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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Screening of Endophytic Fungi Having Ability for Antioxidative and α -Glucosidase Inhibitor Activities Isolated from *Taxus sumatrana*

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Abstract: Endophytic microbes are considered as an important source of natural products. They show antibiotic, anticancer, antioxidative and antidiabetic activities. Therefore, there are many reports on the isolation and bioactivity screening of endophytic fungi from various plants including *Taxus* species. *Taxus sumatrana* (Miq.) de Laub is found in Indonesia. The objective of this study is to conduct an *in vitro* screening of 14 endophytic fungi isolated from *Taxus sumatrana* having antioxidative and α -glucosidase inhibitor activities. Each endophytic fungus was cultured for 7 days and the fungal mycelium and medium were extracted with methanol and ethyl acetate, respectively, to produce each extract. The antioxidative activity of each extract was tested by DPPH free radical scavenging activity and β -carotene bleaching assays, whereas antidiabetic activity was tested based on α -glucosidase inhibitor activity. The screening results showed that fungal mycelia of TSC 13 had the best α -glucosidase inhibitor activity and TSC 24 had the best antioxidative activity. Isolation of bioactive compounds from TSC 13 and TSC 24 is being conducted. This is the first report that endophytic fungi isolated from *T. sumatrana* exhibited anti α -glucosidase inhibitory and anti oxidative activities.

Key words: Endophytic fungi, DPPH free radical scavenging activity, β -carotene bleaching assay, α -glucosidase inhibitor activity

INTRODUCTION

Endophytic microbes are considered an important source of natural products. They exhibit a variety of biological activities such as antibiotic, anticancer, antioxidative and antidiabetic effects. Therefore, there are many reports on the isolation and bioactivity screening of endophytic fungi from various plants including *Taxus* species (Schulz *et al.*, 2002; Strobel, 2003, 2006; Suriyanarayanan *et al.*, 2009). Taxol is an anticancer agent first isolated from *Taxus brevifolia* (Wani *et al.*, 1971). The production of taxol was also reported in endophytic fungi from various *Taxus* species. *Taxomyces andreanae* from *Taxus brevifolia* (Stierle *et al.*, 1993), *Pestalotiopsis microspora*, *Sporormia minima* and *Trichothecium* sp. from *Taxus wallichiana* (Shrestha *et al.*, 2001; *Pestalotiopsis versicolor* and *Pestalotiopsis neglecta* from *Taxus cuspidata* (Kumaran *et al.*, 2010) are reported produced taxol. Other biological activities also reported from *Taxus* endophytic fungi. *Fusarium* sp. from *Taxus wallichiana* and *Taxus baccata* showed antifungal (Gogoi *et al.*, 2008) and antibacterial properties

(Gogoi *et al.*, 2008; Tayung and Jha, 2010). *Taxus sumatrana* (Miq.) de Laub is a *Taxus* species grown in Indonesia. Although, it is also reported to grow in Afghanistan, Tibet, Nepal, India, Bhutan, Burma, China, Vietnam, the Philippines and Taiwan (Books, 2010; Surhone *et al.*, 2011). There are several publication about compounds isolated from *T. sumatrana* grown in Taiwan such as taxol (Kitagawa *et al.*, 1995); 9,13-diacetyltaxumairol W; 10,13-dibenzoyltaxacustin; 7,13-deacetylwallifoliol; 7,13-dibenzoylwallifoliol; 7,9-dibenzoyl-taxumairolP (Shen *et al.*, 2002); taxumairol Q 13-O-acetyl walliofoliol (Shen *et al.*, 2002); tasumatrol A and B (Shen *et al.*, 2003) and tasumatrol E, F and G. (Shen *et al.*, 2005). Here we will report the screening of endophytic fungi with antioxidative and antidiabetic inhibitor activities isolated from this species.

MATERIALS AND METHODS

This research was conducted at the Faculty of Agriculture, Ehime University and Research Centre for Chemistry, Indonesian Institute of Sciences (LIPI) in 2010.

Fungi used for tests: Endophytic fungi were isolated from the stem and leaves of *T. sumatrana* according to the method in Tanaka *et al.* (1999). The plant materials were surface sterilized and placed in a petri dish containing cornmeal-malt extract agar (CMM) containing chloramphenicol 50 g L⁻¹. Fungi were isolated and transferred to fresh media. Isolated endophytic fungi were maintained on Potato Dextrose Agar (PDA) agar slants. The fourteen endophytic fungi used in this experiment (TSC 1, 2, 3, 7, 8, 12, 13, 14, 17, 22, 23, 24, 26 and 28) were isolated by Ms. Harmastini Sukiman (Research Centre for Biotechnology, Indonesian Institute of Sciences) from *Taxus sumatrana* (Miq.) de Laub grown in Cibodas Botanical Garden (LIPI), Indonesia, collected in 2005.

Fermentation: Endophytic fungi stock cultures grown on agar slants of PDA (potato dextrose agar) medium (Tanaka *et al.*, 1999) were transferred to petri dishes containing the same medium and cultured for 7 days. Three agar dishes were transferred to 30 mL of PDB (potato dextrose broth) medium in a 150 mL flask and cultured for 7 days for screening of antioxidative and antidiabetic activities.

Sample preparation: Each culture incubated for 7 days was filtered to separate the mycelium and filtrate (filtered medium). The mycelium was air dried and then extracted with 50 mL of methanol for 3 days to give the mycelium extract. The filtered medium was directly used for antioxidative and antidiabetic assays. Furthermore, the medium was also extracted with an equal volume of ethyl acetate for 3 days. The extracted solution was evaporated under reduced pressure by rotary evaporator to give 2 mL of concentrate.

Assay for 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity: The assay for DPPH free radical-scavenging was conducted according to Lelono *et al.* (2009). The control, standard and samples (media, each extract of mycelium and medium) were individually added to 3 mL of 0.004% MeOH solution of DPPH. Absorbance at 517 nm was measured under constant mixing at room temperature after 30 min and percent inhibitory activity (free radical scavenging activity) was calculated from:

$$\frac{(A_0 - A_1)}{A_0} \times 100$$

where, A₀ is the absorbance of the control (MeOH) and A₁ is the absorbance of the samples or standard

(in MeOH). The standards used for positive DPPH free radical scavengers were Vitamin C and quercetin at 1 mg mL⁻¹.

Beta-carotene bleaching assay: The beta carotene bleaching assay was conducted according to Lelono *et al.* (2009). The absorbance was measured at 470 nm against a blank which was an emulsion without β-carotene. Incubation at 50°C for 60 min was followed by absorbance reading at 470 nm. The control was MeOH and the standard samples used were Vitamin C and quercetin.

Alpha-glucosidase inhibitory assay: The alpha-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 20 mM pNPG (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 (10 mg mL⁻¹) followed by 15 min incubation at 37°C. The reaction was stopped by addition of 2.5 mL of 200 mM Na₂CO₃. The absorbance of p-nitrophenol released from PNPG at 400 nm was measured with a spectrophotometer. The standard sample used for the positive α-glucosidase inhibitor was quercetin (1 mg mL⁻¹).

RESULTS AND DISCUSSION

Antioxidative activity: For this study, the antioxidative activity of the endophytic fungi extract was measured using DPPH free radical scavenging activity and β-carotene bleaching assay. The DPPH method is based on the ability to scavenge the free radical DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the non radical form DPPH-H (Shon *et al.*, 2003). The β-carotene method is based on the presence of an antioxidant that can delay β-carotene bleaching by neutralizing the linoleic acid radicals that could attack the highly unsaturated β-carotene (Shon *et al.*, 2003). Vitamin C and quercetin (a flavonoid), two natural antioxidants, were used as standards. The medium from all endophytic cultures tested directly as sample did not show any antioxidative activities in the DPPH and β-carotene assays. Table 1 shows the screening results of antioxidative activities of mycelium and medium extracts of all the endophytic fungi tested. Figures 1 and 2 show DPPH and β-carotene assays of fungal mycelium and medium extracts of TSC13 and TSC24 at different sample concentrations. As shown in Table 1, both standards have high antioxidative activity to scavenge DPPH free radicals, on the contrary, only quercetin show good antioxidative activity in the β-carotene assay. Under the

Table 1: The results of antioxidant and antidiabetic activities of endophytic fungi from *Taxus sumatrana*

Samples	DPPH		β -Carotene		α -Glucosidase	
	% inhibition		% inhibition		% inhibition	
	FB	M	FB	M	FB	M
TSC1	24.7 \pm 3.3	nd	64.3 \pm 3.1	67.2 \pm 2.4	nd	nd
TSC2	14.8 \pm 2.1	10.1 \pm 1.23	44.8 \pm 2.6	42.8 \pm 2.6	nd	nd
TSC3	18.3 \pm 3.1	16.1 \pm 2.17	71.2 \pm 3.5	55.4 \pm 1.84	2.7 \pm 0.94	nd
TSC7	15.7 \pm 1.0	nd	64.6 \pm 2.1	69.9 \pm 2.18	nd \pm	nd
TSC8	6.3 \pm 1.2	nd	55.9 \pm 2.4	43.4 \pm 2.27	nd	nd
TSC12	2.5 \pm 1.1	nd	73.9 \pm 3.2	43.6 \pm 2.41	55.3 \pm 2.31	nd
TSC13	2.3 \pm 1.2	3.9 \pm 1.09	61.1 \pm 1.4	64.9 \pm 2.08	79.5 \pm 2.62	18.2 \pm 1.67
TSC14	5.2 \pm 1.0	nd	75.8 \pm 4.0	54.4 \pm 1.35	45.3 \pm 1.3	nd
TSC17	11.8 \pm 2.4	nd	55.6 \pm 2.3	63.5 \pm 2.66	68.4 \pm 2.15	nd
TSC22	10.4 \pm 1.4	nd	74.3 \pm 2.5	78.7 \pm 3.08	nd	nd
TSC23	0.7 \pm 0.8	nd	65.4 \pm 1.9	50.1 \pm 02.1	66.3 \pm 1.88	18.6 \pm 2.04
TSC24	69.6 \pm 3.01	36.2 \pm 2.67	85.9 \pm 2.6	86.8 \pm 2.33	4.7 \pm 1.02	nd
TSC26	18.6 \pm 2.05	nd	77.6 \pm 1.5	39.1 \pm 1.27	nd	nd
TSC28	6.6 \pm 1.05	nd	48.0 \pm 1.0	79.2 \pm 1.01	2.4 \pm 1.25	nd
Vitamin C	92.7 \pm 2.06	-	nd	-	-	-
Quercetin	93.8 \pm 1.18	-	56.8 \pm 2.12	-	56.5 \pm 1.33	-

Antioxidant activities showed DPPH free radical scavenging activity and β -carotene bleaching assay. Antidiabetic activity showed α -glucosidase inhibitory activity. Value is shown as average of duplicate measurements \pm standard deviation. FB: Fungal mycelium MeOH extract, M: Fungal medium EtAc extract, nd: Not detected)

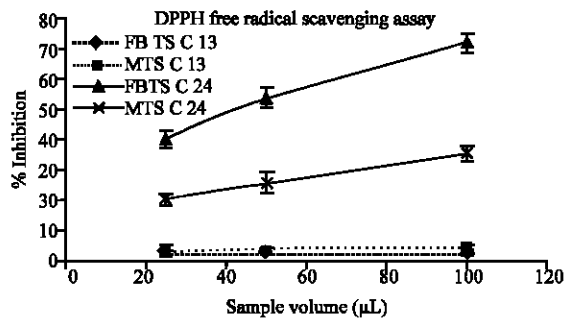


Fig. 1: DPPH assay of TSC13 and TSC24 Note: FB and M refer to Table 1

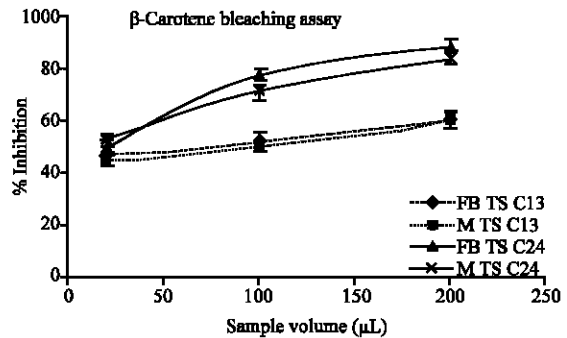


Fig. 2: β -Carotene bleaching assay of TSC13 and TSC24 Note: FB and M refer to Table 1

same culture conditions, the antioxidative activity of the endophytic fungi showed very high variation for both DPPH and β -carotene assays. In the DPPH assay, the fungal mycelium MeOH extract showed very high variation (0.7-69.6 %) in activity and only 4 samples of fungal medium EtAc extract showed activity. This result suggested that in most cases, antioxidants that have the ability to scavenge DPPH were not released into the medium. In the β -carotene bleaching assay, both the fungal mycelium MeOH extract (44.8 - 85.9%) and fungal medium EtAc extract (39.1 -86.8%) showed high activity. This result suggested that endophytic fungi produce antioxidants that could delay the β -carotene bleaching. Both antioxidants in the mycelium and fungal medium might also be released into the medium, or the compounds released into the medium could be different to remain in the mycelium. Figure 1 shows that only TSC24 had good antioxidative activity as a DPPH free radical scavenger.

However, in Fig. 2 both TSC13 and TSC24 have significant antioxidative activity that could delay β -carotene bleaching. Antioxidative activity also has been reported from various endophytic fungi such as *Pestalotiopsis microspora* isolated from *Terminalia catapa* (Strobel *et al.*, 2002); *Corynespora cassiicola* L36 (Chomcheon *et al.*, 2009); endophytic fungi isolated from Ginkgo biloba *Xylaria* sp. (Liu *et al.*, 2007) and *Aspergillus nidulans* and *A. oryzae* (Qiu *et al.*, 2010). The endophytic fungus TSC24 showed the highest DPPH free radical scavenging activity (69.6% for mycelium and 36.2% for medium) and also the highest activity for β -carotene (85.9% for mycelium and 86.2% for medium). Therefore, TSC 24 was selected for further study to isolate the antioxidants of this culture.

Antidiabetic activity: For this study antidiabetic activity of the endophytic fungi extract was measured using the

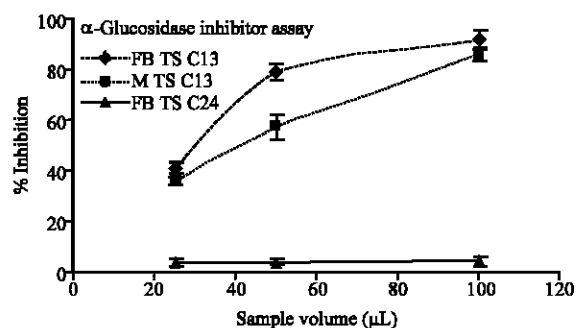


Fig. 3: α -Glucosidase inhibitor assay of TSC13 and TSC24 Note: FB and M refer to Table 1

α -glucosidase inhibitor assay. Medium from all endophytic cultures tested directly as sample did not show any inhibitory activity toward α -glucosidase activity. Table 1 shows the screening results of antidiabetic activities of mycelium and medium extracts of all the endophytic fungi tested. Figure 3 shows α -glucosidase inhibitor assay of fungal mycelium and medium extracts of TSC13 and mycelial extracts of TSC24 at different sample concentrations. Under the same culture conditions, α -glucosidase inhibitor activity of the endophytic fungi showed only in the extracts of 8 fungal mycelia (2.4- 89.5%) and 2 fungal media (18.2 and 18.6%). This result suggested that α -glucosidase inhibitor compounds present mainly in the mycelium did not released or had only a very limited release into the medium. α -Glucosidase inhibitors have been isolated from microbes including fungi such as *Aspergillus terreus* (Dewi *et al.*, 2007); *Bacillus subtilis* B2 (Zhu *et al.*, 2008); *Aspergillus aculeatus* (Ingavat *et al.*, 2009). Although, many studies for α -glucosidase screening also conducted from various plant species, a commercial α -glucosidase inhibitor, acarbose (Glucobay or Precose), is produced as a secondary metabolite on a large scale from fermentation cultures of *Actinoplanes* sp. SE-50. (De Melo *et al.*, 2006). As the endophytic fungus TSC13 showed the highest α -glucosidase inhibitory activity (79.5% for mycelium and 18.2% for medium), it was selected for further study to isolate the potential antidiabetes compounds of this culture.

ACKNOWLEDGMENTS

The authors would like to thank JSPS (The Japan Society for the Promotion of Science) under the Ronpaku Dissertation Ph.D. program for providing fellowship during Fiscal Year 2010 to conduct research in the Faculty of Agriculture, Ehime University, Japan and the Research Centre for Chemistry-LIPI, Indonesia.

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