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Isolation of α-glucosidase Inhibitors Produced by an Endophytic Fungus, *Colletotrichum* sp. TSC13 from *Taxus sumatrana*

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Abstract: *Colletotrichum* sp. have potential to act as antidiabetic agent, due to its α-glucosidase inhibitory. Therefore, the objective of present study was to isolate and identify the bioactive compounds responsible for the α-glucosidase inhibitory activity in *Colletotrichum* sp. TSC13. The methanol extract of TSC13 mycelia, was partitioned with *n*-hexane, chloroform and ethyl acetate. The *n*-hexane fraction exhibited the strongest α-glucosidase inhibitory activity. Column chromatography of this fraction resulted in 8 sub-fractions (F1-8). Fraction 3 (F3) which showed 71.4±2.4% inhibition was analysed further. Analysis using GC-MS after methylation of F3 and comparison to spectra databases and confirmation using authentic sample standards showed that F3 had two saturated fatty acid methyl esters, palmitic acid and stearic acid methyl esters and three unsaturated fatty acid methyl esters, oleic acid, linoleic acid and linoleinic acid methyl esters. Unsaturated fatty acids showed higher activity than the saturated fatty acids and the methyl esters form of unsaturated fatty acids showed slightly less active than the free acids. Further analysis using an ethyl acetate extract, it was confirmed that most of the fatty acids were present in the form of free acids. Therefore, it was concluded that the α-glucosidase inhibitor compounds in *Colletotrichum* sp. TSC13 were unsaturated fatty acids. This is the first report that a *Colletotrichum* sp. from *T. sumatrana* has α-glucosidase inhibitory activity.

Key words: Endophytic fungi, α-glucosidase inhibitory activity, *Colletotrichum* sp., fatty acids

INTRODUCTION

Diabetes Mellitus (DM) is a common metabolic disease affecting numerous people in the world. According to International Diabetes Federation, globally, there were estimated 366 million people with DM in 2011 and predicted to increase to 552 million by 2030 (Whiting *et al*., 2011). Therefore, it is important to continue study for finding medicines for DM treatments. Isolation of many bioactive natural products for their antibiotic, anticancer, antioxidative and antidiabetic constituents from endophytic microbes were reported (Schulz *et al*., 2002; Strobel, 2003, 2006; Suriyanarayanan *et al*., 2009). Hence, endophytic microbes are an excellent natural source for screening of useful compounds potential for drug discovery including DM.

In vitro screening of antidiabetic activity from natural products can be conducted using the α-glucosidase inhibitory assay (Kim *et al*., 2004). In human digestive system, α-glucosidase is one of the enzymes responsible for the breakdown of carbohydrate into glucose. Inhibitors of this enzyme such as commercial antidiabetic drug, acarbose is a microbial product to control postprandial hyperglycemia for people suffered with DM (Kim *et al*., 2004). Previously, on screening 14 endophytic fungi isolated from *Taxus sumatrana* for antidiabetic activity, fungal isolate TSC13 was found to have a strong α-glucosidase inhibitory activity (Artanti *et al*., 2011). This TSC13 fungus was identified as a *Colletotrichum* sp. Other fungi isolates were found less active than TSC13. In current study, the isolation of α-glucosidase inhibitors from the methanol extract of this fungus was reported.

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MATERIALS AND METHODS

This study was conducted at the Faculty of Agriculture Ehime University, Japan and Research Centre for Chemistry, Indonesian Institute of Sciences (LIPI), Indonesia, in 2011-2012.

Fungi used for tests: The endophytic fungus TSC13 was isolated by Ms. Harmastini Sukiman (Research Centre for Biotechnology, Indonesian Institute of Sciences) from *Taxus sumatrana* (Miq.) de Laub grown in Cibodas Botanical Gardens (LIPI), Indonesia, collected in 2005 and maintained in Potato Dextrose Agar (PDA) agar slants (Artanti *et al*., 2011). "TSC" is the code given to the collection of endophytic fungi isolated from *T. sumatrana*.

Identification of potential endophytic fungi: The identification was conducted by Techno Suruga Laboratory Co., Ltd. according to standard procedures. TSC 13 was found to be a *Colletotrichum* sp. (anamorph).

Fermentation: *Colletorichum* sp. TSC13 grown on agar slants of PDA (potato dextrose agar) medium (Tanaka *et al*., 1999) was transferred to petri dishes containing the same medium and cultured for 7 days. Liquid culture experiments were conducted in 1000 mL Erlenmeyer-flasks containing 300 mL of PDB (potato dextrose broth) medium. The flasks were incubated at 25°C with shaking at 100 rpm for seven days.

Sample preparation: Each culture incubated for 7 days was filtered to separate the mycelium and filtrate (filtered medium). The mycelium was extracted with 1000 mL of methanol for 1 day, 3 times. The extracted solution was evaporated under reduced pressure by rotary evaporator.

Isolation of the α-glucosidase inhibitor compounds: The methanol (MeOH) extract obtained from the liquid fermentation of *Colletotricum* sp. TSC13 was partition using *n*-hexane, chloroform, ethyl acetate (EtOAc) and MeOH. The *n*-Hexane fraction (100 mg) which had the most α-glucosidase inhibitory activity, was subjected to Silica Gel 60 column chromatography using solvent *n*-hexane: ethyl acetate 4:1 (v/v).

Alpha-glucosidase inhibitory assay: The α-glucosidase inhibitory assay was conducted according to Kim *et al*. (2004). Sample (0.1 mL) was added to a test tube containing 0.1 mL of 3 mM *p*NPG (*p*-Nitrophenyl α-D-glucopyranoside) and 2.2 mL of 100 mM phosphate buffer at pH 7.0, then incubated for 5 min at 37°C. The reaction was initiated by addition of 0.1 mL of enzyme solution (1 mg/0.1 mL) followed by a 15 min incubation at 37°C. The reaction was stopped by addition of 2.5 mL of 200 mM Na_2CO_3. The absorbance of *p*-nitrophenol released from *p*NPG at 400 nm was measured with a spectrophotometer.

Analysis of bioactive compounds: Samples were methylated using methanol acidified with sulphuric acid at 64°C for 30 min to form Fatty Acid Methyl Esters (FAME) for determination of fatty acid content. Methanol was evaporated to a small volume and water was added. FAME was extracted from the water fraction using EtOAc. Chemical analysis was conducted using gas chromatography coupled with mass spectrometry (GC-MS Shimadzu QP-2010) and a GC Shimadzu-QP-2014 equipped with a SPB-50 column (30 m×0.25 mm I.D., 0.25 μm film thickness). The analysis was performed according to the method of Yamamoto *et al*. (2008); column temperature, 235°C; carrier gas, helium (linear gas velocity, 30 cm sec^{-1}; split ratio, 1/30; ion source temperature, 200°C and interface temperature, 280°C. The identification of chemicals was performed in comparison with database (NIST08 library) and confirmed using authentic standard samples. Quantification of FAME content in the samples was conducted by comparing the peak area of the samples with curves for FAME standards.

RESULTS AND DISCUSSION

Identification of endophytic fungi: Figure 1 shows mycelia of TSC 13, identified as a *Colletotrichum* sp. (anamorph). *Colletotrichum* sp. a common endophytic fungus found in many species such as *Colletotrichum* sp. *Artemisia annua* (Lu *et al*., 2000), *Ocimum sanctum* (Dey *et al*., 2011) and *Cephalotaxus hainanensis* (Lu *et al*., 2012),

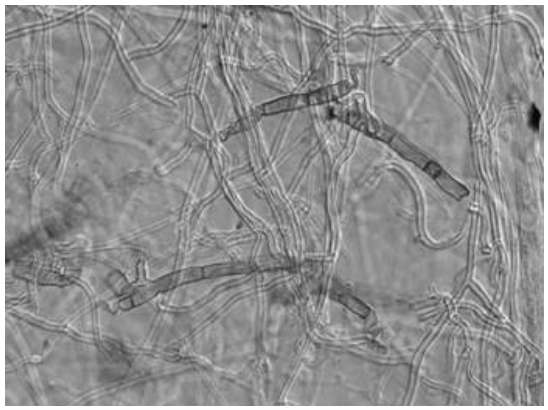

Fig. 1: Micrograph of *Colletrotrichum* sp. TSC13 (x400)

C. gloeosporioides from *Justicia gandarusa* (Gangadevi and Muthusamy, 2008), *Vitex negundo* (Arivudainambi *et al*., 2011), *C. boninense*, *Maytenus ilicifolia* (Pileggi *et al*., 2009) and *C. capsici* from *Capsicum annuum* (Kumaran *et al*., 2011). This is the first report of a *Colletotrichum* sp. isolated from *Taxus* spp.

Isolation of α-glucosidase inhibitory active compounds: Previous screening showed that the highest α-glucosidase inhibitory activity of TSC13 was from the methanol extract (Artanti *et al*., 2011), therefore, the methanol extract was selected for further study. The α-glucosidase inhibitory activities of the TSC13 MeOH extract after partition with various solvents are shown in Table 1. The *n*-hexane fraction exhibited the highest α-glucosidase inhibitory activity (68.4±4.1% inhibition). Further column chromatography separation of this fraction resulted in 8 sub-fractions (Fr 1-8). The antidiabetic activity of these sub-fractions is shown in Table 2. F3 and F4 had the highest α-glucosidase inhibitory activity with 71.4±2.4 and 78.5±5.1% inhibition, respectively. F3 was chosen for further identification because of its higher yield. Analysis using GC-MS and comparison with spectrum database and confirmation using authentic sample standards showed that F3 had two saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0) methyl esters and three unsaturated fatty acids, namely, oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) methyl esters (Fig. 2, 3, Table 3). This result suggested that FAMEs were confirmed as the α-glucosidase inhibitor compounds in F3. Since for GLC and GC-MS of the samples were methylated, Table 4 shows the α-glucosidase activity of individual fatty acids and the mixture as free fatty acids or methyl ester forms. The results showed that unsaturated fatty acids as free acids or methyl esters had higher inhibitory activity as compared to the saturated fatty acids and their methyl esters. At sample concentration of 10 μg mL^{-1}, α-glucosidase inhibitory activity was range from 69.7±4.8

Table 1: Alpha-glucosidase inhibitory activity of *Colletotricum* sp. TSC13 MeOH extract after partition with various solvents

Fractions	Inhibition (%)
n-hexane	68.4±4.1
Chloroform	39.7±2.9
EtOAc	41.1±3.3
MeOH	3.8±1.6

Values are Mean±SD, n = 2

Table 2: Yield of F-Hx fractions from *Colletotrichum* sp. TSC13 obtained by silica gel chromatography and their α-glucosidase inhibitory

Fractions	Yield (mg)	Inhibition (%)
F1	0.9	4.8±1.2
F2	7.6	27.0±3.6
F3	16.2	71.4±2.4
F4	6.0	78.5±5.1
F5	3.6	26.7±2.8
F6	5.3	19.7±4.3
F7	4.5	12.2±3.0
F8	4.0	9.2±3.8

Values are Mean±SD, n = 2, F-HX: *n*-hexane fraction from the MeOH extract of *Colletotrichum* sp. TSC 13 mycelia

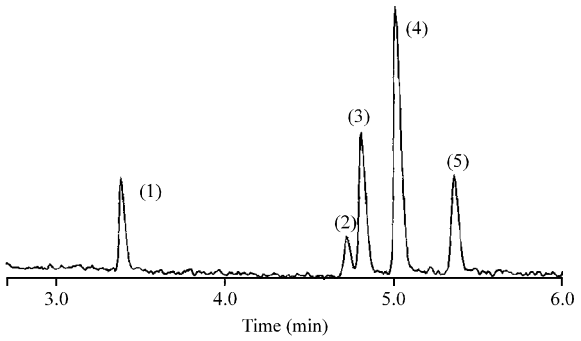

Fig. 2: Total ion chromatogram (TIC) of F3 (the active fraction obtained from column chromatography of *Colletotrichum* sp. TSC 13 *n*-hexane fraction), 1: Palmitic acid methyl ester, 2: Stearic acid methyl ester, 3: Oleic acid methyl ester, 4: Linoleic acid methyl ester, 5: Linolenic acid methyl ester

Table 3: Fatty acid methyl ester content of F3

Fatty acid methyl ester	Content (%)
Palmitic acid methyl ester	1.3
Stearic acid methyl ester	0.3
Oleic acid methyl ester	3.0
Linoleic acid methyl ester	5.7
Linolenic acid methyl ester	1.7

The active fraction obtained from column chromatography of *Colletotrichum* sp. TSC 13 *n*-hexane fraction

Table 4: The α-glucosidase inhibitory activity of fatty acids and fatty acid methyl esters standards and methylated F3 α-glucosidase assay at 10 μg mL^{-1} sample

Samples	Inhibition (%)
Palmitic acid	8.9±2.1
Stearic acid	5.3±1.6
Oleic acid	97.4±5.3
Linoleic acid	96.2±6.8
Linolenic acid	82.4±6.2
Mixed acids	88.9±8.8
Palmitic acid methyl ester	6.4±1.9
Stearic acid methyl ester	3.0±2.2
Oleic acid methyl ester	83.1±6.4
Linoleic acid methyl ester	78.1±5.2
Linolenic acid methyl ester	69.7±4.8
Mixed methyl esters	73.5±6.7
Methylated F3	71.7±3.9

Values are Mean±SD, Mixed acids: Mixture of palmitic acid, stearic acid, oleic acid, linoleic acid and linoleinic acid, Mixed methyl esters: Mixture of palmitica acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester and linoleinic acid methyl ester, F3: The active fraction obtained from column chromatography of *Colletotrichum* sp. TSC 13 *n*-hexane fraction

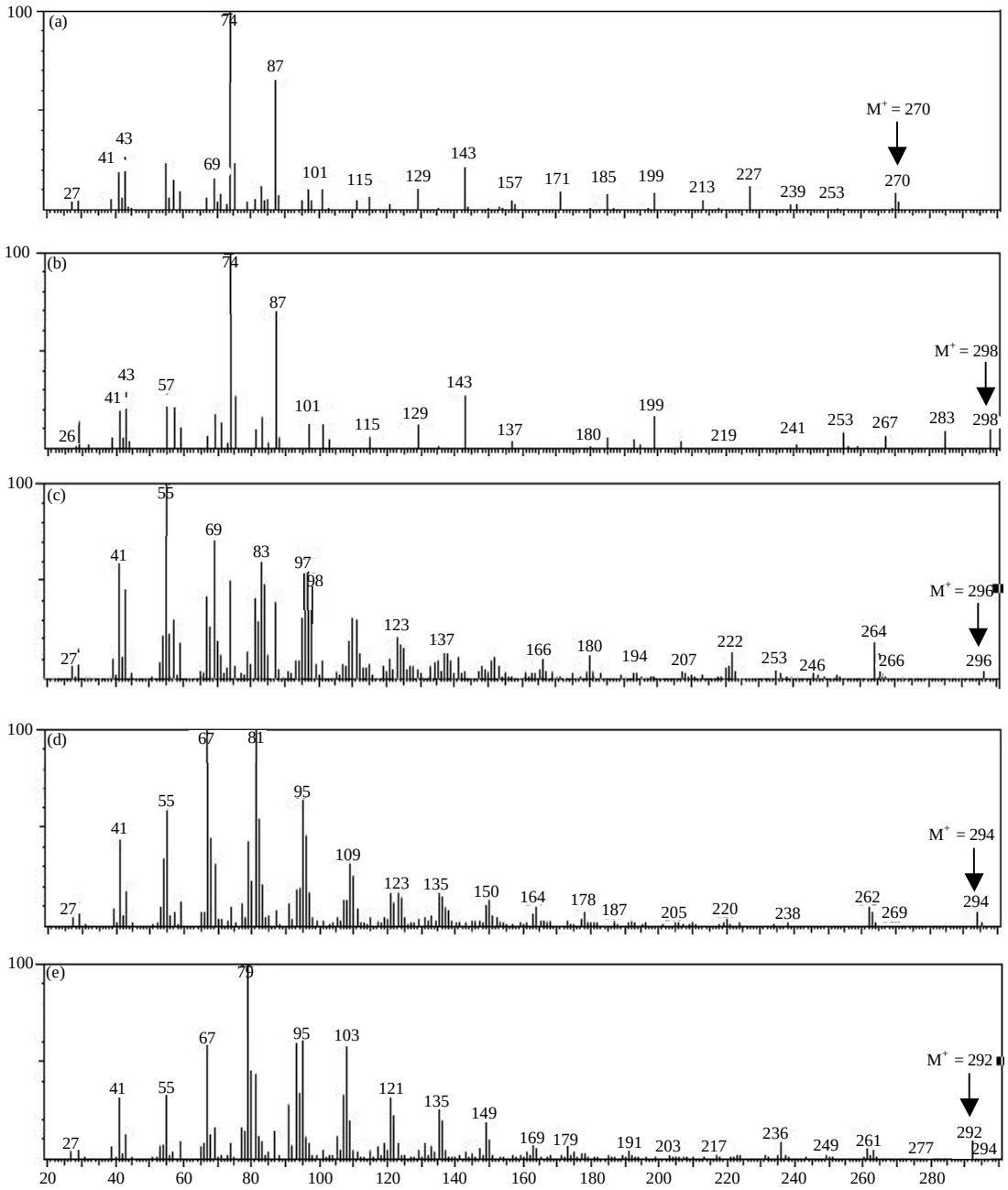

Fig. 3(a-e): MS spectra of fatty acid (a) Palmitic acid methyl ester (m/z 270), (b) Stearic acid methyl ester(m/z 298), (c) Oleic acid methyl ester (m/z 296), (d) Linoleic acid methyl ester (m/z 294) and (e)Linoleinic acid methyl ester (m/z 292) in F3 (the active fraction obtained from column chromatography of *Colletotrichum* sp. TSC 13 *n*-hexane fraction)

to 97.4±5.3% and for unsaturated fatty acid as free acids and methyl esters, whereas for saturated fatty acids, it was range from 3.0±2.2 to 8.9±2.1%. These three unsaturated fatty acids are commonly found in human diets. Conjugated linoleic acid may be important for treating obesity since it had suppressive effects on energy intake, showing inhibitory activity against adipocytes and induced apoptosis of maturing 3T3-L1 pre-adipocytes (Yun, 2010). Other unsaturated fatty acids reported to have α-glucosidase activity were

7(Z)-octadecenoic acid and 7(Z), 10(Z)-octadecadienoic acid from sea cucumber (Nguyen *et al*., 2011); 10-hydroxy-8(E)-octadecenoic acid: an intermediate of the bioconversion of oleic acid by *Pseudomonas aeruginosa* to 7,10-dihydroxy-8(E)-octadecenoic acid (Paul *et al*., 2010) and 1,2-dilinoleoylglycerol-3-phosphate and 1-palmitoyl-2-linoleoyl-glycero-3-phosphate from wheat germ (Liu *et al*., 2011).

The Thin Layer Chromatography (TLC) analysis showed that the methyl ester was present even before the methylation of the samples (MeOH extracts, *n*-hexane fraction and F3. This suggests that the mycelium might have both the methyl ester and free fatty acid forms. However, the methyl ester could be an artifact of the extraction process conducted using MeOH. This possibility was verified by extraction with *n*-hexane and EtOAc. TLC showed that the *n*-hexane extract did not have a methyl ester spot, however, the EtOAc extract did. Total Ion Chromatogram (TIC) profiles of the methylated *n*-hexane and EtOAc extracts are shown in Fig. 4. The TIC profiles showed different major peaks between *n*-hexane extract and EtOAc extract. The major peak in *n*-hexane extract is palmitic acid methyl ester whereas the major peak in EtOAc extract is linoleic acid methyl ester which as same as the major peak found in F3 (Fig. 2). The linolenic acid methyl ester peak is absent in *n*-hexane extract but present in EtOAc extract. Therefore, different solvent extraction may result in the extraction of different in fatty acids obtained. Further analysis of the amount and α-glucosidase inhibitory activity of unsaturated fatty acids from *Colletotrichum* sp. TSC13 by preparative TLC of the EtOAc extract is shown in Fig. 5, Table 5 and 6. Figure 5 shows the positions of the predicted methyl ester fraction and free acid fraction. Table 5 shows the percentage of fatty acids content of each fraction after methylation. The most FAME are present in F3 (46.8% to 77.8%) with the predicted free acid fraction, although, lesser amounts are also present in F1 (the predicted methyl ester fraction) and F2. The results are also supported by the α-glucosidase inhibitor assay results (Table 6). Therefore it could be suggested that the α-glucosidase inhibitor compounds in *Colletotrichum* sp.

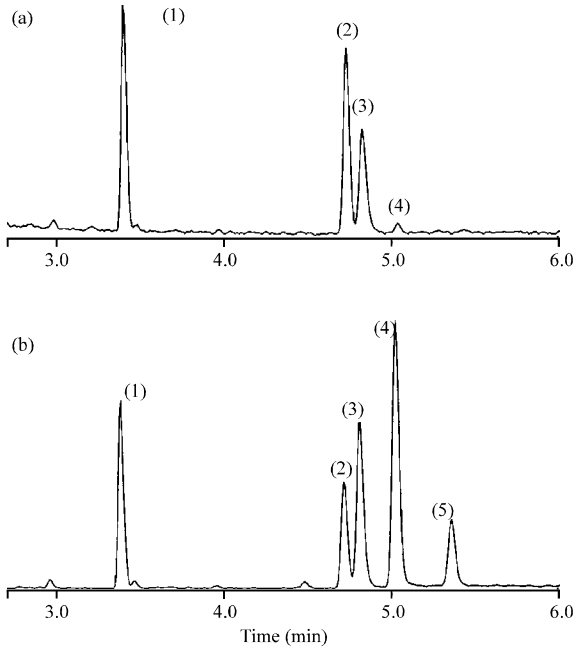

Fig. 4(a-b): Total ion chromatogram (TIC) of (a) *n*-hexane and (b) EtOAc extracts; 1: Palmitic acid methyl ester, 2: Stearic acid methyl ester, 3: Oleic acid methyl ester, 4: Linoleic acid methyl ester, 5: Linolenic acid methyl ester

Table 5: Fatty acid methyl esters in F1-4 of the EtOAc extract from *Colletotrichum* sp. TSC13

	Fatty acid methyl ester content (%)*				
Fractions	Pme	Sme	Ome	Lme	Llme
F1	15.0	20.9	19.8	19.3	18.5
F2	13.0	29.5	17.5	3.0	2.1
F3	67.8	46.8	58.8	76.1	77.8
F4	4.2	2.8	3.9	1.6	1.6

Pme: Palmitic acid methyl ester, Sme: Stearic acid methyl ester, Ome: Oleic acid methyl ester, Lme: Linoleic acid methyl ester, Llme: Linoleinic acid methyl ester, *Percentage of fatty acid methyl ester content in each fraction to fatty acid methyl ester content in EtOAc extract

Table 6: The α-glucosidase inhibitory activity of the EtOAc extract (EEA) and its fractions

Samples	Inhibition (%)
EEA	84.5±1.4
F1	82.5±1.0
F2	69.7±0.6
F3	90.3±0.8
F4	45.2±3.6

Values are Mean±SD, n = 2

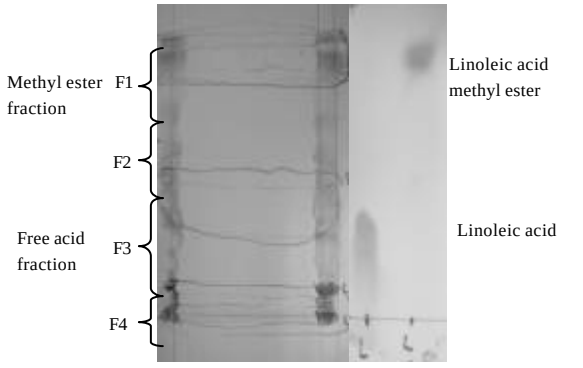

Fig. 5: Preparative TLC profile of the EtOAc extract from *Colletotrichum* sp. TSC 13 mycelium and TLC of linoleic acid and linoleic acid methyl ester standards, Visualization: H_2SO_4 spray+heating, Mobile phase: *n*-hexane:EtOAc = 4:1

TSC13 are the unsaturated fatty acid oleic acid, linoleic acid and linolenic acid mostly in the form of free acids and a lesser amount in the form of methyl esters which have potential as antidiabetic agents.

There are relatively few *Colletotrichum* studies on bioactive compounds as compared to publications on this species as a pathogen causing anthracnose disease in plants. Bioactive compounds isolated from *Colletotrichum* was reported being antimicrobial active against multidrug-resistant *Staphylococcus aureus* (Arivudainambi *et al*., 2011); a capthpesins B and L inhibitor (Otsuka *et al*., 1999a, b); a radical scavenger (Femenia-Rios *et al*., 2006; Tianpanich *et al*., 2011); colletotric acid as an antimicrobial (Zou *et al*., 2000); taxol (Gangadevi and Muthusamy, 2008; Kumaran *et al*., 2011); and monorden and monocillins I, II, III as antifungal agents (Wicklow *et al*., 2009). However, there is no report found about *Colletotrichum* sp. having antidiabetic activity or containing α-glucosidase inhibitors.

CONCLUSION

The results showed that the α-glucosidase inhibitors in *Colletotrichum* sp. TSC13 were the unsaturated fatty acids, namely, oleic acid, linoleic acid and linolenic acid. To best our knowledge, this is the first report of a-glucosidase inhibitors from endophytic fungus *Colletotrichum* sp. TSC13 isolated from *T. sumatrana*. Study on the media compositions that might affect the unsaturated fatty acid production in this fungus is currently being conducted.

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