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# Bioactivity guided isolation and identification of potential antidiabetic components of ethyl acetate extract obtained from Syzygium cumini endophytic actinobacterium

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## Abstract

In India, the medicinal plants have been used for centuries to combat diseases. The present study was carried out to characterize and evaluate antidiabetic, antioxidant and free radical scavenging activities of the actinobacterium (J-7) obtained from roots of Indian medicinal plant *Syzygium cumini*. The ethyl acetate extract of J-7 showed anti starch degradation activity with $IC_{50}$ value of 83.52 ± 0.19 and 148.52 ± 5.92 µg/ml against α-amylase and α-glucosidase respectively. It showed significant antioxidant potential when tested using β-carotene linoleate model ($IC_{50}$= 631.05 ± 0.13 µg/ml) and also scavenged DPPH radical ($IC_{50}$= 861.17 ± 1.94 µg/ml). The extract also displayed reductive ability in line with BHT. The total phenolic content was 47.92 ± 2.98 and 6.94 ± 0.93 mg of catechol and gallic acid equivalents/ gram extract respectively. The purified extract exhibited pure competitive inhibition against α-amylase and near competitive inhibition against α-glucosidase. Liquid chromatography mass spectrometry analysis of the purified α-amylase inhibitor revealed the presence of 2 major compounds *i.e.* 6-hydroxyl-4-methyl coumarin and quercetin. The results of this study indicate that J-7 could be probed further for isolating some medically useful compounds.

***Keywords:*** Antidiabetic; Antioxidant; Endophytic Actinobacteria; J-7; *Syzygium Cumini*



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## 1. Introduction

A study by the World Health Organization (WHO) estimated that diabetes will be the 7th leading cause of deaths in 2030. In spite of the regular introduction and extensive utilization of hypoglycemic agents, it remains to be a major health constraint worldwide, affecting 9% of the population all over the world (WHO, 2017). Diabetes mellitus is a group of metabolic alterations, which is characterized by hyperglycemia, lipoprotein abnormalities (Scoppola et al., 2001) and excessive generation of reactive oxygen species. These lead to a series of secondary complications including polyurea, polyphasia, ketosis, neuropathy, retinopathy as well as cardiovascular disorder (Huang et al., 2016). The major factors which contribute to its increased risk include high family aggregation, obesity, insulin resistance, nutritional status, age and lifestyle change due to urbanization (Nanditha et al., 2016). A successful treatment for the management of diabetes is yet to be discovered. The oral hypoglycemic agents currently in use like metformin, sulphonylureas, biguanides, thiazolidinediones, meglitinide derivatives are posing several side effects as liver problems, lactic acidosis, diarrhea and risks of secondary failure. Thus, there is a need to re-investigate the traditionally involved herbs and natural products in the management of diabetes mellitus. Hypoglycemic activity of the plants is attributed to inhibition of the intestinal absorption of glucose (Nistor Baldea et al., 2010).

*Syzygium cumini* (family Myrtaceae) has been widely used to treat diabetes by the traditional practitioners for hundreds of years (Freitas et al., 2015). This medicinal species is widely distributed throughout India, Sri Lanka, Australia, Thailand and Philippines. This is an evergreen tree growing up to 30 m high. The fruits are oval to elliptical 1.5-3.5 cm long, dark purple or nearly black, luscious, fleshy and edible (Sharma et al., 2012). Various parts of this tree are known to be rich in phyoconstituents such as quercetin, kaempferol, bergenins, eugenin, fatty acid esters, flavonoids, tannins, β-sitosterol (Ivan, 2006), gallic acid, ellagic acid, resin (Chaudhary and Mukhopadhyay, 2012), myrecetin and phytosterols. The presence of such biochemicals has imparted distinct medicinal properties to *S. cumini* including antiallergic (Brito et al., 2007), antibacterial, antidiabetic (Tripathi and Kohli, 2014), anti-inflammatory (Aravindan et al., 2008), antimalarial, antioxidant (Jacob and Titus, 2009), blood cholesterol lowering, chemopreventive and vibriocidal (Paul et al., 2011) activities.

The microbiota belonging to the inner tissues of plants (endophytes) is known to reflect the inherent properties of its host (Taghavi et al. 2005). Actinobacteria from well-known Indian medicinal plants have been isolated, identified and tested for their antimicrobial (Saini et al., 2016) and antidiabetic properties (Saini et al., 2015). Thus, in continuation to these works, a study has been carried out to produce bioactive compounds, with better functioning and quality. These metabolites have been reported for the first time from an endophytic actinobacterium obtained from *S. cumini*.

## 2. Materials and methods

### 2.1. Isolation of endophytic actinobacterium

The actinobacterium discussed in this report has been isolated from root tissues of a healthy *Syzygium cumini tree* from Ludhiana, Punjab (30.9° North and 75.85° East; Elevation, 247 m). This was followed by

washing the samples in running tap water and sequential immersion in 70% (v/v) ethanol (5 min), sodium hypochlorite solution (0.9%, w/v, 20 min) and sterile water (3-5 times). It was followed by soaking in 10% (w/v) sodium bicarbonate solution to retard the growth of endophytic fungi (Cao et al., 2004). A 0.1 ml volume of the suspension obtained after maceration, was spread onto Petri plates containing Starch Casein Agar media (SCA) and incubated (28 ± 2 °C; 7-14 days). Further, cycloheximide (50 µg/ml) was added to the medium to suppress fungal growth. The growth of endophytic actinobacterium was observed fourth day onwards.

## 2.2. Fermentation and extraction of secondary metabolites

Well grown slant culture was inoculated into 50 ml medium in a 250 ml Erlenmeyer flask containing the SCB production medium and incubated for 7 days in rotary shaker (200 rpm) at 28 ºC. Thereafter the broth was centrifuged (10,000 rpm, 15 min, 10 ºC). The supernatant was extracted twice with ethyl acetate: methanol (4:1). The extract was then lyophilized using a freeze drier at -130º C. Different concentrations of the ethyl acetate extracts were made in 10% dimethyl sulphoxide (DMSO) by weighing the crude material obtained after lyophilization.

## 2.3. Determination of α-amylase and α-glucosidase inhibitory activity

The α-amylase inhibition potential was determined using the method of Kazeem et al. (2013). The reaction was started by the addition of 250 µl of J-7 extract (10-1000 µg/ml in 10% DMSO) and 250 µl of α-amylase (0.05 mg/ml in 0.02 M sodium phosphate buffer; pH 6.9). After 10 min of incubation at 25 °C, 250 µl of 1% starch solution was added to initiate the reaction. After incubating for 10 min at 25 °C, the reaction was arrested by the addition of 500 µl of dinitrosalicylate (DNS) reagent. It was followed by keeping the reaction mixture in a boiling water bath for 5 min, cooling and diluting to 5 ml with water. α- amylase activity was determined by reading the absorbance of the mixture at 540 nm. Acarbose was taken as a positive standard.

Inhibition of α-glucosidase was examined according to previous researcher (Anam *et al*., 2009), taking voglibose as a comparison. As a substrate, p-nitrophenyl- alpha-D-gluco pyranoside (pNPG, 0.015 g/ml) was dissolved in 0.1 M phosphate buffer pH 7. The mixture of reaction contained 200 µl of enzyme (0.5 mg/ml in 0.1 M phosphate buffer; pH 7) and 50 µl J-7 extract. After this the reaction mixture was incubated at 25 ºC for 10 minutes, followed by addition of 100 µl substrate and incubation for 10 minutes at 25 ºC. The reaction was stopped by adding 0.1 M of 1000 µl sodium carbonate and p-nitrophenol (pNP) produced was measured at 405 nm. A standard curve was prepared by using different concentrations of pNP in phosphate buffer with pH 7.

## 2.4. Antioxidant production and free radical scavenging activities *in vitro*

### *2.4.1. 2,2 Diphenyl 1- Picryl Hydrazyl (DPPH) Radical Scavenging Activity*

This was measured according to Hanato et al. (1988). Methanol DPPH solution (0.1 mM) was mixed with serial dilutions (100-1000 µg/ml) of the sample and after 10 min, the absorbance was read at 515 nm. The antiradical activity was expressed as $IC_{50}$ (µg/ml). Vitamin C was used as a standard to compare.

### *2.4.2. Hydroxyl Radical Scavenging Activity*

This was performed as described by the method of Sridevi et al. (2014). To 500 µl of different concentrations (100-1000 µg/ml) of the extract, 100 µl of 28 mM 2-deoxy-2-ribose, 200 µl of 200 µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl $H_2O_2$ (1 mM), and 100 µl ascorbic acid (1 mM) were poured. After an incubation period of 1 hr. at 37 ºC, the extent of deoxyribose degradation was measured by the Thio Barbituric Acid (TBA) reaction. The absorbance was measured at 532 nm. Vitamin C was again taken as a positive control. In all of the above cases, inhibitory activity was determined using the following equation:

$$\text{Percent inhibition} = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) \times 100}{\text{Abs}_{\text{Control}}}$$

### *2.4.3. β-Carotene Photobleaching Assay*

A β-carotene solution was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform. Out of this solution 2 ml was pipetted into a 100 ml round bottom flask. After the removal of chloroform under vacuum, 40 mg purified linoleic acid, 400 mg 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 J-7 extract (100-500 µg/ml). Ascorbic acid was used for comparative purposes. Soon after the addition of emulsion in each tube, the zero time absorbance was measured at 470 nm. Measurement of absorbance was redone after incubation at 50 ºC in a water bath for 2 hours. A blank, devoid of β-carotene, was prepared for background subtraction (Sharma and Kaur, 2015).

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

$$AA = \frac{100[1-(A_0-A_t)]}{A_0^\circ - A_t^\circ}$$

where, $A_0$ and $A_t$ = Absorbance of test initially and absorbance of test after time t; and, $A_0^\circ$ and $A_t^\circ$ = Absorbance of control initially and absorbance of control after time t.

### *2.4.4. Reducing Power Activity*

For reducing power activity, different concentrations of extract (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 ºC. After incubation, 2.5 ml of 10% trichloroacetic acid was added to each mixture followed by centrifugation at 3,000 rpm for 10 min. The upper layer (5 ml) was then mixed with 5 ml of

distilled water and 1 ml of 0.1% ferric chloride (Verma et al., 2014). The absorbance of the resultant solution was measured at 700 nm and was compared with Butylated Hydroxy Toluene (BHT), taken as standard.

#### *2.4.5. Total Phenolic Content (TPC) estimation*

This was done by the addition of 0.1 ml of ethyl acetate extract (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 $Na_2CO_3$ added, followed by an incubation period of 2 h at 25 ºC and taking absorbance at 765 nm. Standard curves were prepared using different concentrations of catechol and gallic acid using same reagents and conditions. The TPC was expressed as mg of catechol and gallic acid equivalents per gram of extract (Mehni and Shahdadi, 2014).

### 2.5. Preparation of the extract for purification

The samples for α-amylase inhibitors were prepared by fermenting the cultures in SCB for 7-10 days in rotary shaker (200 rpm) at 28 ºC. Thereafter, the broth (500 ml) was centrifuged at 10,000 rpm for 15 min at 10 ºC and the filtrate was separated. The supernatant was extracted twice with ethyl acetate: methanol (4:1). The extract was then concentrated using a hot air oven at <50 ºC. Similar protocol was used for α-glucosidase inhibitor; however ammonium sulphate (30-80%) was used for concentration of the protein of interest.

### 2.6. Purification of the potent enzyme inhibitors by silica gel column chromatography

Approximately 300 g of silica gel absorbent, 60-120 mesh was taken and mixed in chloroform so as to thoroughly wet and to remove bubbles from the silica gel. A glass funnel was set at the top of and silica gel mixture was poured with gentle shaking (5 cm × 60 cm). In order to pre-elute the column, chloroform: ethanol (5: 95) was added to the top of the silica gel. Dry powder of sample in silica was transferred to the top of prepared column. The loaded portion was eluted with a stepwise gradient of chloroform: ethanol fraction (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 and 55:45). The separation of compounds was stated as colored band travelled towards the bottom of the column. The fractions obtained with various elution gradients were collected and analyzed for the presence of α-amylase and α-glucosidase inhibitors using spectrophotometric method. The inhibitor enriched fractions were identified, pooled together, concentrated and termed as J-7$^{P\text{-}\alpha\text{-}Amy}$ and J-7$^{P\text{-}\alpha\text{-}Glu}$.

A pictorial representation of the above mentioned steps has been given in Figure 1.

### 2.7. Kinetic study of purified α-amylase and α-glucosidase inhibitors

The mode of inhibition of α-amylase by J-7$^{P\text{-}\alpha\text{-}Amy}$ was conducted according to the modified method described by Kazeem et al. (2013). Briefly, 250 µl of J-7$^{P\text{-}\alpha\text{-}Amy}$ (2 mg/ml) was preincubated with 250 µl of α-amylase solution for 10 min at 25 ºC in one set of tubes. In another set of tubes 250 µl of phosphate buffer (pH 6.9) was preincubated with α-amylase. Then, 250 µl of starch solution at increasing concentrations (0.50-5.0 mg/ml) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25 ºC and then boiled for 5 min after the addition of 500 µl of DNS to stop the reaction. The amount of reducing sugars released was determined calorimetrically using a maltose standard curve and converted to reaction velocities.

The mode of inhibition of α-glucosidase by the purified compound was conducted according to the modified method described by Kim et al. (2005). A 50 µl volume of J-7$^{P\text{-}\alpha\text{-}Glu}$ (5 mg/ml) was preincubated with 200 µl of α-glucosidase solution (0.5 mg/ml) for 10 min at 25 ºC in one set of tubes. In another set of tubes, α-glucosidase was preincubated with 50 µl of phosphate buffer (pH 7.0). Then, 100 µl of pNPG solution at increasing concentrations (0.50-5.0 mg/ml) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25 ºC and 1 ml 0.1 M $Na_2CO_3$ to stop the reaction. The amount of pNP released was determined calorimetrically using pNP standard curve and converted to reaction velocities.

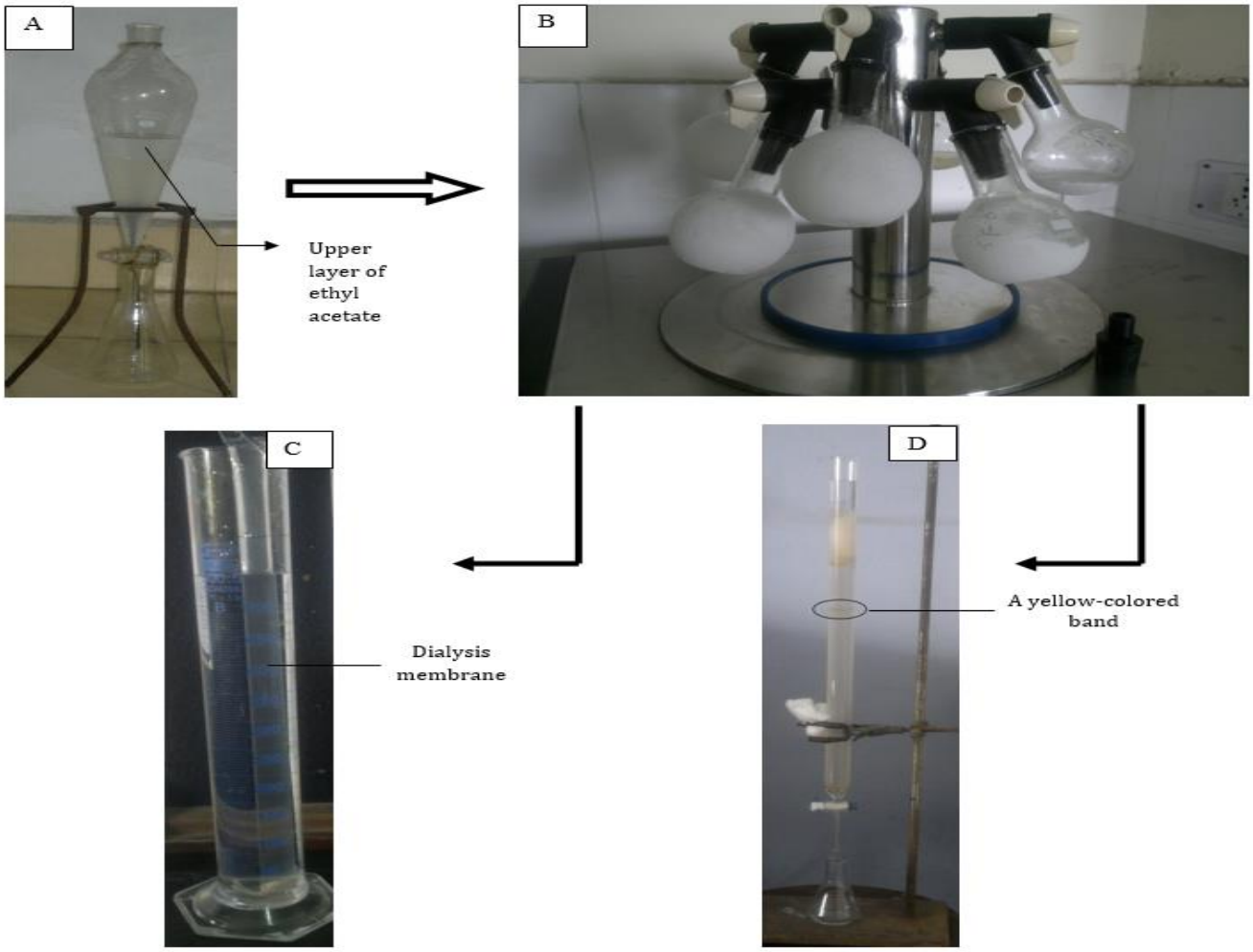


**Figure 1.** Schematic diagram of experimental settings:

(A) Extraction of the supernatant with ethyl acetate: methanol (40:10) in a separatory funnel

(B) Concentration of the extract at -130 °C using lyophilizer

(C) Dialysis of J-7 ethyl acetate extract after ammonium sulphate precipitation

(D) Yellow colored band obtained while carrying out silica gel column chromatography of alpha amylase inhibitors

A double reciprocal plot (1/v versus 1/[S]) was plotted, where v is reaction velocity and [S] is substrate concentration. The mode of inhibition of the crude extract on α-amylase and α-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics (Nelson and Cox, 2008). In all the tests performed, 10% DMSO was used as a control, in order to nullify effects produced due to its presence.

## 2.8. Statistical analysis

The data for biochemical and physiologic parameters were analyzed and expressed as mean ± SD. The 50% inhibitory values were calculated from linear regression analysis using computer program, Microsoft Excel, 2007.

## 2.9. Liquid Chromatography- Mass Spectrometry (LC-MS) of the purified extract

Thin layer chromatography of the purified compound obtained after column chromatography was performed using chloroform: methanol as the solvent system. The plate was let dry and visualized using UV light. Using the edge of a spatula, the bands were scraped off and scrapings were sent for characterization by LC-MS. The whole procedure was carried out at Sophisticated Analytical and Instrumentation Facility, Panjab University, Chandigarh. The centre is equipped with a Waters Micromass Q-Tof Micro. The Instrument is hybrid quadrupole time of flight mass spectrometer equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APcI) sources. The Mass Spectrometer is coupled with Waters 2795 HPLC having quaternary pumping configured for flow rates from 0.05- 5.0 ml/min. The auto sampler is configured with a 100 µl syringe. The conditions which were used for operating the system were as described by Krishna Kumar et al. (2014). Experiment was performed at ambient temperature.

| **HPLC conditions** | | | |
|---|---|---|---|
| Analytical column | C18 column, 3 µm, 100×4.6 mm | | |
| Injection volume | 5 µl | | |
| Flow rate | 300 µl/min | | |
| Mobile Phase: | | | |
| Solvent-A | 0.1% Formic Acid | | |
| Solvent-B | Acetonitrile | | |
| Gradient program | Time (min) | Solvent A | Solvent B |

| | | | |
|---|---|---|---|
| | 00.01 | 75 | 25 |
| | 02.00 | 70 | 30 |
| | 08.00 | 40 | 60 |
| | 12.00 | 40 | 60 |
| | 15.00 | 75 | 25 |
| | 20.00 | 75 | 25 |
| **Mass spectrometry experiment conditions** | | | |
| Ion probe | ESI | | |
| Sheath gas | (22) Nitrogen | | |
| Auxiliary gas | (8) Nitrogen | | |
| Discharge current | 5.0 K μA | | |
| Ionization mode | Negative | | |
| Capillary volt | -16 V | | |
| Tube Lens | 55 V | | |

## 2.10. Characterization of the endophytic actinobacterium

### *2.10.1. Cultural characteristics*

The isolate was grown on 4 different media *viz*., SCA, Nutrient Agar (NA), Glycerol Yeast Extract agar (GYEA) and Yeast Malt Extract Agar (YMEA). The plates were observed for the production of diffusible pigments and color of substrate and aerial mycelium.

### *2.10.2. Staining and biochemical characteristics*

Staining techniques *viz*., Gram's and Acid-fast staining and biochemical tests *viz*., decomposition of tyrosine and hydrolysis of starch (1%), esculin, NaCl (5%), sodium azide (0.03%), tween 20, tween 80 utilization were performed.

### *2.10.3. Microscopic characteristic*

The cover slip method was followed to observe characteristic spore arrangement. The culture was inoculated all over the NA agar surface using sterile inoculation loop. A cover slip was placed over the streaked zone,

and the slide was placed in a sterile moist chamber and incubated until good growth of the isolate was observed. The cover slip was removed, placed on a drop of dilute crystal violet stain on a clean glass slide and observed under oil immersion objective in order to study the arrangement of spores.

## 3. Results

### 3.1. Determination of α-amylase and α-glucosidase inhibitory activity

As evident from Table 1, the ethyl acetate extract of J-7 was exhibiting a dose dependent increase in inhibitory activity ($IC_{50}^{J-7}$ = 83.52 ± 0.19 µg/ml). The activity of Acarbose, was almost constant at various concentrations tested ($IC_{50}^{Acarbose}$ = 10.64 ± 1.33 µg/ml).

Both the tested samples (J-7 and voglibose) displayed similar trend with respect to inhibitory activity. After reaching a maximum (97%) at 250 µg/ml, α-glucosidase inhibitory potential of J-7 exhibited a decrease with increase in concentration further (Table 1).

**Table 1.** Anti-starch degradation properties of the ethyl acetate extract J-7

| **Concentration (µg/ml)** | **Inhibitory property** | | | |
|---|---|---|---|---|
| | **α-amylase** | | **α-glucosidase** | |
| | **J-7** | **Acarbose** | **J-7** | **Voglibose** |
| 10 | 15.07±0.09 | 49.53±0.80 | - | - |
| 25 | 30.55±0.14 | 49.07±0.80 | - | - |
| 50 | 41.03±0.28 | 46.75±0.80 | 10.24±0.38 | 12.60±0.12 |
| 75 | 49.07±0.06 | 44.90±0.80 | 18.63±1.96 | 21.82±0.04 |
| 100 | 52.41±0.00 | 41.66±1.39 | 45.77±0.01 | 25.07±0.08 |
| 250 | 57.24±0.00 | - | 97.44±0.02 | 63.34±0.10 |
| 500 | 57.43±0.02 | - | 75.09±0.22 | - |
| 750 | 57.91±0.02 | - | 61.04±0.79 | - |
| 1000 | 54.55±0.06 | - | 62.17±0.46 | - |
| $IC_{50}$ | 83.52±0.19 | 10.64±1.33 | 148.52±5.92 | 196.84±0.16 |

### 3.2. Antioxidant production and free radical scavenging activities *in vitro*

#### *3.2.1. DPPH radical Scavenging Activity*

The extract exhibited significant dose dependent inhibition of DPPH activity with a 50% inhibition at a concentration of 861.17 ± 1.94 µg/ml. The results are given in (Fig. 2(a)). The $IC_{50}$ value of vitamin C was

178.37 ± 0.13 µg/ml. It is interesting to point here that after reaching a maximum of 78.64% at 500 µg/ml, the inhibitory activity of vitamin C started decreasing gradually.

### *3.2.2. Hydroxyl Radical Scavenging Activity*

As depicted in Fig. 2 (b), the hydroxyl radical scavenging effects of J-7 extract was significantly high with >60% inhibition at all the tested concentrations.

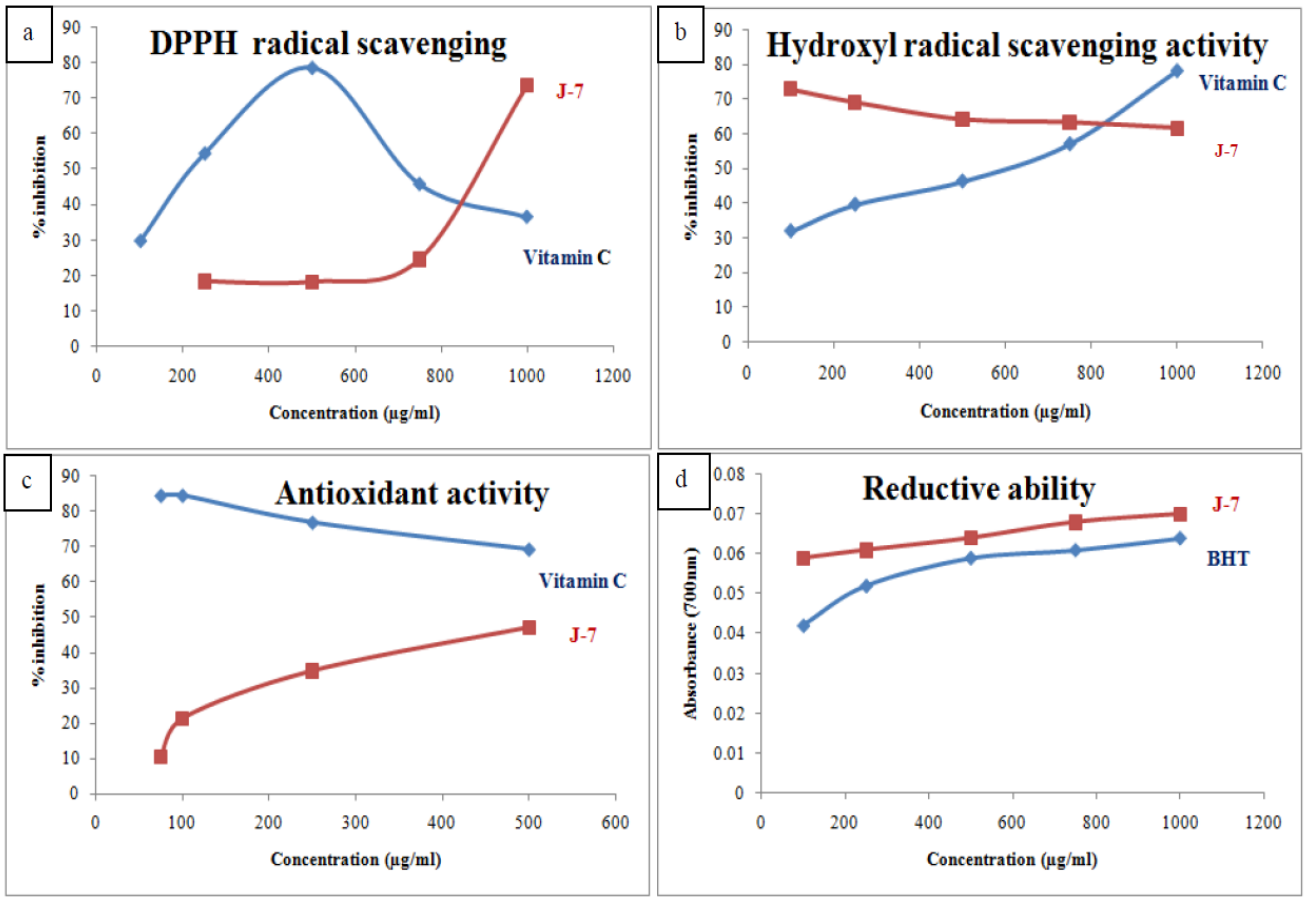


**Figure 2.** (a) DPPH scavenging effect of different concentrations (100-1000 µg/ml) of J-7 and vitamin C. Each value represents Mean±SEM of triplicate experiments.

(b) Hydroxyl radical scavenging effect of different concentrations (100-1000 µg/ml) of J-7 and vitamin C. Each value represents Mean±SEM of triplicate experiments.

(c) Antioxidant activity of different concentrations (100-500 µg/ml) of J-7 in the β-carotene bleaching assay and vitamin C. Each value represents Mean±SEM of triplicate experiments.

(d) Reductive ability of different concentrations (100-1000 µg/ml) of J-7 and BHT. Each value represents Mean±SEM of triplicate experiments.

### *3.2.3. β-Carotene Photobleaching Assay*

The extract hindered the bleaching of β-carotene in a dose-dependent manner. After 120 min of incubation, 50% inhibition was observed at 631.05 ± 0.13 µg/ml and the value for vitamin C was 1030.27±0.00 µg/ml. The reason for such high $IC_{50}$ values was the high degrees of inhibition at 100 µg/ml, that showed a gradual decrease afterwards (Fig. 2 (c)).

### *3.2.4. Reducing Power activity*

Figure 2 (d) shows the reductive capabilities of the extract compared to the standard BHT. The reducing power of J-7 extract increased with increasing quantity of the sample. Interestingly, the absorbance of the extract was higher as compared to BHT at all the concentrations tested.

### *3.2.5. Total Phenolic Content estimation*

The TPC summed up to 47.92 ± 2.98 and 6.94 ± 0.93 mg/g of the ethyl acetate extract for the catechol and gallic acid equivalents.

## 3.3. Kinetic study of purified α-amylase and α-glucosidase inhibitors

Ethyl acetate extract J-7 exerted a competitive inhibition vis-à-vis α-amylase showing the same and different compared to the control (Fig. 3). Same was the case for α-glucosidase inhibitory activity, whereby the purified extract exerted near competitive inhibition (Fig. 4). The Km value for α-amylase control was 0.55 mM, whereas in the presence of inhibitor an increase was observed (0.62 mM). Similarly, for α-glucosidase, the Km factor displayed an increase from 2.00 to 2.80 mM. As evident in case of competitive inhibitions, their respective Vmax values were almost constant (0.008 and 0.070 mM/ min for α-amylase and α-glucosidase respectively).

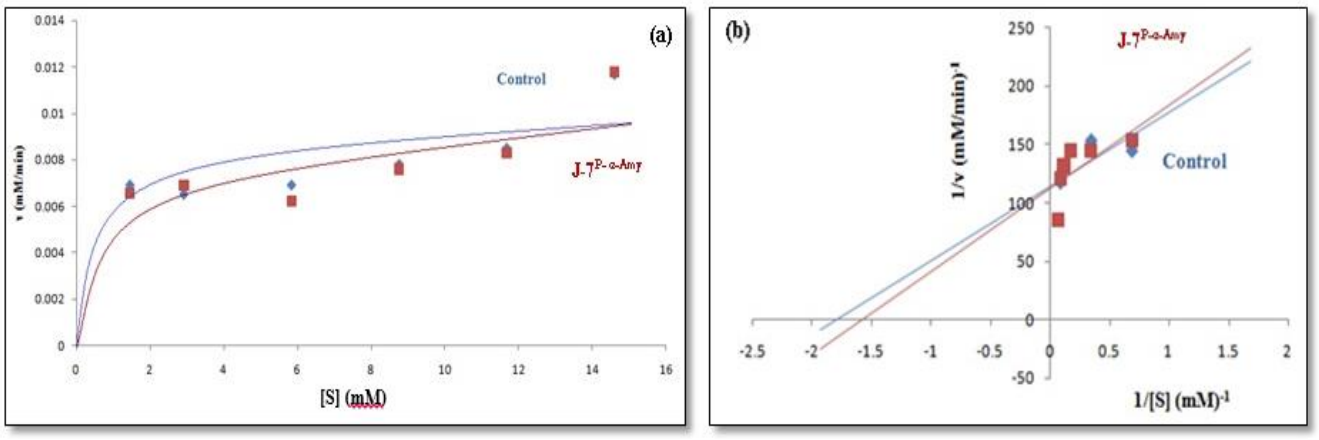


**Figure 3.** Competitive mode of inhibition of α- amylase by purified extract of J-7 (J-$7^{P\text{-}\alpha\text{-}Amy}$)

(a) Michaelis Menten plot.
(b) Lineweaver-Burk plot.

## 3.4. Liquid Chromatography- Mass Spectrometry of the purified extract

The chemical constituent of the purified extract was determined using LC-MS analysis. The resolved chemical entities were then identified using online software i.e., MassBank which is a public repository for sharing mass spectral data. Two major peaks (m/z= 175.00 and 304.98) were detected in the extract (Fig 5). The obtained values of m/z were provided to the software which it read and gave results in form of number of matched formulae which includes mass (m/z) values and its chemical formulae. The compounds were then checked for the existing literature showing positive antidiabetic and antioxidant activity. Hence, our finding reveals two major compounds *viz.* 6-hydroxyl-4-methyl coumarin (m/z= 175.00) and dihydroquercetin (m/z= 175.00 and 304.98). It is interesting to acknowledge that both of these compounds are derivatives of the basic structure chromene, which is also known as benzopyran.

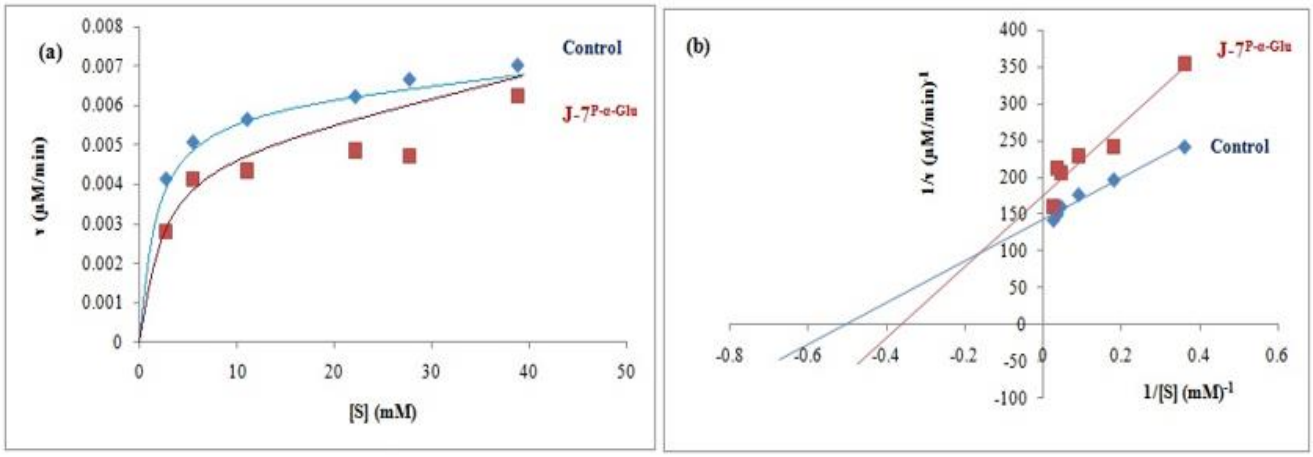


**Figure 4.** Near competitive mode of inhibition of $\alpha$-glucosidase by purified extract of J-7 (J-$7^{P\text{-}\alpha\text{-}Glu}$)
(a) Michaelis-Menten plot and
(b) Lineweaver-Burk plot.

## 3.5. Characterization of the endophytic actinobacterium isolate

Isolate J-7 was identified by morphological, biochemical and physiological characteristics. Table 2 shows the morphological features of the strain. It grew well on all media used and had a grey to white aerial mycelium. However, no diffusible pigments were detected on the media tested. On NA and YMEA media, yellow colony color was established. Table 3 shows the physiological properties of J-7. Optimal growth of strain was observed 5% NaCl. The culture was able to produce enzymes such as amylase, protease, tyrosinase, and lipase. Under the compound microscope, the culture was observed to have chain type spore arrangement. Hence, based on the morphological and physiological characteristics, J-7 was tentatively assigned to the genus *Streptomyces*.

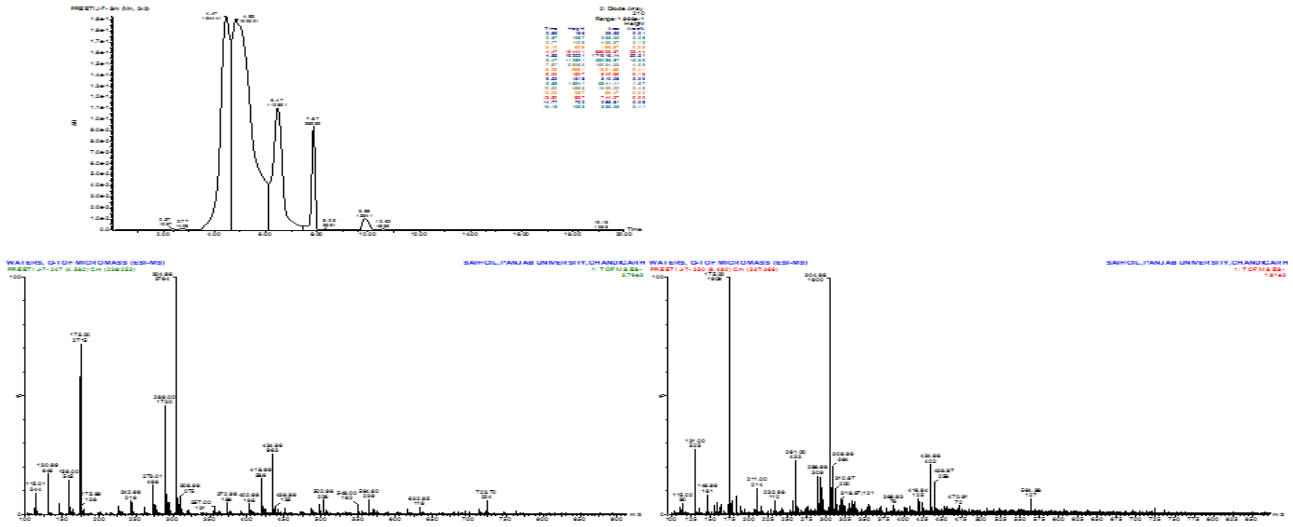

**Figure 5.** LC-MS profile of purified ethyl acetate extract J-7 (J-7$^{P\text{-}\alpha\text{-}Amy}$)

**Table 2.** Morphological features of J-7 on different media

| Medium | Mycelium colour | Diffusible pigment | Reverse side |
|---|---|---|---|
| **Glycerol yeast extract agar** | Gray white | - | White |
| **Nutrient agar** | White | - | Yellow |
| **Starch casein nitrate agar** | Grey | - | Buff |
| **Yeast malt extract agar** | Grey white | - | Yellow |

## 4. Discussion

Plants are the natural resources for various enzymatic as well as enzyme inhibitory activities. Some animal studies have suggested the role of plant extracts in preventing an excessive postprandial rise of blood glucose levels (Karau et al., 2012). The use of antioxidants along with carbohydrate-degradation inhibiting compounds has been widely reported in reducing the levels of oxidative stress and in slowing down or preventing the development of complications associated with chronic diseases (Oyenihi et al., 2015; Zhang et al., 2015).

**Table 3.** Physiological and biochemical characteristics of J-7

| **Characteristics** | **Results** |
|---|---|
| Gram staining | Positive |
| Shape and growth | Filamentous and aerial |
| Growth in the presence of 5% NaCl | Positive |
| **Hydrolysis of** | |
| Casein (10%) | Positive |
| Esculin (0.1%) | Positive |
| Starch (1%) | Positive |
| T-20 (1%) | Positive |
| T-80 (1%) | Positive |
| **Decomposition of** | |
| Tyrosine (0.5%) | Production of zone and brown color |

In the present study, the strain J-7, isolated from the root tissues of *Syzygium cumini*, was presumptively identified to be belonging to *Streptomyces* sp. The ethyl acetate extract of endophytic actinobacterium strain was thus tested for different *in vitro* starch degradation inhibition and antioxidant properties. The ethyl acetate extract of J-7 effectively controlled α-amylase and α-glucosidase based reactions. Likewise, J-7 extract was able to inhibit DPPH free radicals *in vitro*. The method is based on the conversion of DPPH free radical in the presence of a hydrogen-donating antioxidant to a non-radical form by the reaction (Christhudas et al., 2013). Hydroxyl radical scavenging capacity of a compound has been related to its antioxidant activity (Shukla et al., 2009). Ethyl acetate extract of J-7 prevented the free radical-mediated deoxyribose damage by reacting with hydroxyl free radicals. In the β-carotene linoleate model system, coupled oxidation of β-carotene and linoleic acid takes place, which generates free radicals. As a result, the system loses its chromophore and characteristic orange color is produced, which can be monitored spectrophotometrically (Christhudas et al., 2013). In our study, the inhibition of β-carotene bleaching by J-7 extract showed almost equivalent activity with vitamin C (at 500 µg/ml concentration). For measurement of reductive ability, the conversion of $Fe^{3+}$ to $Fe^{2+}$ in the presence of extract was investigated. The reducing power increased with increasing concentration of the extract and was found to be higher as compared to BHT. The reducing capacity of a compound may be an indicator of its potential antioxidant activity (Meir et al., 1995).

Competitive inhibition refers to that mode in which substrate affinity of the enzyme is hindered by the presence of another molecule with similar symmetry but higher affinity towards the enzyme. The results of present study indicated that the extract exhibited competitive modes of inhibition towards both the enzymes tested. Thus, it may be assumed that providing doses of extract *in vivo* at regular intervals will result in decreased exposure of the enzyme to the carbohydrate moiety resulting in suppression of secondary effects of diabetes.

The antidiabetic, antioxidant and scavenging are believed to be due to the presence of measurable amount of phenolics as estimated by Folin-Ciocalteau method (Moradi-Afrapoli et al., 2012). Polyphenols obtained from plants have been demonstrated to have significant antioxidant activity. This activity is believed to be mainly due to their redox properties which are regarded important for adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Upadhyay et al., 2010).

Our assumptions are further supported by the results of LC-MS analysis, which indicated the occurrence of chromene family of compounds. Chromenes have been widely demonstrated to exhibit a remarkable array of biochemical and pharmacological activities. They constitute the basic structural back bone of many types of tannin and polyphenols widely present in plants e.g. green tea, fruits and vegetables (Rensburg et al., 1997). The presence of the chromene-containing structure is correlated with the capability to prevent several diseases (Kennedy and Thornes, 1997). Synthetic analogues have attracted considerable attention due to their useful biological and pharmacological properties including antimicrobial (El-Saghier et al., 2007; Kumar et al., 2009), antioxidant (Alvey et al., 2008), anticancer (Kemnitzer et al., 2008), anticoagulant, antidiabetic (Vishnu et al., 2015), hypotensive (Tandon et al., 1991), local anesthetic (Longobardi et al., 1990) and central nervous system activities. In addition, these also bear potential for treatment of Alzheimer's disease (Bruhlmann et al., 2001) and Schizophrenia disorder (Kesten et al., 1999). Compounds, like hydroxyl-coumarins (Hoult and Paya, 1996), directly recombine free radicals and interrupt the initiation and/or propagation of the induced chain reactions, thus acting as potent metal chelators and free radical scavengers, resulting in a powerful antioxidant effect (Traykova and Kostova, 2005).

To exhibit antioxidant activity, a coumarin (derivative) has to possess at least one hydroxyl group (Shukla et al., 2009). The evaluation of coumarin isolates from *Geranium wallichianum* (Ismail et al., 2009) and Korean medicinal plants (Tandon et al., 1991) point towards the presence of catechol moiety and oxygen containing scaffold at C-6 and C-7 positions of the coumarin core for antioxidant activity. Also, the α-pyrone coumarin ring uplifts free radical scavenging activity, antilipid peroxidation ability and also has a suppressive effect on enzymes (Tandon et al., 1991). Synthetic compounds, 4-methylcoumarins (Cavar et al., 2009), substituted 7- or 8-hydroxybenzo[f]-coumarins, 6-hydroxybenzo[h]-coumarins and 7-azomethinecoumarins were tested for their antioxidant ability *in vitro* (Kontogiorgis and Hadjipavlou-Litina, 2004) and for their ability to interact with DPPH stable free radical (Al-Amiery et al., 2015 a), scavenging of hydrogen peroxide (Al-Amiery et al., 2015 b), superoxide anion and inhibition of lipid peroxidation, too (Verma et al., 2014).

## 5. Conclusion

This study suggested that endophytic actinobacteria from *Syzygium cumini* possessed antidiabetic, antioxidant and reducing potentials. It also confirms the fact that just like the host plant an endophyte is also having the capability to combat development of various oxidative stress-related chronic diseases. The spore-forming, robust endophytic actinobacteria could be a potential source of bioactive metabolites while their host plants face a survival issue. Further investigation on the animal models may lead to chemical entities for clinical use.

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