Original Article

In Vitro Pharmacological Activity of Endophyte *Aspergillus austwickii* isolated from the Leaves of *Premna serratifolia*

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ABSTRACT

Diabetes mellitus is a widely occurring non-communicable disease that is rapidly spreading worldwide. It results from the dysregulation of glucose in the bloodstream or the improper functioning of the enzymes α-amylase and α-glucosidase. Endophytes are microorganisms that reside symbiotically within the living tissues of plants. Endophytic fungi possess the ability to synthesize various important bioactive metabolites. The current study aims to explore the less-reported endophytic fungus *Aspergillus austwickii*, isolated from the ethnobotanical medicinal plant *Premna serratifolia* L. The methanolic extract of the endophytic fungus was subjected to *in vitro* antioxidant assays, anti-inflammatory assays, and antidiabetic assays, in addition to analyzing the total phenolic and total flavonoid content, along with the phytochemicals present. The results revealed that *Aspergillus austwickii* contains phytoconstituents such as alkaloids, phenols, flavonoids, tannins, and carbohydrates. The total phenolic content and total flavonoid content of the fungus were found to be 22.048 µg GAE/g and 18.828 µg QE/g, respectively. The crude extract demonstrated 46.20±0.53% antioxidant activity, with an IC50 value of 128.69 µg/mL for radical scavenging as determined by the DPPH assay. It also exhibited 71.86±0.34% anti-inflammatory activity in the protein denaturation assay. Notably, it displayed antidiabetic activity against both α-amylase and α-glucosidase, with percentage inhibitions of 68.22±0.17% and 73.72±0.18%, and IC50 values of 178.10 µg/mL and 166.16 µg/mL, respectively. The current study indicates that the methanolic extract of *Aspergillus austwickii* possesses significant antioxidant, anti-inflammatory, and antidiabetic properties. The findings of this research can inform future studies aimed at discovering new natural drugs to combat various disorders.

Keywords: Antioxidant, Alpha (α) amylase, Alpha (α) glucosidase, Anti-Inflammatory Activity.

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1. Introduction

The World Health Organization (WHO) recognizes diabetes mellitus as one of the most prevalent chronic non-communicable diseases. It is considered a significant lifestyle disorder that severely impacts overall health and is regarded as a global epidemic, particularly affecting urban populations (1). In India, approximately 40.9 million individuals are affected, with estimates suggesting this number may rise to nearly 70 million by the year 2025 (2). Diabetes mellitus is a metabolic disorder characterized primarily by elevated blood sugar levels, which, over time, can lead to serious damage to vital organs, including the heart, eyes, kidneys, blood vessels, and nerves. A major challenge in treating this disease is the normalization of blood glucose levels in affected patients. Achieving a disease-free state requires the control or prevention of monosaccharide absorption and the regulation of carbohydrate-hydrolyzing enzymes. Two key enzymes, α-amylase and α-glucosidase, are essential for converting complex dietary sugars into simpler sugars that can be readily absorbed by the digestive tract. These enzymes help reduce elevated postprandial blood glucose levels, thereby regulating insulin action (3). Current treatments typically involve synthetic drugs, which are often associated with side effects. Therefore, there is a pressing need to explore safer alternatives to synthetic medications. Recently, plants with biological activities have emerged as promising candidates for addressing these concerns. Given the significant health implications, there is a need to identify alternative sources of secondary metabolites that are both beneficial and have fewer side effects. Endophytic fungi are microorganisms that inhabit plants, forming a symbiotic relationship with the host. They spend part or all of their life cycle within the host plant, obtaining nutrients while promoting the host's growth through the synthesis of bioactive compounds (4, 5). These interactions enable endophytes to produce a wide array of bioactive compounds with antimicrobial (6), antiviral (7), antioxidant (8), and antidiabetic properties (9). As such, these bioactive compounds are gaining prominence in the pharmaceutical, medical, and agricultural industries. Given these attributes, this study aims to explore the properties of the less-reported endophytic fungus *Aspergillus austwickii* for its antioxidant and *in vitro* biological activities.

2. Materials and Methods

2.1. Isolation, Molecular Identification and Mass Cultivation

The leaves of *Premna serratifolia* L. were collected, thoroughly washed in gently running tap water to remove adhering dirt particles, and then cut into small pieces. These pieces were incubated on solid media for 2-5 days (10). The cultured organism was undertaken for molecular identification using 18S rRNA sequencing. A phylogenetic tree was constructed using MEGA X software by comparing the homologous sequence with the query sequence in NCBI BLAST (11). The endophytic fungus was mass cultured by growing it in liquid media for 21 days. The mycelium was separated and subjected to solvent extraction using methanol. The extract was obtained by evaporating the solvent in a rotary evaporator at 40°C and 60 rpm, then stored for further use.

2.2. Phytochemical Analysis

The endophytic fungal extract was screened for the presence of phytochemicals and secondary metabolites, including alkaloids, flavonoids, phenolics, saponins, cardiac glycosides, etc., following the protocol described by Devi et al. (12).

2.3. Determination of Total Phenolic Content

The total phenolic content of the methanolic extract was assessed using the Folin-Ciocalteu Reagent (FCR) method (13). A mixture containing 0.5 mL of extract and 2.5 mL of FCR was allowed to stand at room temperature for 5 minutes. Sodium carbonate was then added to achieve a final volume of 10 mL. This mixture was incubated in the dark for 30 minutes at room temperature. Gallic acid was used as the standard, and absorbance was measured at 765 nm. Total phenolic content (TPC) is expressed in Gallic acid equivalents (µg GAE/g).

2.4. Determination of Total Flavonoid Content

The total flavonoid content was determined using the protocol described by (14), with quercetin as the standard. The mixture included 10% aluminum chloride and sodium acetate, combined with 0.5 mL of the endophytic fungal extract and methanol. The absorbance was measured at 415 nm after a 30-minute incubation period. Results are expressed as µg QE/g.

2.5. *In vitro* Antioxidant Activities

The endophytic fungal extract was evaluated for antioxidant capacity using various assays, including the Ferric Reducing Antioxidant Power (FRAP) assay, Phosphomolybdenum assay, and DPPH radical scavenging assay.

2.5.1. Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing power of the endophytic fungal extract was determined using ascorbic acid as the standard. The antioxidants in the extract reduce Fe^{3+} to Fe^{2+}. A mixture of 2.5 mL of phosphate buffer and 1% potassium ferricyanide was combined with different concentrations of the endophytic fungal extracts. After a 30-minute incubation, 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. To the supernatant, 2.5 mL of distilled water and 0.5 mL of ferric chloride were added, and absorbance was measured at 700 nm (15).

2.5.2. Phosphomolybdenum (PM) Assay

The methanolic endophytic fungal extract was assessed for total antioxidant power using a mixture of ammonium molybdate and sodium phosphate, with ascorbic acid as the standard. Different concentrations of the extract were treated with equal volumes of the mixture and incubated for 90 minutes at 95°C. After cooling, absorbance was measured at 695 nm (16).

2.5.3. Radical Scavenging Assay by DPPH

Radical scavenging activity was determined using the DPPH method as described by Yadav et al. (13). Various concentrations of 1 mL of methanolic extract and standard drug ascorbic acid were treated with 1 mL of 0.1 mM DPPH. The reaction mixture was incubated for 20 minutes in the dark at room temperature, and absorbance was measured at 517 nm. The percentage of inhibition was calculated using the formula:

% inhibition of DPPH = (1- As/Ac) x 100

2.6. In Vitro Anti-Inflammatory Assay

2.6.1. Protein Denaturation Assay

To assess the anti-inflammatory potential of the methanolic extract from endophytic fungi, a protein denaturation assay was conducted (17). Varying concentrations of the fungal extracts and the standard drug, diclofenac sodium, were combined with 1.8 mL of 1% BSA solution. The pH of the mixture was adjusted to 6.5 before incubation at room temperature for 20 minutes, followed by heating at 60°C for 15 minutes. After cooling, absorbance was measured at 660 nm. The percentage inhibition of protein denaturation was calculated using the formula: Protein inhibition percentage = (1 - As/Ac) x 100, where As and Ac represent the absorbance of the sample and control, respectively.

2.7. Antidiabetic Activity

2.7.1. α-Amylase Inhibition Assay

This assay was performed using pancreatic α-amylase as per standard protocol (18). To a solution containing 0.1 mL of PBS and 0.2 mL of starch (5 mg/mL), various concentrations of the methanolic fungal extract (0.5 mL) and the standard drug, metformin, were added and incubated at 25°C for 10 minutes. DNS reagent (0.4 mL) was subsequently added and further incubated at 90°C for 5 minutes. Absorbance was measured at 500 nm, and α-amylase inhibition percentage was calculated as follows:

% inhibition = (Ac - As) / Ac x 100.

where Ac is the absorbance of the control and As is that of the sample.

2.7.2. α-Glucosidase Inhibition Assay

In this assay, 100µL of α-glucosidase enzyme was mixed with various concentrations of fungal extract (200µL), acarbose (standard), and 500 µL of phosphate buffer, followed by incubation at 37°C for 5 minutes. A solution of p-nitrophenyl-α-D-glucopyranoside (200µL) was then added and incubated for an additional 30 minutes at 37°C. Afterward, 500µL of Na_2CO_3 was added, and the absorbance was measured at 405 nm. Inhibition percentage was calculated using:

% inhibition = (Ac - As) / Ac x 100 (19).

2.8. Statistical Analysis

Results are expressed as mean ± standard error, with experiments performed in triplicate. Data were analyzed using one-way ANOVA and Dunnett's multiple comparison test. Statistical significance was set at $p<0.05$.

3. Results

3.1. Isolation, Molecular Identification and Mass Cultivation

Premna serratifolia L. serves as a valuable source of endophytes. The isolated endophytic fungus, identified phylogenetically as *Aspergillus austwickii* (GenBank ID: ON490470), was used in the current study. Phylogenetic analysis was conducted using 18S rRNA sequence data with the neighbor-joining method. Methanolic extracts from *A. austwickii*, prepared through maceration and concentration, were employed for further experiments, as shown in Figure 1.

3.2. Phytochemical Analysis

Phytochemical analysis of the methanolic extract revealed primary and secondary metabolites, including alkaloids, flavonoids, phenols, steroids, tannins, cardiac glycosides, and carbohydrates, with terpenoids, amino acids, and coumarins absent (Table 1). Total phenolic and flavonoid contents are summarized in Table 2.

3.3. In Vitro Antioxidant Activities

The methanolic extract demonstrated reducing power, as indicated by the reduction of Fe^{3+} to Fe^{2+} in a concentration-dependent manner (0.04 to 0.183) (Figure 2). Similarly, the total antioxidant capacity using phosphomolybdate assay also showed increased optical density with extract concentration (0.023 to 0.083) (Figure 3). The radical scavenging assay using DPPH demonstrated activity from 17.31% to 46.20% relative to standard ascorbic acid, with IC50 values in Figure 4 and Table 3.

3.4. In Vitro Anti-inflammatory Activity

3.4.1. Protein Denaturation Assay

BSA denaturation inhibition by the fungal extract ranged from 22.20 % to 71.86%, compared to diclofenac sodium (45.95% to 89.67%) as shown in Figure 5. IC50 values are reported in Table 3.

3.5. Antidiabetic Activity

3.5.1. α-amylase Inhibition Assay and α-glucosidase Inhibition Assay

The fungal extract showed hypoglycemic activity in the α-amylase inhibition assay, with inhibition ranging from 21.53% to 68.22% (IC50 = 178.10 µg/mL) compared to metformin (40.27% to 88.25%). In the α-glucosidase inhibition assay, the fungal extract inhibition ranged from 19% to 73% (IC50=166.16µg/mL), with acarbose showing inhibition from 48% to 88% (IC50 = 47.68 µg/mL) (Figures 6 and 7, Table 3).

4. Discussion

The secondary metabolites synthesized by the organism impart pharmaceutical properties, highlighting it as a valuable source of secondary metabolites for use in pharmaceutical and agricultural application uses (20). The results obtained in the current study are in accordance with those Yadav, et al. (13) and Khalil, et al. (23), who reported the presence of alkaloids, steroids, tannins, phenolic and flavonoid compounds. These phenols and flavonoids are known to exhibit strong antioxidant properties, antibacterial activity, and lipid peroxidation (21). Previous studies suggest a relation between phenolic content and antioxidant activity (22).

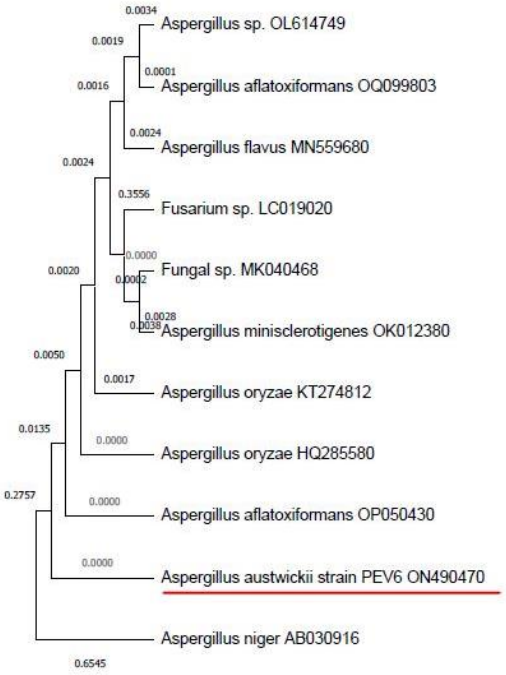

Figure 1. Phylogenetic tree of *Aspergillus austwickii* (**ON490470**) constructed by neighbour joining method indicating the bootstrap value.

Table 1. Phytochemical analysis of endophytic fungus.

Tests	**Endophytic fungus**
Secondary metabolites	***Aspergillus austwickii***
Alkaloids	+
Flavonoids	+
Phenols	+
Terpenoids	-
Tannins	+
Saponins	-
Cardiac glycosides	-
Amino acids	-
Carbohydrates	+
Coumarin	-

Table 2. Total Phenolic and Flavonoid content

	Total Phenolic Content (µg GAE/g)	**Total Flavonoid Content (µg QE/g)**
Aspergillus austwickii	22.048	18.828

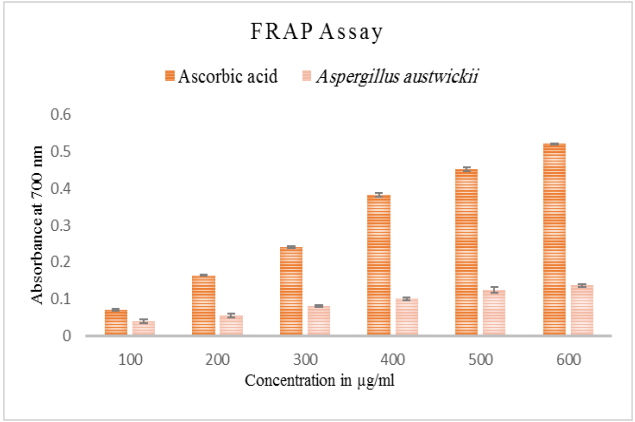

Figure 2. Reducing Power Assay (FRAP) showing concentration dependent antioxidant activity. Data are expressed as mean ± SE.

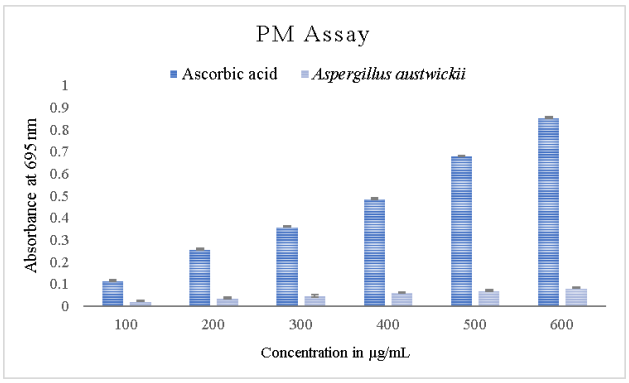

Figure 3. PM assay: Increased antioxidant activity of the extract was seen on increasing the extract concentration. Data are expressed as mean ± SE.

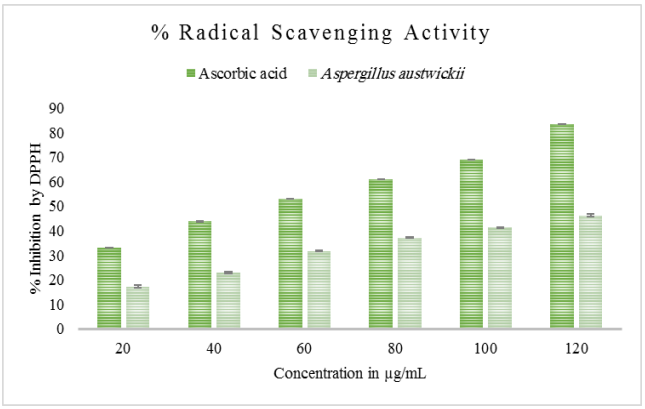

Figure 4. Free Radical Scavenging activity (DPPH). The graph depicts dose dependent increase in the % inhibition of DPPH. Values are noted as mean ± S.E.

Table 3. IC50 values of samples.

IC50 values µg/mL				
Samples	**DPPH**	**Anti-inflammatory**	**Alpha amylase**	**Alpha glucosidase**
Aspergillus austwickii	128.69	160.98	178.10	166.16
Ascorbic acid	54.88	-	-	-
Diclofenac sodium	-	60.72	-	-
Metformin	-	-	79.71	-
Acarbose	-	-	-	47.68

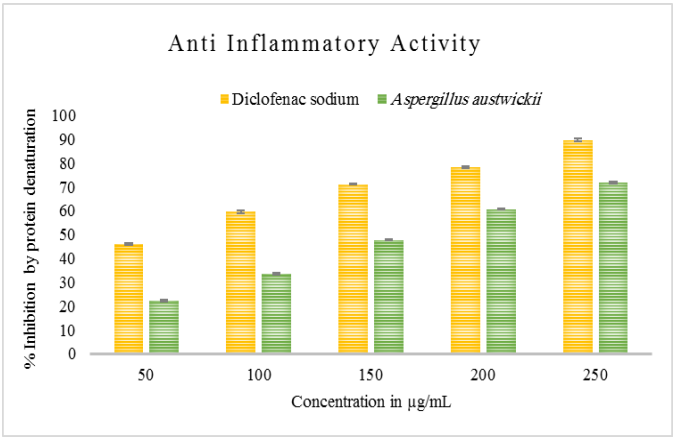

Figure 5. *In vitro* anti-inflammatory activity by protein denaturation method. Values are noted as mean ± S.E.

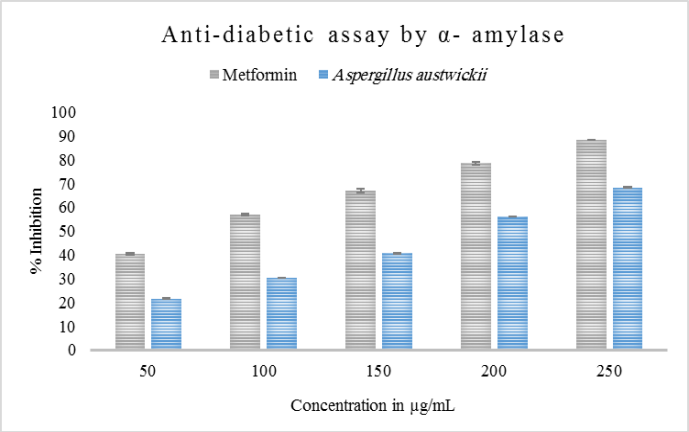

Figure 6. *In vitro* anti-diabetic assay of α-amylase inhibition activity. Values are noted as mean ± S.E.

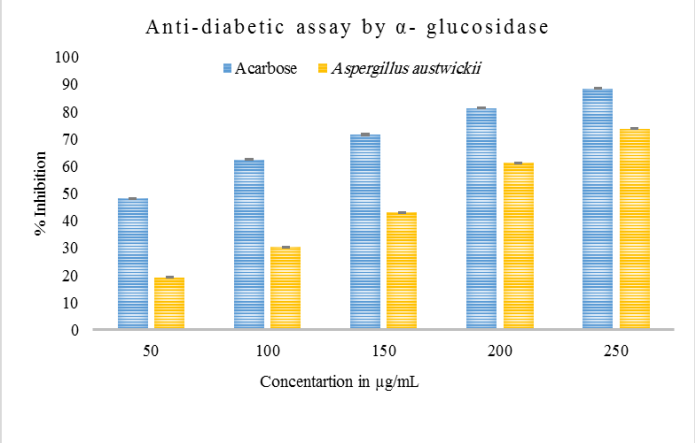

Figure 7. *In vitro* anti-diabetic assay of α-glucosidase inhibition activity, showing values are noted as mean ± S.E.

The phytochemical analysis detected in the present study shows phenols, flavonoids, and tannins, aligning with results reported by Satari et al. for various Aspergillus species (26). Our results indicate that the phenolic and flavonoid contents were nearly similar, which contrasts with findings from Khalil, et al. (23). However, *in vitro* antioxidant assays, including the reducing power assay and total antioxidant capacity, indicate that this organism has substantial antioxidant potential. DPPH radical scavenging activity reached 41.34 ± 0.18% with an IC50 of 128.69 µg/mL, outperforming *Aspergillus nidulans* and *Aspergillus flavus* as reported by Sharaf et al. (27). The presence of tannins in the extract further suggests anti-inflammatory activity (28). *In vitro* biological activities demonstrated anti-inflammatory effects through protein denaturation inhibition and antidiabetic effects via α-amylase and α-glucosidase inhibition. The IC50 for protein denaturation was 160.98 µg/mL, with a 71.86 ± 0.34% inhibition rate, comparable to findings by Moharram et al. across *Aspergillus* species (65–79.9%) (25). Govindappa et al. reported that most *Aspergillus* species exhibited around 87% inhibition, *Aspergillus flavus*, which recorded 44% (17). Our hypoglycaemic activity findings were consistent with those for *Aspergillus* species isolated, showing 91 ± 0.06% inhibition for α-amylase and 43 ± 0.01% inhibition for α-glucosidase (24). The present study highlights *Aspergillus austwickii*, isolated from the medicinal plant *Premna serratifolia* as one of the promising source of endophytes. The endophytic fungus produces secondary metabolites, including alkaloids, phenolics, flavonoids, and tannins, and demonstrates significant antioxidant, anti-inflammatory, and antidiabetic properties. This work suggests that *A. austwickii* is a valuable source of bioactive compounds, with substantial biological activity. Future research should focus on isolating and structurally elucidating potent compounds from *A. austwickii*, potentially providing new drug candidates for treating various disorders.

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Authors' Contribution
V.SL. conducted the experiments and drafted the data; A.KS. Contributed to methodology standardization and project execution; J.HH. Provided editorial support, and V.AB. Finalized the draft for submission.

Ethics
Not applicable

Conflict of Interest
The Authors declare conflict of interest as Nil

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The present work didn't receive any funding.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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