CHAPTER 3

Screening of plant and actinomycete extracts for α-amylase inhibitory activity

3.1 INTRODUCTION

The plant kingdom is a rich source of structural biodiversity and offers a variety of natural products. The novel bioactive compounds possess a broad range of structural diversity bearing biological activities, which includes alkaloids, phenolics, terpenoids, steroids etc. Various types of medicines are used in the form of crude drugs such as tinctures, poultices, powders and other herbal formulations (Samuelsson, 1992). Ethnopharmacologists, botanists, microorganisms and natural product chemists are reforming different strategies to develop phytochemicals which could provide effective treatment for various diseases.

Most plants are hosts to one or more endophytic microorganisms. Endophytes are the microorganisms that colonize the living cells of host with no overt tissue damage. In recent years, bioactively potential actinomycete endophytes have introduced radical change in natural product research. One of the most interesting endophytic bacteria is the member of actinomycetes, the soil dwelling Gram-positive bacteria. The actinomycetes, especially *Streptomyces* spp. are biotechnologically important, providing over two third of antibiotics and bioactive compounds used these days (Baltz, 2008). Streptomyces are believed to be a rich source of new and useful compounds, and up to 40% of known microbial metabolites are derived from this group (Nagai et al., 2011). The endophytic actinobacteria are also considered as the important natural source to obtain a variety of secondary metabolites (Qin et al., 2011).

Enzyme inhibition is a reaction between a molecule and an enzyme that blocks the action of the enzyme, either temporarily or permanently, depending on the type of enzyme inhibitor. This process occurs in the natural world all the time, and it has a number of applications for humans, including in the formulation of pharmaceuticals and the development of certain products. There are several types of enzyme inhibition involving different types of molecules and processes (Horton et al., 2006).
α-amylase inhibitors are also known as starch blockers. Starches are complex carbohydrates that cannot be absorbed unless they are first broken down by the digestive enzyme amylase and other secondary enzymes. The inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of absorption of the simple sugars. Two of these inhibitors are: Acarbose™ and Quercetin. Acarbose™ is an anti-diabetic drug and Quercetin is a flavonoid found in plants. Many plants have been investigated for their potential to reduce the production of simple sugars from carbohydrates (Raj et al., 2008). They play an important role in the treatment and management of diabetes, particularly in developing countries where most people have limited access to primary health care (Ali et al., 2006).

The inhibition of α-amylase enzyme is a target in drug-design in the development of compounds for the treatment of diabetes, obesity and hyperlipidemia (Franco et al., 2002). The search for appropriate anti-hyperglycemic agents is focused on plants used in traditional medicine because natural products may be a better option than currently used drugs (Hu et al., 2001). A number of medicinal plants and their formulations are used for treating diabetes in the traditional Indian Ayurvedic system as well as in the ethnomedicinal practices.

The inhibition of α-amylase, the key enzyme involved in the hydrolysis of starch by soil actinomycetes have been documented (Mahmud, 2003). Culture broths of Streptomyces hygroscopicus subsp. limonatus (Iwasa et al., 1970 and 1971), S. diastaticus subsp. amylostaticus and S. calvus (Namiki et al., 1982a and 1982b) have yielded inhibitors of α-amylase. Most α-amylase inhibitors are of microbial origin and are isolated from marine actinomycetes (Suthindhiran et al. 2009). The endophytic sources of α-amylase inhibitors have not been documented.

Acarbose™ and Voglibose are currently used as α-amylase and α-glucosidase inhibitors, but are known to induce side effects such as bloating, flatulence and diarrhoea (Rhabaso-Lloret and Chiasson, 2007). It has been suggested that such adverse effects might be caused by the excessive inhibition pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the
colon (Bischoff, 1994). Therefore, screening of natural α-amylase inhibitors from the dietary plants is effective therapy for post prandial hyperglycemia with minimal side effects. The present chapter deals with the screening of plant and endophytic actinomycete extracts for the inhibition of α-amylase enzyme.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of plant and actinomycete extracts

The preparation of plant and actinomycete extracts have been described in the sections 2.2.1 and 2.2.3 of Chapter II, respectively. Further, these extracts were screened for the α-amylase inhibition activity by *in vitro* methods.

3.2.2 Screening of plant and actinomycete extracts for α-amylase inhibitory activity

3.2.2a α-amylase inhibition assay

3.2.2a (i) Starch-Iodine colour assay

*Preparation of 0.02 M sodium phosphate buffer:* 138 g of NaH$_2$PO$_4$ (monobasic) and 142 g of Na$_2$HPO$_4$ (dibasic) was dissolved in 1 L of distilled water to make 1 M sodium phosphate buffer.

Screening of both plant and actinomycete extracts for α-amylase inhibitors was carried out in a 96-well microtitre plate (Tarsons®) based on the starch-iodine test (Xiao *et al*., 2006). The total assay mixture composed of sodium phosphate buffer (0.02 M, pH 6.9) containing sodium chloride (6 mM), enzyme solution and different concentrations of plant and actinomycete extracts. The mixture was incubated at 37° C for 10 min. Soluble starch (1%, w/v) was added to each reaction well and incubated at 37° C for 15 min. Hydrochloric acid (1 M, 20 μl) was added to stop the enzymatic reaction, followed by the addition of 100 μl of iodine reagent. The color change was noted. The control reaction was represented by 100% enzyme activity, and did not contain any extract. The known α-amylase inhibitor, Acarbose™, was used as the positive control. A dark-blue colour indicates the presence of starch; a yellow colour indicates the absence of starch while a brownish color indicates partially degraded starch in the reaction mixture.
3.2.2a (ii) 3, 5-dinitrosalicylic acid assay

The inhibition assay was performed using the chromogenic dinitrosalicylic acid (DNSA) method (Suthindhiran et al., 2009). 500 µl of the extracts and 500 µl of sodium phosphate buffer (0.02 M, pH 6.9 with 0.006 M sodium chloride) containing α-amylase from Aspergillus oryzae (Sigma-Aldrich Co., St. Louis, Mo.) were incubated for 10 min at 25º C. After pre-incubation, 500 µl of starch solution (1%) in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5 sec intervals. The reaction mixtures were then incubated at 25º C for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after the addition of 10 ml distilled water and the absorbance was measured at 540 nm. The known α-amylase inhibitor Acarbose™ was used as a positive control. The IC₅₀ values were determined from plots of percent inhibition versus the inhibitor concentration and calculated by the logarithmic regression analysis from the mean inhibitory values

\[
\% \text{ Inhibition} = \frac{(A_{540 \text{ Control}} - B_{540 \text{ Extract}})}{A_{540 \text{ Control}}} \times 100
\]

3.2.2b Evaluation of anti-diabetic activity by the uptake of glucose by hemi diaphragm

The anti-diabetic activity was tested by two different experiments using hemi diaphragm as a model. The term hemi diaphragm refers to a lateral half of diaphragm with normal muscle development on one side only. Since, muscle is the peripheral site of insulin action (Katsumata et al. 2007), porcine hemi diaphragm, obtained from the animal slaughter house was considered. Fresh samples were collected and washed thoroughly with saline (0.85%, w/v), until a clear pink color was obtained. In one set of experiments, both plant and actinomycete extracts (10-50 µg of 1 mg/ml) were added directly onto porcine hemi diaphragm to detect the uptake of glucose by hemi diaphragm, while in the second set of experiments, the extracts were added to porcine pancreas to determine the insulin-releasing ability of the extracts.

3.2.2b (i) Release of the insulin using pancreas

Fresh porcine pancreas, obtained from the animal slaughter house, Mysore was washed thoroughly with saline (0.85%, w/v). 300 mg of fresh porcine pancreas
was incubated in saline (0.85%) contained in 12-well culture plates (Nes™, Shangai, China), for 10 min (Fig. 3.1). Both plant and the actinomycete extracts (1 mg/ml) were added separately to pancreas at 10-50 µg concentration, followed by the addition of 0.5 ml of glucose (10 mM) and incubated for an hour. After the incubation, 0.8 ml aliquots were collected and used for the estimation of glucose uptake.

![Image](12-well culture plate and Porcine pancreas)

**Figure 3.1 Release of insulin using pancreas**

### 3.2.2b (ii) Estimation of glucose-uptake by the isolated hemi diaphragm

The porcine hemi diaphragms (300 mg, fresh weight) were incubated with insulin-glucose solution while the other diaphragm was incubated with glucose alone as a control. At the end of the experiment, the glucose left in the incubation medium was determined in order to evaluate the rate of uptake of glucose by the isolated muscle cells. The porcine diaphragm (300 mg) was taken in 1.0 ml of saline and different concentrations of the extracts (10-50 µg) - plus 0.5 ml of glucose (10 mM) was added. The aliquots were collected at different time intervals (0, 5, 10, 20, 30 and 60 min). 50 µl of the aliquots were transferred to plates and subsequently 200 µl of GOD-POD reagents (Autospan®, Tokyo, Japan) was added and read spectrophotometrically at 505 nm. 0-1U insulin was used instead of the extracts as a positive control (Vallance-Owen and Hurlock, 1954). The uptake of glucose was determined by measuring the disappearance of glucose from the medium, colorimetrically with glucose oxidase and peroxidase.
3.3 Data analysis

All analyses of the extracts were carried out in triplicates. The reported value for each test was calculated as the mean of three measurements and represented as mean ± Standard Error of the Mean. The IC\textsubscript{50} values were calculated from linear regression analysis. The results were processed by SPSS software (version 16.0).

3.4 RESULTS
3.4a \(\alpha\)-amylase inhibition assay

Among the solvent extracts of medicinal plants considered for the study, the methanolic extract of \textit{L. ciliata} showed positive results in the initial screening for the inhibition of \(\alpha\)-amylase by the Starch-Iodine color assay. The extract was further quantified by chromogenic DNSA method. The methanolic extract of \textit{L. ciliata}, showed 72.9\% inhibition (200 \(\mu\)g/ml) with an IC\textsubscript{50} value of 138.7± 3.47 \(\mu\)g/ml. The standard inhibitor Acarbose\textsuperscript{TM} showed 82.1\% inhibition with an IC\textsubscript{50} value of 96.29± 2.89 \(\mu\)g/ml (Fig. 3.2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.2.png}
\caption{Per cent inhibition of \(\alpha\)–amylase activity of Acarbose\textsuperscript{TM} and methanolic extract of \textit{L. ciliata}}
\end{figure}

\textit{L. ciliata} methanol extract and the standard Acarbose\textsuperscript{TM} were prepared at 1 mg/ ml concentration. 200 \(\mu\)g/ ml extracts were tested for the inhibition of \(\alpha\)–amylase activity. The absorbance was read at 540 nm spectrophotometrically. Per cent inhibition was calculated against blank and represented. Each value represents the mean ± SEM of triplicate experiments.
Eight endophytic isolates, representing five from \textit{L. ciliata} and three from \textit{R. densiflora} showed positive results in the initial screening for the inhibition of \(\alpha\)-amylase activity by Starch-iodine color assay. Among the positive isolates, the extract of \textit{Streptomyces longisporoflavus} (JX965948) isolated from the stem fragments of \textit{L. ciliata} showed 60.2\% inhibition (200 \(\mu\)g/ ml) (Fig. 3.3). The IC\(_{50}\) value was 162.3±1.05 \(\mu\)g/ ml, in comparison to the standard inhibitor Acarbose™ (73.1±1.12 \(\mu\)g/ ml). \textit{Streptomyces} sp., isolated from \textit{R. densiflora} was negative for \(\alpha\)-amylase inhibition assay. The IC\(_{50}\) values were determined from plots of percent inhibition versus inhibitor concentration and calculated by logarithmic regression analysis from the mean inhibitory values.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3.3.png}
\caption{Per cent inhibition of \(\alpha\)-amylase activity of Acarbose™ and \textit{S. longisporoflavus} extract}
\end{figure}

\textit{S. longisporoflavus} extract and the standard Acarbose™ were prepared at 1 mg/ ml concentration. 200 \(\mu\)g/ ml extracts were tested for the inhibition of \(\alpha\)-amylase activity. The absorbance was read at 540 nm spectrophotometrically. Per cent inhibition was calculated against blank and represented. Each value represents the mean ± SEM of triplicate experiments.
3.4b Evaluation of the anti-diabetic activity by the uptake of glucose by hemi diaphragm

Both *S. longisporoflavus* and *Streptomyces* sp. (Fig. 3.4) extracts did not possess the insulin-releasing ability but, *Streptomyces* sp. extract improved the insulin's ability to move glucose into muscle cells. No plant extracts in the study, showed the anti-diabetic activity by the uptake of glucose by hemi diaphragm.

3.4b (i) Release of insulin using pancreas

The experiment on the release of insulin by pancreas, in which *S. longisporoflavus* and *Streptomyces* sp. extracts were added, indicated the lack of release of insulin's ability by the isolated porcine pancreas. The release of insulin was therefore evaluated by the uptake of glucose through the hemi diaphragm. The uptake of glucose by the hemi diaphragm in both controls as well as the test samples were same, but glucose uptake was observed in the insulin added test sample only (Fig. 3.5). There was no increase in the rate of glucose uptake in the test samples to which the actinomycete extracts were added. Therefore, this experiment indicates that the actinomycete extracts do not facilitate the release of insulin by the pancreas.

![Figure 3.4 Morphology of actinomycete endophytes on agar medium, light microscopy (100X) and SEM (20,000X).](image)

1- *Streptomyces longisporoflavus*, 2- *Streptomyces* sp. a- light microscopy image (100X), b- scanning electron microscopy image (20,000X)
Actinomycete extract concentrations were prepared at 1 mg/ml. The extracts (50 µg/ml) were tested for release of insulin by pancreas. Glucose uptake was determined by measuring the disappearance of glucose from the medium at 505 nm spectrophotometrically. Each value represents the mean± SEM of triplicate experiments.

3.4b (ii) Estimation of glucose uptake by the isolated hemi diaphragm

Among the three test samples, i.e., insulin, *S. longisporoflavus* and *Streptomyces* sp. extract added samples, only *Streptomyces* sp. extract and insulin test samples showed an increase in glucose utilization by hemi diaphragm during one hour of incubation, whereas *S. longisporoflavus* extract added sample did not indicate glucose uptake. All rates of glucose utilization were expressed as mg/g of wet hemi diaphragm (Fig. 3.6). Results indicated that *Streptomyces* sp., extract has anti-diabetic activity.
Figure 3.6 Direct effects of the actinomycete extract on the uptake of glucose by hemi diaphragm.

Actinomycete extract concentrations were prepared at 1 mg/ml. The extract (50 µg/ml) was tested for uptake of glucose by hemi diaphragm. The absorbance was measured at 505 nm spectrophotometrically. Glucose uptake was determined by measuring the disappearance of glucose from the medium. Each value represents the mean ± SEM of triplicate experiments.

3.5 DISCUSSION

α-amylase inhibitors are known as starch blockers because they are known to prevent dietary starch from being digested and absorbed by the body. This could be useful for treating obesity and diabetes mellitus – a metabolic disorder characterized by chronic hyperglycemia resulting from defects in secretion of the insulin (Ali et al., 2006). Diabetes is increasing rapidly and consumes vast amount of resources in all countries. The oxidative products, mainly superoxide anion radical during oxidative stress lead researchers to give more attention to protective functions of naturally occurring antioxidants and α-amylase inhibitors in the cells of organisms containing them (Conforti et al., 2005).

Plants play an important role in the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have
access to modern treatment. α-amylase, a salivary or pancreatic enzyme plays an important role in the early breakdown of complex carbohydrates into simple molecules. Modulation of α-amylase activity affects the utilization of carbohydrates as an energy source and stronger is this modulation; more significant is the reduction in the breakdown of complex carbohydrates. Currently, methods to determine the levels of α-amylase inhibitor are based on the measurement of α-amylase activity resulting by the differential iodine staining power in the presence or absence of an inhibitor during the action of the enzyme on soluble starch or by using an alkaline reactive whose brown reduction products are determined photometrically (Mosca et al., 2008).

In the present study, the extracts of four medicinal plant species and the extracts of actinomycetes were screened for the inhibition of α-amylase activity. Among the plant extracts screened for α-amylase inhibition activity, the methanolic extract of L. ciliata showed 72.9% inhibition with IC$_{50}$ value of 138.7± 3.47 µg/ml in comparison to the standard inhibitor Acarbose™ (82.1% inhibition with IC$_{50}$ value of 96.29± 2.89 µg/ml). Similarly other researchers also have screened medicinal plant extracts for α-amylase activity. Funke and Melzig (2006) demonstrated that the aqueous extracts of Mitragyna inermis leaves showed 90% α-amylase inhibition (200 mg/ml) and leaves of Tamarindus indica showed 50% inhibition at 200 mg/ml. Sudha et al. (2011) reported that the methanolic extract of Morus alba leaves (IC$_{50}$=1.44 mg/ml) and the acetone extract of Ocimum tenuiflorum (IC$_{50}$=8.9 µg/ml) exhibited α-amylase inhibitory activity. The ethanolic extract of Andrographis paniculata showed the α-glucosidase inhibitory effect in a concentration-dependent manner (IC$_{50}$ = 17.2 ± 0.15 mg/ml) and a weak α-amylase inhibitory activity (IC$_{50}$ = 50.9 ± 0.17 mg/ml) (Subramanian et al., 2008).

Preliminary screening for the α-amylase inhibition was performed based on the formation of starch-iodine color complex. A dark blue color indicates the presence of starch; a yellow color indicates the absence of starch while, a brownish color indicates partially degraded starch in the reaction mixture. Our study revealed that the reaction mixture containing methanolic extract of L. ciliata leaves showed dark blue color complex thus indicating the presence of inhibitors. Inhibitors of this enzyme
delay the absorption of ingested carbohydrates and thereby reduce the increase in blood glucose levels. Therefore, investigations on such agents from new, unexplored sources are important.

The actinomycetes are Gram-positive, bacteria, which occupy a wide range of habitats. The primary and secondary metabolites produced by these organisms are highly potent, biologically active and remain a powerful source for pharmaceutical discovery (Balagurunathan and Radhakrishnan, 2007). The terrestrial sources of new enzyme inhibitors seem to be rare and different species of microorganisms may produce structurally identical inhibitors (Imada, 2004) and thereby provides limited options for enzyme inhibition activities. There is a need to find new, safe and effective therapeutic agents for the treatment of many diseases and disorders associated with the carbohydrate metabolism.

To date, most amylase inhibitors of microbial origin have been isolated from terrestrial actinomycetes and few reports are available from marine microorganisms. Of the nearly 5000 isolates from various marine habitats, only strain no. 178, from sediment collected from Aburatsubo Inlet in Kanagawa Prefecture at a depth of 5 m, was found to produce an inhibitor. Due to their purported ability to prevent the breakdown of starch and absorption, the α-amylase inhibitors have been used for weight loss in humans (Bailey, 2003). Imada (2005) has reported the α-amylase enzyme inhibitor-producing marine actinomycete *S. corichorusii* subsp. *rhodomarinus* subsp. nov. Suthindhiran *et al.* (2009) isolated *Micromonospora* sp. with α-glucosidase and α-amylase inhibitory activities from the marine sediment sample. Raja *et al.* (2010) have reported marine actinobacteria producing amylase inhibitors against both prokaryotic and eukaryotic amylases isolated from mangrove rhizosphere of *Rhizophora mucronata* in Vellar estuary, East coast, India. There are no reports of α-amylase inhibitor-producing endophytic actinomycetes. Therefore, in this study, the extracts of actinomycetes were screened for the α-amylase inhibition. Our study revealed that the reaction mixture containing the extract of *S. longisporoflavus* (JX965948) showed dark blue color complex thus indicating the presence of inhibitors.
In 2010, the global prevalence of diabetes was estimated to have reached 285 million and it is predicted to reach 438 million in 2030. Available agents provide imperfect control of the disease, and the medical need for better therapies is widely recognized. About 90 to 95% of patients have non-insulin dependent diabetes mellitus (NIDDM) or type-2 diabetes and the standard therapy for the treatment of NIDDM has its own limitations (Norman, 2010). Diet, oral hypoglycaemic drugs and insulin are the standard modes of treatment. Insulin resistance in skeletal muscle cells is known to play a pivotal role in the development of diabetes. So, we evaluated the potency of the extracts in inducing glucose by using muscle cells as a model. The release of insulin using pancreas experiment indicated that the actinomycete extracts do not facilitate the release of insulin by the pancreas. Our approach was to explore microbial extracts with anti-diabetic property that stimulate the uptake of glucose in hemi diaphragm. The uptake of glucose by isolated hemi diaphragm showed that Streptomyces sp. extract does have anti-diabetic activity. This extract decreases the level of glucose in blood directly by acting on hemi diaphragm, but not through the release of insulin by pancreas. Kulkarni-Almeida et al. (2011) discovered a novel anti-diabetic compound NFAT-133 from a Streptomyces strain PM032466, an isolate of arid soil which expressed a secondary metabolite that induced glucose uptake in skeletal muscle cells.

Both S. longisporoflavus (JX965948) and Streptomyces sp. (JQ926174) extracts showed anti-diabetic activity by inhibiting the α-amylase activity and improving the ability of insulin to move glucose into hemi diaphragm respectively. There are different principles involved in the oral medications for diabetes such as the stimulation of β-cells to produce more insulin, improving the ability of insulin to move glucose into muscle cells and by blocking α-glucosidase and α-amylase enzymes. These enzymes help in the digestion of starch thereby slowing the rise of blood glucose levels (Rang et al., 2003). In our opinion, both actinomycete extracts, probably stimulated the uptake of glucose via the insulin-receptor signaling pathway in which, the insulin binds to its receptor, and in turn starts many protein-activation cascades. These include the translocation of Glut4 transporter to the plasma membrane and influx of glucose, glycogen synthesis, glycolysis and fatty acid synthesis (De Meyts et al., 1973).
Among the microbial resources, the actinomycetes are the sources of anti-diabetic compounds. Acarbose™, Voglibose, Valienamine, Adiposin-1, and Trestatin-B were reported from *Actinoplanes utahensis* (Schmidt *et al.*, 1977), *Streptomyces hygroscopicus-limoneus* (De Melo *et al.*, 2006; Kameda *et al.*, 1980), *S. calvus* (Mahmud, 2003; Truscheit *et al.*, 1981), and *S. dimorphogenes* (Yokose *et al.*, 1983 and 1984) respectively. Acarbose™ is an oral α-glucosidase and α-amylase inhibitor that was first launched by Bayer (Switzerland) in 1989 for the oral treatment of type-2 diabetes mellitus (Schmidt *et al.*, 1977). Drugs sources are known from the marine and soil actinomycetes, whereas no endophytic source is a producer of α-amylase inhibitor.

The biological activities of the leaf extracts in the considered plant species have implications in the treatment of diabetes. Traditional plant remedies or herbal formulations exist from ancient times and are still widely used, despite all the controversy concerning their efficacy and safety (Fugh-Berman and Ernst, 2001), to treat hypoglycemic and hyperglycemic conditions all over the world. It must be noted that many ethnobotanical surveys of medicinal plants used by the local population have been performed in different parts of the world and there is a considerable number of plants described as anti-diabetic. Further studies are needed to ascertain the ‘leads’ to develop into clinically useful medicines.

Therefore, our results for the first time provide information on the α-amylase inhibitor-producing endophytic actinomycetes from the anti-diabetic plants, as well as its ability to augment glucose-uptake. Overall, the study provides a promising approach to employ endophytes as sources of anti-diabetic agents.

In the current scenario, most of modern drugs have been isolated from natural sources such as medicinal plants containing a wide range of chemical compounds that serve as leads to the development of novel anti-diabetic agents. Numerous challenges are encountered on the way of drug discovery from both plants and endophytes which includes the procurement and authentication of plant materials, implementation of high-throughput screening bioassays and scale-up of bioactive lead compounds.