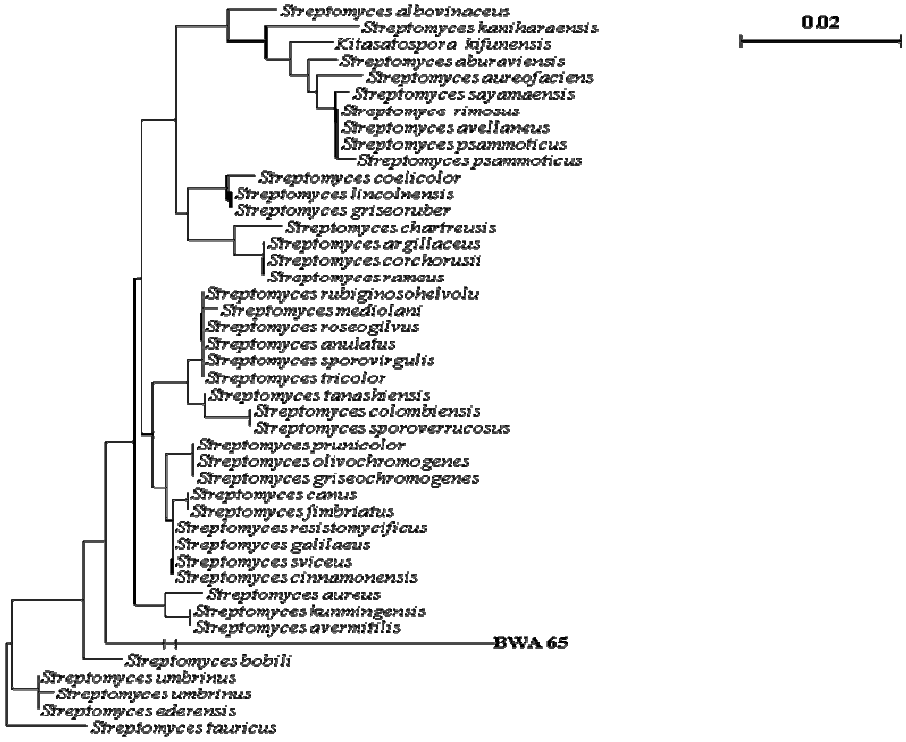

Fig. 5: Phylogenetic tree of endophytic actinomycete of isolate BWA65 based on the 16S rDNA sequences

CONCLUSION

In this study, 65 endophytic actinomycetes have been isolated from various diabetic medicinal plants. Endophytic actinomycete isolate BWA65 from *Tinospora crispa* has high ability to inhibit the alpha glucosidase activity. Production of alpha-glucosidase inhibitor compounds in this plants largely due to the contribution of its actinomycetes endophytes. Isolate BWA65 has 92% similarity with *Streptomyces olivochromogenes*.

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