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Alpha-Amylase Inhibition Activity of Endophytic Fungi Isolated from Leaves of *Artocarpus heterophyllus*

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Abstract: ***The present study aimed to investigate the alpha-amylase inhibition activity of endophytic fungi isolated from the senescent leaves of Artocarpus heterophyllus. Surface sterilized leaf segments were placed on potato dextrose agar (PDA) and malt extract agar base (MEAB) media. Primary fungal cultures were sub-cultured to obtain the pure cultures. Ethyl acetate fungal extracts were tested using the alpha-amylase inhibition assay. The highest active fungal extract was subjected to bio assay guided fractionation and the percentages of alpha-amylase inhibition of those fractions were also determined. Three different endophytic fungi were isolated using the PDA and MEAB. The highest antidiabetic active fungal extract showed 89±2.0% alpha-amylase inhibition activity at 5000 ppm which was higher than that of the standard drug acarbose (71± 2.5%) at 5000 ppm. The highest antidiabetic active fungal extract also showed an increasing alpha-amylase inhibition activity when concentration was increased from 1000 ppm to 5000 ppm. Four fractions were obtained by normal phase silica gel column chromatography, based on the TLC profiles. Out of them three fractions showed more than 50% alpha-amylase inhibition activity at 5000 ppm concentration.This study showed that the ethyl acetatecrude extract of isolated endophytic fungus showed promising antidiabetic activity.***

Keywords: endophytes, fungal extract, antidiabetic, alpha-amylase inhibition, *Artocarpus heterophyllus*

1. Introduction

1.1 Endophytes

Endophytes are endosymbiotic organisms, which colonize within the healthy internal tissues of the host plant for at least part of its life cycle without causing any apparent harm to the host[1]. Edophytes are physiologically and ecologically important to the host plant and they are rich sources of secondary metabolites. Endophytes can produce a wide variety of bioactive compounds that might be useful as biopharmaceuticals such as antimicrobial, antioxidant, anticancer and antidiabetic agents. Isolation of bioactive compounds from endophytic fungi plays a significant role in drug discovery. Drug production by the endophytic fungi gives more advantages because endophytic fungi are unlimited, reproducible and season independent [2].

1.2 Diabetes mellitus

Diabetes mellitus is one of the most common metabolic disorders which affect hundreds of millions of people in the world today. The inherited or acquired deficiency in production of insulin by the pancreas and the ineffective insulin production by the pancreas are the major reasons for diabetes mellitus. These reasons can lead to poor secretion of insulin hormone or poor response of target cells to insulin, or combination of both factors. Control of the carbohydrate digestion and monosaccharide absorption can be used to treat the diabetes mellitus [3]. The alpha-amylase and alpha-glycosidase inhibitors are anti-nutritive agents that can be used to prevent the digestion and absorption of the carbohydrates. Acarbose is one of the most commonly used synthetic alpha-amylase and alpha-glycosidase inhibitors that can delay the digestion of the carbohydrates [4].

1.3 Artocarpus heterophyllus

Artocarpus heterophyllus is a Moreceae family plant which can be used as a rich source of antidiabetic, antimicrobial, antioxidant and antibacterial agents [5].Matured leaves of *Artocarpus heterophyllus*are used in Ayurvedic medicine in Sri Lanka as an antidiabetic medicine.

The different studies have carried out to determine the antidiabetic activity of *A. heterophyllus*. Chackrewarthy et al. [6]conducted studies to determine the antidiabetic activity of ethyl acetate and aqueous extracts of *A. heterophyllus* leaves using the streptozotocin-induced diabetic rats. They reported that ethyl acetate and aqueous fractions of *A. heterophyllus* leaves can significantly lower fasting blood glucose levels by 42.5% and 28.7%, respectively.

According to the results of another study conducted by Omar et al.[3], the ethanolic and n-butanol leaf extracts of *A. heterophyllus* exert a significant antidiabetic effect in streptozotocin-induced rats. They have isolated antidiabetic active flavnoid, isoquercitrin from the leaf extract and it was confirmed using the spectrophotometric analysis.

Another study has been carried out by Chandrika et al. [7] to determine the hypoglycemic action of the flavonoid fraction of *A. heterophyllus* leaves. This group has investigated that the effective dose of the flavonoid fraction of the leaf extract was 50 mg/kg for normal and alloxan-diabetic rats and the therapeutic effect was obtained after 2 hours of administration. This group has determined the toxicological effects which can occur due to the long term administration of the ethyl acetate fraction of the leaf extract and observed that there was no significant effect on liver, kidney and heart function. Therefore, this group has claimed that flavonoid fraction of *A. heterophyllus* leaves is a safe hyperglycemic active extract.

2. Materials and Methods

2.1 Collection of samples

The senescent leaves of *A.heterophyllus* obtained from Rukmalgama, Sri lanka was used for isolation of endophytic fungi.

2.2 Isolation of endophytic fungi from the senescent leaves of *A. heterophyllus*

The senescent leaves of *A. heterophyllus* were surface sterilized as follows. Leaves were washed thoroughly under running tap water for 10 minutes. After draining off the water plant leaves were immersed in 70% ethanol for 1 minute while gently agitating. Then the leaves were immersed in 5% NaOCl household bleach solution. After that, leaves were again immersed in 70% ethanol for 30 seconds and excess ethanol was drained off. Finally the leaves were washed thoroughly with sterile distilled water for several times and placed on sterile filter paper to dry.

Leaves were cut into 0.5 cm^2 segments and kept in the petri plates with potato dextrose agar (PDA) and malt extrct agar base (MEAB) media. Petriplates were sealed using the parafilm and incubated at room temperature. To obtain pure cultures, primary cultures were subcultured in growth media.

2.3 Microscopic observation of endophytic fungi

A drop of lactophenol cotton blue stain was placed on a clean microscopic slide. Using an inoculating loop, a loopful culture of mycelium was collected from the petri plate and placed it on the drop of lactophenol cotton blue on a slide. A cover slip was placed over the specimen and slide was observed under the light microscope.

2.4 Preparation of crude extract

The fungal mycelia and the agar media were cut into small pieces and transferred into a beaker containing ethyl acetate. It was subjected to sonication in an ultrasonic bath at 110 V for 30 minutes. Extracts were filtered through cotton wool into a conical flask and the extraction was repeated twice using the same procedure. All three extracts were combined and concentrated by rotary evaporation below 40 °C till they become completely dry and then weight of the dried residues were obtained.

2.5 Alpha-amylase inhibition assay

Antidiabetic activity of each sample was determined using the alpha-amylase inhibition assay. Alpha-amylase solution (1 mg/ml) was prepared using 0.02 M buffer solution. Fungal extracts (5 mg) were dissolved in distilled water with 1% DMSO (10 ml) (5000 ppm) separately. Alpha-amylase solution (100 µl) was mixed with fungal extract (100 µl) and incubated at 25 °C for 30 minutes. After incubation, above solution was (100 µl) with 1 mg/ml concentration was mixed with 0.5% w/v starch solution (100 µl) and incubated at 25 °C for 3 minutes. DNSA reagent was added to the solution and incubated at 85 °Cin a water bath for 15 minutes. Then this solution was allowed to cool to room temperature and diluted with distilled water (900 µl). Negative controls were conducted in the same manner replacing plant extracts with 1% DMSO (100 µl) in distilled water.

Blanks were prepared by adding DNSA reagent prior to the addition of starch solution to denature the enzyme. It was also kept in 85 °C water bath for 15 min and then diluted with distilled water (900 µl) as before. Absorbance was measured at 540 nm wavelength. Acarbose (5000 ppm) was used as the positive control. The percentage inhibition of alpha-amylase activity was calculated using the following equation [8].

$$\text{Inhibition percentage} = \frac{A^{\circ} - A}{A^{\circ}} \times 100\%$$

A° - Absorbance of the control; A - Absorbance of the sample

The highest antidiabetic active fungal extract was identified and that fungus was grown in large scale for further studies. The percentage of alpha-amylase inhibition of the highest antidiabetic fungal extract was also determined for concentration series from 1000 ppm to 5000 ppm compared with standard drug acarbose.

2.6 Separation of the highest antidiabeticative crude extract using bio assay guided fractionation

The highest active fungal extract was subjected to bio assay guided fractionation using the normal phase column chromatography(ethyl acetate: dichloromethane: methanol: formic acid; 58:38:2:2). Fractions with similar TLC profiles were combined (Fraction A, B, C, D) and alpha-amylase inhibition activity was determined.

3. Results and Discussion

Two fungi (F1, F2) were isolated in the PDA media and three fungi (F1, F2, and F3) were isolated using the MEAB media. According to the morphology and microscopic view, two fungi grown in MEAB media were appeared as same as F1 and F2 isolated from the PDA media. In addition to those two fungi, fungus F3 was also isolated in MEAB media.

3.1 Macroscopic view of endophytic fungi isolated from *A. heterophyllus*

F1 fungus culture contained dense, white and greencolour mycelia with concentric zonation. Smooth margins were present in F1 and green colour pigmentations were also appeared (Figure:1a). F2 fungus culture had cottony, white to pale pink mycelium with orange conidial masses produced on the colony. Smooth margins were present in F2 fungus without the zonation and pink colour pigmentations were also present(Figure: 1b). F3 fungus culture contained dense, blackish white mycelia with few orange colour conidial masses near to the inoculum point. In F3 culture concentric zonation with smooth margins and black colour pigmentations were also present (Figure: 1c).

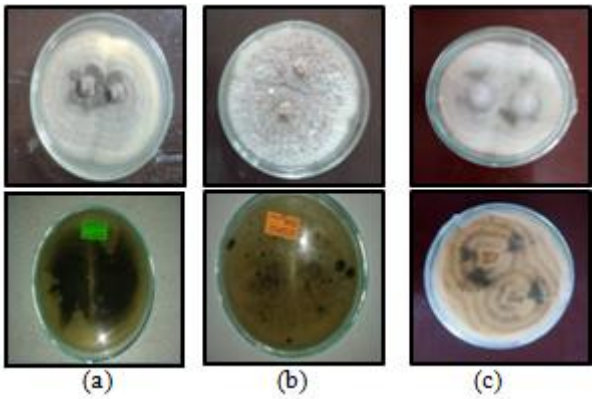

Figure 1: Macroscopic view of the endophytic fungi isolated from senescent leaves of *Artocarpus heterophyllus* after two weeks; (a) F1, (b) F2 (c) F3

3.2 Microscopic view of endophytic fungi isolated from *A. heterophyllus*

Under the microscopic appearance F1 fungus showed hyaline, branched, septatehyphae. Further, there were septate conidiophores with pigmented oblong shaped spores (Figure: 2a).Under the microscopic appearance F2 fungus showed pigmented, branched, aseptatehyphae. Further, there were cylindrical pigmented conidia (Figure; 2b). According to the morphological characteristics reported in literature, F2 fungus was preliminary identified as a fungus belongs to the genus *Colletotrichum* [9].Under the microscopic appearance F3 fungus showed pigmented, branched, aseptate hyphae. Further, there were cylindrical pigmented conidia (Figure: 2c).

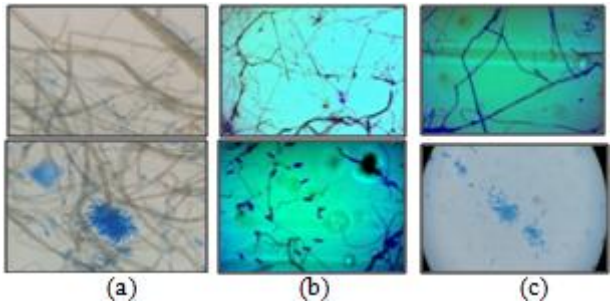

Figure 2: Microscopic view of the lactophenol cotton blue stained fungi: (a) F1(b) F2 (c) F3 (magnification 10×40)

3.3 Alpha-amylase inhibition assay

The highest percentage of alpha-amylase inhibition activity was shown by F2 at 5000 ppm. F3 showed the next highest percentage inhibition of alpha amylase activity at 5000 ppm. However, F1 showed a minute percentage of alpha-amylase inhibition compared to the other two fungi. F2 showed higher percentage of alpha-amylase activity than the standard drug acarbose at 5000 ppm. F1 and F3 showed lower percentage of alpha-amylase inhibition activity than the standard drug acarbose at 5000 ppm (Table 1).

Table 1: Percentage inhibition of the alpha-amylase inhibitory activity of the fungal crude extracts at 5000 ppm

Fungi type	Average % inhibition
F1	4.7 ± 2.1
F2	89 ± 2.0
F3	34 ± 4.0
Acarbose (positive control)	71 ± 2.5

F2 fungal extract and acarbose showed a increasing alpha-amylase inhibition activity when the concentration was increased from 1000 ppm to 5000 ppm. At the 3000 ppm, 4000 ppm and 5000 ppm concentrations, the alpha-amylase inhibition activity of F2 extract was higher than that of acarbose(Table 2). However, 1000 ppm and 2000 ppm concentrations the alpha-amylase inhibition activity of fungal extract is lower than that of acarbose.

Table 2: The percentage inhibition of alpha-amylase activity of F2 extract and acarbose at different concentrations from 1000 ppm to 5000 ppm

Concentration (ppm)	Average % inhibition of fungal extract	Average % inhibition of Acarbose
1000	6.87 ± 2.32	10.3 ± 0.509
2000	10.9 ± 1.42	25 ± 1.5
3000	70 ± 3.0	48 ± 1.5
4000	80 ± 4.6	67 ± 1.7
5000	89 ± 2.0	71 ± 2.5

3.4 Bio-assay guided fractionation

Normal phase column chromatography was conducted using 500 mg of the crude extract of F2. Twenty five fractions were collected after running the column using ethyl acetate:dichloromethane:methanol:formic acid (58:38:2:2) solvent system. Four fractions (A-D) were obtained after combining the fractions with similar TLC profiles. An intense spot with R_f = 0.95 appeared in fraction A under 254 nm UV wavelength. Fraction B showed several spots in the TLC. Two intense spots with R_f = 0.68, 0.58 were appeared in fraction C under 254 nm wavelength. Further three intense spots were appeared in the fraction D with R_f = 0.68, 0.58, 0.49 values (Table 3).

Table 3: Results obtained from two trials of column chromatography

	Trial 1			Trial 2		
	Dry weight/ mg	Appearance	R_f value	Dry weight/ mg	Appearance	R_f value
A	98	Pale yellow oily solid	0.95	103	Pale yellow oily solid	0.95
B	108	Dark yellow solid	Complicated TLC profile	110	Dark yellow solid	Complicated TLC profile
C	46	Brown solid	0.68	68	Brown solid	0.68
			0.58			0.58
D	67	Dark yellow solid	0.68	72	Dark yellow solid	0.68
			0.58			0.58
			0.49			0.49

According to the results in Table 4, fractions B showed the highest and fraction D showed the lowest percentage of alpha-amylase inhibition activity. The percentage of alpha-amylase inhibition activities of fraction A and C were nearly equal. Fraction B showed higher antidiabetic activity than that of the standard drug acarbose where as other fractions showed lower percentage of alpha-amylase inhibition activity.

Table 3: Percentage alpha-amylase inhibition activity of fractions A-D obtained from the column chromatography of F2 at 5000 ppm concentration

Fraction	Average % of Inhibition
A	65 ± 3.1
B	89 ± 1.5
C	65 ± 2.0
D	27 ± 3.5
Positive control (Acarbose)	71 ± 2.5

4. Conclusion

Three morphologically different endophytic fungi could be able to isolate from the senescent leaves of *Artocarpus heterophyllus* using PDA and MEAB media. The crude extract of F2 showed the highest alpha-amylase inhibition activity among the isolated fungi. The crude extract of endophytic F2 could be considered as a promising candidate for isolation of antidiabetic active secondary metabolites. Further studies should be conducted to isolate the active pure compounds from the fungal extract and identify them using the spectroscopic analysis.

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