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In vitro enzyme inhibitory and free radical scavenging potentials of an *Aegle marmelos* endophytic actinomycete extract

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Abstract

Actinomycetes are potential sources to discover new biologically active metabolites. While Aegle marmelos has been widely studied for the bioactive potentials of its endophytic fungi and bacteria, the endophytic actinomycetes from this plant have been far lesser exploited. An endophytic actinomycete obtained from Aegle marmelos was tested for its in vitro enzyme inhibitory capabilities. The supernatant was extracted in ethyl acetate to prepare various concentrations (100-1000µg/ml). The extract was found to exhibit 50% inhibition (IC_{50}) of alpha-amylase and alpha-glucosidase, at concentrations of 1950.71 ± 0.11µg/ml and 391.38± 0.09µg/ml, respectively. Ethyl acetate extracts were also tested for their reducing activity and free radical scavenging (hydroxyl radical, superoxide anion, nitric oxide free radical) abilities. Total phenol contents were determined to be 42.11±1.88mg/g of the extract (catechol equivalents) and 6.47±0.95mg/g of the extract (gallic acid equivalents). New acquisitions in these fields will be fundamental in order to exploit actinomycetal strains from Aegle marmelos, which can exhibit bioactive properties.

Keywords: *Aegle marmelos*, endophytic actinomycete, Alpha-amylase inhibition, Alpha-glucosidase inhibition, Reducing power, Enzyme inhibitory activity, Free radical scavenging potential, Total phenol content.

Introduction

The most vandalizing among the present day concerns are the advent of drug-resistant bugs and associated diseases, along with the unwanted side effects of existing drugs. Many synthetic agents are constantly targeted for removal from the market due to safety and environmental concerns. Working toward the creation of safe and secure moieties that are sustainable, readily available and environment friendly, is therefore vital.

As per World Health Organization (WHO), the number of people diagnosed with diabetes (420million in 2021) is likely to rise by 570million in 2030[1]. Type II diabetes also known as non-insulin-dependent diabetes mellitus, accounts for more than 95% of all diabetic cases[2]. Insulin resistance, a disease in which the body is unable to adequately utilise insulin, is the cause of type II diabetes. Thus, this disease cannot be cured without modifications at genetic/molecular level, but can be controlled. Biguanide, sulfonylureas, and thiazolidinedione inhibitors, which are available, do not appreciably modify the track of diabetic problems. These agents are now being used infrequently owing to their adverse responses and secondary failure rates that are extremely high[3].

Enzyme inhibitors are biochemical tools that can be used to investigate biological activities and ailments. Two most frequently analysed enzyme inhibitors in the case of diabetes are against alpha-amylase and alpha-glucosidase. These lead to suppression of postprandial hyperglycemia by delaying the hydrolysis of starch to simple sugars prior to absorption. In addition, free radicals also have been associated with the development of oxidative stress-related diseases like diabetes, Alzheimer's, and stomach cancers caused by *Helicobacter pylori*. Antioxidantsare crucial in this regard as these can prevent or retard the substrate oxidation in a chain reaction.

Every civilization on the globe has used herbal and natural folk medicinal products for centuries. Fruit, shell, seeds, and leaves are all parts of *Aegle marmelos* that have been shown to have antidiabetic and antioxidant properties[4-6]. Biochemicals such as aegelin, citronellal, eugenol, cineol, lupeol, cuminaldehyde, mermelosin, citral, skimmianine, and others have been obtained from different parts of this plant.

Endophytes that colonize the inner tissues of plants improve the host's fitness by creating a plethora of biologically active compounds and safeguarding it. In exchange, host plants usually provide nourishment and protection. Actinomycetes are Gram-positive bacteria that possess the potential to create anexpansive range of secondary metabolites that are useful to the pharmacy sector.

Furthermore, most medicinal plant endophytic actinomycetes can produce important chemicals, some of which are novel chemical structures[7]. In terms of profitability, medicine synthesis using microbes will be more profitable because it will be faster, repeatable, infinite, and climate independent.

There is a plethora of literature pertaining to bioactive compound production from endophytic fungi and bacteria of *A. marmelos*. However, endophytic actinomycetes from this plant have been far lesser exploited.

Keeping the abovementioned points in view, the present investigation has been undertaken to screen an isolate (*Microbispora* sp., B-8) for synthesis of enzyme inhibitors, antioxidants and free radical scavengers.

As per our knowledge, this is one of the few initial reports pertaining to bioactive potential of endophytic actinomycetes from *A. marmelos*. This will not only help in generating the literature but also explicit the active involvement of actinomycetes from less studied Indian medicinal plants.

Materials and methods

Procurement of the isolate: The Microbiology department of Punjab Agricultural University was the collection point for *A. marmelos* endophytic actinomycete (B-8) belonging to genus *Microbispora*.

Preparation of ethyl acetate extracts: In a 250ml Erlenmeyer flask, a loopful of fresh inoculum was seeded into 50ml Starch Casein Nitrate broth and cultured for 7 days (28ºC at 200rpm). To collect the supernatant, centrifugation of the culture was performed at 10,000 rpm for 15 minutes at 10ºC. Ethyl acetate: methanol (4:1) was used to extract the supernatant twice. This was followed by lyophilization of the supernatant at -130°C using a freeze dryer. Dissolving the crude extract in 10% dimethyl sulfoxideyielded following concentrations: 100, 250, 500, 750, and 1000µg/ml.

Evaluation of enzyme inhibitory activity *in vitro:* Evaluation of α-amylase inhibitory activity: A 250µl of α-amylase solution (0.05mg/ml) was poured to 250µl of the ethyl acetate extracts (100-1000µg/ml). Thereafter, incubation at 25°C was provided for 10 min. In order to initiate the reaction, starch solution (1%, 250µl) was poured. Enzyme action was stopped by addition of dinitrosalicylate reagent (500µl) after incubating for 10 minutes at 25°C. This was followed by 5 minutes incubation in a water bath maintained at 100°C. Solution was allowed to cool and diluted to 5000µl with distilled water. To evaluate α-amylase activity, the absorbance was measured at 540 nm[8].

Evaluation of α-glucosidase inhibitory activity: To 200µl α-glucosidase (0.5mg/ml), 50µl extract was added. After incubating the mixture for 10 minutes at 25ºC, 100µl of pNPG substrate (0.015g/ml) was also added, and was allowed to react again at 25ºC for 10 minutes. In order to halt the reaction 0.1M of 1000µl sodium carbonate was added. The α-glucosidase activity was evaluated at 405 nm[9].

To figure out the % inhibition of enzymatic activity for both of reactions, the formula below was used.

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100 \quad (1)$$

Evaluation of antioxidant and free radical scavenging potentials *in vitro:* Test for hydroxyl radical scavenging activity: To perform this test, 100µl each of 2-deoxy-2-ribose (28mM), H_2O_2 (1mM) and ascorbic acid (1mM), along with 200 µl of $FeCl_3$ (200µM), were poured into sample (500µl) and EDTA (1:1v/v, 1.04mM). The degree of deoxyribose breakdown was analyzed at 532nm after one-hour incubation at 37°C[10]. Blank was used as control. In order to determine the scavenging activity, the formula below was used.

$$\text{Scavenging activity (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (2)$$

Test for nitric oxide scavenging activity: The 500µl sample was incubated with sodium nitroprusside (2000µl, 10mM) in phosphate buffer saline (500 µl), at 25ºC for 150 min. This was followed by pouring 500µl of the reaction mixture in 1000 µl of sulphanilic acid reagent (0.33%). After letting the solution react for 5min, 1000µl of 5% 1-naphthyl ethylenediaminedi hydrochloride (NEDH) was added to it. After 30 minutes of incubation, at 540nm the chromophore's absorbance was measured[11]. The formula (2) was used to calculate the scavenging activity.

Sodium nitrite (5-75µM) in phosphate buffer (pH 7.0) was used to prepare a standard curve, using 1% NEDH solution and reading the absorbance at 540nm.

Test for superoxide scavenging activity: This was performed by generating superoxide radicals in a pre-incubated mixture containing Tris - HCl buffer (20mM), along with phenazineme thosulphate (0.05mM) as well as sample. This was followed by adding NADH (0.078mM) and reading the absorbance 560nm. The scavenging activity was calculated with the help of the formula (2).

Test for reducing activity: Toa 5ml solution containing potassium ferricyanide (2.5ml, 1%) and phosphate buffer (2.5 ml, 200mM), sample was poured. Incubation at 50°C for 20 minutes followed by the addition of 2.5ml of 10% trichloroacetic acid, were the next steps in the procedure. Centrifugation period was 10 min at 3,000 rpm. Five ml each of supernatant and distilled water were poured into 1 ml of ferric chloride (0.1%). Afterwards, absorbance was read at 700 nm[12].

Test for total phenol content: To 100µl of the sample, 1000µl Folin-Ciocalteau reagent and 1900µl distilled water were added. Following that, 1000µl of sodium carbonate was poured into the test tube. After incubating the mixture for 120 minutes at 25ºC, at 765nm the absorbance was measured[13].

Standard curves for catechol and gallic acid were prepared using Folin-Ciocalteau reagent and measuring absorbance at 765nm.

Results and discussion

The endophyte, like its host plant, is capable of combating enzymatic activity and oxidative stress (Table-1). A beautiful uphill trend can be observed in the α-amylase inhibition results by the extract with increase in concentration. On the other hand, a downward trend was observed with the use of extract for inhibition of α-glucosidase. The reason for this could be the presence of retarding factors in the sample, as it was not purified[14]. The low values of 50% inhibitory concentrations for α-amylase and α-glucosidase (1950.71±0.11µg/ml and 391.38 ± 0.09µg/ml) point at the enhanced potential of crude extract to overcome the postprandial glucose concentrations in blood. It may prove as an effective remedy against starch degrading enzymes, after purification.

Extremely reactive, oxygen-containing compounds, such as, hydroxyl radical, hydrogen peroxide, hypochlorite radical, superoxide anion, nitric oxide radical, singlet oxygen, and different lipid peroxides, are examples of free radicals. Physiological/biochemical processes, endogenous sources, or pollutants can all produce free radicals. These can cause cellular damage by reacting with biomolecules, enzymes, as well as other tiny molecules[15].

Hydroxyl radical scavenging activity of *Microbispora* sp., B-8 ethyl acetate extract was higher than its nitric oxide and superoxide scavenging effects. More than 60% scavenging effects were produced by the extract against hydroxyl radicals, at all the concentrations studied. Cross-linking between apo-B monomers is induced by the hydroxyl radical, which causes oxidative damage in diabetic population[16]. Results indicate that the extract may prove to be an essential remedy in this scenario.

Antioxidants protect the human system by actively and synergistically eliminating free radicals. Free radical scavengers like alkaloids, amines, stilbenes, betalains, phenolic acids, coumarins, flavanoids, vitamins, etc. with plenty of antioxidant activity can be obtained from plants[17]. Reducing power has been linked to antioxidant activity. Reducing compounds are electron donors which may convert oxidized intermediates into primary and secondary antioxidants[18]. All the concentrations of endophytic actinomycete extract exhibited the reductive potential, as suggested by their absorbance (range = 0.024 – 0.032, Table-1).

Phenols have a wide spectrum of activities, *viz*. anti-carcinogenic, antioxidant, antimutagenic, capacity to change the expression of genes, etc[19]. Phenolics are the most abundant phytochemicals in plants and plant products, accounting for the majority of antioxidant action[20]. Two types of phenolic compounds were detected in the sample, *i.e.* catechol (42.11 ± 1.88mg/g of the extract) and gallic acid (6.47±0.95mg/g of the extract) equivalents. The amount of phenolic compounds and free radical scavenging capability are also linked positively[21].

Conclusion

This study suggested that endophytic actinomycetes from *Aegle marmelos* may be used to manage postprandial blood glucose levels, without recourse to artificial α-amylase and α-glucosidase inhibitors. Additional research in animal models may assist in the identification of chemical entities suitable for clinical usage. The utilization of microbes as reservoirs of bioactive substances can also be beneficial to the pharmaceutical industry, which relies on easily available inoculums for its operations.

Table-1: Bioactive properties of the endophytic actinomycete (*Microbispora* sp., B-8) ethyl acetate extract.

Test	Results expressed as	Concentrations of ethyl acetate extract (µg/ml)					IC_{50} (µg/ml)
		100	250	500	750	1000	
Alpha-amylase inhibition	% activity	24.44 ± 0.30	26.21 ± 0.00	30.56 ± 0.00	32.62 ± 0.05	37.96 ± 0.11	1950.71 ± 0.11
Alpha-glucosidase inhibition		69.03 ± 0.01	58.33 ± 0.00	39.31 ± 0.03	30.91 ± 0.04	13.09 ± 0.00	391.38 ± 0.09
Hydroxyl radical scavenging		61.73 ± 0.02	76.54 ± 0.03	92.64 ± 0.05	82.69 ± 0.11	79.12 ± 0.11	18.91 ± 0.61
Nitric oxide scavenging		18.83 ± 0.54	18.21 ± 0.91	17.18 ± 0.64	19.25 ± 0.39	24.35 ± 0.04	2330.47 ± 8.79
Superoxide anions scavenging		-	-	2.13 ± 0.13	3.17 ± 0.02	4.87 ± 0.00	10183.06 ± 5.83
Reductive ability	Absorbance	0.032 ± 0.000	0.024 ± 0.001	0.026 ± 0.000	0.026 ± 0.001	0.027 ± 0.000	-

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