In vitro studies on antidiabetic, antioxidant and phytochemical activities of endophytic actinomycete from Azadirachta indica A. Juss.

Preeti Saini¹, Madhurama Gangwar¹ and Amrinder Kaur²

¹Department of Microbiology, PAU, Ludhiana-141004, Punjab, India.
²Department of Plant Pathology, PAU, Ludhiana-141004, Punjab, India.

*Correspondence at saini.preeti7777@gmail.com

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Abstract

Inhibitory effects of root endophytic actinomycete A-12 from Azadirachta indica A. Juss (Indian lilac, neem) against α-amylase and α-glucosidase were studied. The antioxidant capability was assessed through 2,2-diphenyl-picrylhydrazyl (DPPH), hydroxy radical scavenging and reducing power assays. In vitro antidiabetic activity of ethyl acetate extract revealed good inhibition exhibiting IC₅₀ values of 411.70 and 208.92 µg/ml against α-amylase and α-glucosidase respectively. The reducing power of the extract was found to be concentration dependent. The extract revealed measurable total phenolics i.e., 80.22±9.67 (catechol) and 12.48±1.96 mg/g (gallic acid) equivalents. The extract exhibited remarkable scavenging activity on DPPH (IC₅₀=447.95±0.35µg/ml) and hydroxyl radical (IC₅₀=1961.02±0.69µg/ml) and also displayed high and dose-dependent antioxidant capacity. The results are therefore indicative of the possible potentials of ethyl acetate extracts to manage hyperglycemia.

Keywords: Antidiabetic activity, Azadirachta indica A. Juss, DPPH, endophytic actinomycete, hydroxy radical, reducing power.
Introduction

Diabetes mellitus Type II is a chronic endocrine disorder characterized by hyperglycemia, in which blood sugar levels are elevated because cells do not respond to the produced insulin. Since the disease is non insulin dependent type, hence blood glucose levels have to be maintained within normal ranges (80-120mg/dl). This can be achieved by inhibition of carbohydrate hydrolyzing enzymes viz. α-amylase and α-glucosidase. Currently available therapeutic drugs like biguanides and sulphonamides have been reported to impose side effects like secondary failure, when used for longer periods [1]. Therefore alternative sources of these medicines are being searched from natural sources. Further, plant food rich in polyphenols have been reported to cause effects similar to insulin in the utilization of glucose and act as good inhibitors of key enzymes like α-amylase and α-glucosidase associated with type 2 diabetes [2]. Studies have also shown that the bioactivity of polyphenols in plants is linked to their antioxidant activity and many of these plants also possess hypoglycaemic properties [3]. Also the use of synthetic antioxidants must be under strict regulation due to potential health hazards [4]. Azadirachta indica is a species that has been long in use in the ancient Indian practices like Ayurveda for the treatment of this ailment. Earlier reports also documented the presence of flavonoids, xanthine, terpenoids and glycosides in the A. indica [5]. Verma et al. [6] have proven the efficacy of endophytes from this plant for its antimicrobial properties. So the present study was conducted, with an aim to analyze efficacy of its endophytic actinomycete isolate for antidiabetic and antioxidant characteristics.

Materials and Methods

Endophytic actinomycete isolate A-12 was procured from the department of Microbiology, Punjab Agricultural University, Ludhiana. The isolate was reported to be obtained from root tissues of this plant.

Fermentation and extraction of secondary metabolites

Five day old culture was inoculated into 100 ml SCB medium in 250 ml of Erlenmeyer flask and incubated for 7 days in rotary shaker (200rpm) at 28°C. After fermentation the broth was centrifuged at 10,000 rpm for 15 min at 10°C, followed by extraction of the supernatant with equal
volume of ethyl acetate: methanol (4:1). The extract was then concentrated using a freeze drier at -130ºC.

**Screening of the endophytic actinomycete isolate for antidiabetic properties**

Different concentrations of the ethyl acetate extracts (EAE) were prepared by weighing the crude material obtained after lyophilization. Five concentrations viz. 100, 250, 500, 750 and 1000µg/ml of the crude extract were made by dissolving in 10% Dimethyl sulfoxide (DMSO) solution.

(a) **Screening of isolate for the production of alpha-amylase inhibitor**

The alpha amylase inhibition potential was determined by the method of McCue and Shetty [7]. The activity was initiated by the addition of 250 µl of EAE (100-1000 µg/ml in 10% DMSO) and 250µl of α-amylase (0.05mg/ml in 0.02M sodium phosphate buffer; pH 6.9). After 20 min incubation at 25°C, 250µl of 1% starch solution was added to initiate the reaction. After 10 min of incubation at 25°C, the enzyme action was arrested by 500µl of dinitrosalicylate reagent, followed by keeping in a boiling water bath for 5 min, cooling and diluting to 5ml with water. α-amylase activity was determined by measuring the wavelength of the mixture at 540 nm, using Systronics® Double beam Spectrophotometer 2203®smart.

(b) **Screening of isolate for the production of alpha-glucosidase inhibitor**

Inhibition of alpha glucosidase activity was examined according to Anam et al. [8]. Enzyme alpha-glucosidase with a concentration 0.5 mg/ml was dissolved in 0.1 M phosphate buffer pH 7. As a substrate, p-nitrophenyl- alpha-D-gluco pyranoside 0.015 g/ml was dissolved in 0.1 M phosphate buffer at pH 7. The mixture of reaction contained 200 µl of enzyme solution and 50 µl sample. Afterwards the reaction mixture was incubated at 25°C for 10 minutes, 100 µl of substrate was added and incubated for 10 minutes at 25°C. The reaction was then stopped by adding 0.1 M of 1000 µl sodium carbonate. Amount of p-nitrophenol produced was measured at 405 nm.

**In vitro production of antioxidants and free radical scavenging activities of the isolate.**

(c) **Total Phenolic Content (TPC) estimation:** Total phenolic content was assayed according to the Folin-Ciocalteau method [9]. This was done by the addition of 0.1 ml of sample (100-1000 µg/ml), 1.9 ml distilled water, and 1 ml of Folin-Ciocalteau reagent in a tube and then 1 ml of 100 g/L Na₂CO₃ added, followed by an incubation at 25°C for 2 h and taking absorbance at 765 nm.
Total phenolic content of EAE was expressed as milligrams of gallic acid and catechol equivalents per gram of extract.

(d) **Reducing Power Activity:** For reducing power activity the method of Yen and Duh [10] was used, whereby different concentrations (100-1000 µg/ml) were added to 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated for 20 min at 50°C. After incubation, 2.5 ml of 10% trichloroacetic acid was added to each tube followed by centrifugation at 3,000 rpm for 10 min. The upper layer (5ml) was then mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbance of the resultant solution was measured at 700nm.

(e) **2,2-diphenyl-picrylhydrazyl (DPPH) Radical Scavenging Assay:** This was measured according to Hanato et al. [11]. 0.1mM methanol DPPH solution was mixed with serial dilutions (100-1,000 µg/ml) of the sample and after 10 min, the absorbance was read at 515 nm. The antiradical activity was expressed as IC₅₀ (µg/ml).

(f) **Hydroxy Radical Scavenging Activity:** This was performed as described by the method of Elizabeth and Rao [12]. One hundred microliter of 28 mM 2-deoxy-2-ribose, 200 µl of 200 µM FeCl₃ and 1.04 mM EDTA (1:1 v/v), 100 µl H₂O₂ (1mM), and 100 µl ascorbic acid (1mM) were added to 500 µl of different concentrations (100-1,000 µg/ml) of the sample. After an incubation period of 1 h at 37°C, the extent of deoxyribose degradation was measured. The absorbance was read at 532 nm against the blank solution. The scavenging activity was calculated by the formula (1).

In all the above cases mentioned except reducing power, the activities of crude extract were calculated as:

\[
\text{Activity (\%)} = \left[ \frac{(A_0 - A_1)}{A_0} \times 100 \right] \quad (1)
\]

Where \(A_0\) is the absorbance of the control and \(A_1\) is the absorbance of the sample.

(g) **Antioxidant Assay using \(\beta\)-Carotene Linoleate Model System:** The antioxidant activity of EAE was evaluated by the \(\beta\)-carotene linoleate model system. A solution of \(\beta\)-carotene was prepared by dissolving 2 mg of \(\beta\)-carotene in 10 ml of chloroform. Two milliliter of this solution was pipetted into a 100 ml round bottom flask. After chloroform removal under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water was
added to the flask with vigorous shaking. Aliquot (4.8 ml) of this emulsion was transferred into different test tubes containing different concentrations of EAE (100-1,000 µg/ml). Soon after the addition of emulsion in each tube, the zero time absorbance was measured at 470 nm. Tubes were then placed at 50°C in a water bath. Measurement of absorbance was continued until the colour of \( \beta \)-carotene disappears; a blank, devoid of \( \beta \)-carotene, was prepared for background subtraction.

Antioxidant activity (AA) was calculated by the following equation:

\[
AA = 100 \left[ 1 - \frac{A_0 - A_t}{A_0 - A_t} \right]
\]

Results and discussion

Screening of the endophytic actinomycete isolate for antidiabetic properties

As evident from table 1, the crude ethyl acetate extract displayed a dose dependent decrease in the alpha amylase and alpha glucosidase inhibitory activities. IC\(_{50}\) concentrations for \( \alpha \)-amylase and \( \alpha \)-glucosidase were 411.70 and 208.92 µg/ml. Alpha amylase inhibition was giving a slow change in slope with increase in dose, while in case of alpha glucosidase a steep slope was observed. The results indicated that this extract can be used in lower concentrations for \textit{in vivo} tests. Similar results were reported by Akshatha et al. [13], who obtained endophytic actinomycete \textit{Streptomyces longisporoflavus} from \textit{Leucas ciliata}, a medicinal species of the Western Ghats, and screened for the anti-diabetic potential for inhibition of \( \alpha \)-amylase. The results presented by them indicated the inhibition of \( \alpha \)-amylase by \textit{Streptomyces longisporoflavus} extract with IC\(_{50}\) values of 162.3 ± 1.05 µg/ml. Rosaline and Agastian [14] reported that alpha-amylase inhibitory activity of ethyl acetate extract of endophytic actinomycete from \textit{Catharanthus roseus} was 83.29% at 1mg/ml concentration.

\textbf{In vitro production of antioxidants and free radical scavenging activities of the isolate.}

Total phenolic content was determined in terms of gallic acid and catechol equivalents. The ethyl acetate extract yielded 80.22±9.67 and 12.48±1.96 mg/g catechol and gallic acid equivalents respectively. In a study by Shobha et al. [15], concentrated broth of \textit{Streptomyces} species KSRO-04 was found to have 1.23 mg gallic acid equivalents/g of dry metabolite. Christhudas et al. [1] reported 176 mg of catechol equivalents/gram extract total phenolic content of the methanolic extract. The extract was obtained from \textit{Streptomyces} sp. Loyala UGC, an
endophyte of *Datura stramonium* L., a wild-growing medicinal herb known as Jimson weed belongs to the family Solanaceae. The reducing power was estimated in terms of absorbance at 700nm. Higher absorbance indicates better reducing capacity. In the present study, there was a constant increase in the absorbance with rise in concentration. Similar trends were observed by other researchers [1, 15].

Table 1. Antidiabetic, antioxidant, free radical scavenging and reducing properties of endophytic actinomycete A-12. [Values indicate mean±SD of three replicates].

<table>
<thead>
<tr>
<th>Ethyl acetate concentrations</th>
<th>Antidiabetic activity (Inhibition %)</th>
<th>Antioxidant activity (%)</th>
<th>Free Radical Scavenging activity (Inhibition %)</th>
<th>Reducing Power (Absorbance 700nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha amylase</td>
<td>Alpha glucosidase</td>
<td>DPPH Radicals</td>
<td>Hydroxy Radicals</td>
</tr>
<tr>
<td></td>
<td>Beta carotene-linolate model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EA^100</td>
<td>62.75±0.00</td>
<td>65.00±0.01</td>
<td>15.58±0.01</td>
<td>19.54±0.02</td>
</tr>
<tr>
<td>EA^250</td>
<td>56.55±0.00</td>
<td>37.54±0.04</td>
<td>16.42±0.00</td>
<td>34.10±0.02</td>
</tr>
<tr>
<td>EA^500</td>
<td>57.25±0.02</td>
<td>34.98±0.56</td>
<td>54.27±0.01</td>
<td>39.09±0.07</td>
</tr>
<tr>
<td>EA^750</td>
<td>53.09±0.06</td>
<td>25.00±0.01</td>
<td>30.73±0.04</td>
<td>91.13±0.08</td>
</tr>
<tr>
<td>EA^1000</td>
<td>58.62±0.06</td>
<td>27.50±0.00</td>
<td>15.00±0.00</td>
<td>28.77±0.08</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>411.70±0.00</td>
<td>208.92±0.28</td>
<td>315.69±0.48</td>
<td>447.95±0.35</td>
</tr>
</tbody>
</table>

There was a rise in DPPH radical scavenging activity as evident by decrease in absorbance with increase in dose till 750µg/ml, where its activity reached upto 91.13%. Fifty percent inhibition reached at 447.95±0.35 µg/ml. Hydroxy Radical Scavenging Activity also displayed a dose dependent increase in activity with a maximum of 26.34±0.04% at 1000 µg/ml. Antioxidant property was maximum between concentrations of 250-750 µg/ml, indicating that higher concentrations are not required for its activity. Hence, IC₅₀ value was also recorded at 315.69 µg/ml. Christudas et al. [1] also obtained similar results for these free radical scavenging and antioxidant activities. They observed that methanolic extract of endophytic actinomycete *Streptomyces* sp. Loyala UGC, showed remarkable scavenging activity on DPPH (IC₅₀
435.31±1.79µg/ml, hydroxyl radical (IC₅₀ 350.21±1.02µg/ml). Antioxidants of β-carotene linoleate model system revealed that their extract exhibited 50 % inhibition at 420.12±1.50µg/ml after 120-min reaction time.

Conclusion

This study suggested that endophytic actinomycete form *Azadirachta indica* A. Juss. possessed antidiabetic and antioxidant activity which might be helpful in preventing or slowing down the progress of diabetic mellitus.

Conflict of Interest

There is no potential conflict of interest with reference to the current manuscript. All the authors have read the manuscript and agreed to submit the same for publication.
References


