3

EXPERIMENTAL PROCEDURE

Diabetes Mellitus a very common chronic disease is becoming the third “killer” of the health of mankind along with cancer, cardiovascular and cerebrovascular diseases because of its high prevalence, morbidity and mortality. Once diagnosed, it is well regulated by means of various therapeutically effective drugs. Besides, the therapy based on chemotherapeutic agents, the present century has progressed towards naturopathy. Medicinal plants have an ever emerging role to play in the treatment or management of lifelong prolonging diseases like Diabetes Mellitus, especially in developing countries where resources are meagre (Durgeshnandani *et al*., 2014).

Most often the desired biological response is due to not one but a mixture of bioactive compounds. The relative proportion of active compounds can vary from plant to plant of the same species and also in different parts of plants. The active principles present in medicinal plants have been reported to possess various activities such as pancreatic beta cell regeneration, stimulating insulin release from beta cells, showing insulin-like action, fighting the problem of insulin resistance and reducing the uptake, absorption and utilization of glucose (Wadkar *et al.,* 2008). Various approaches have been developed to fight against diabetes and the research is continuously going ahead (Seema and Chanchal, 2014)

Knowledge about medicinal plants could encourage the production of phytotherapeutics from different plants or the isolation of the bioactive molecules with known action mechanism (Trojan-Rodrigues *et al*., 2012). The limitations of currently-available oral antidiabetic agents either in terms of efficacy or safety coupled with the emergence of the disease into a global epidemic have encouraged a concerted effort to discover drugs that can manage Type 2 Diabetes more efficiently (Ranjan and Ramanujam,2002).

For most medicinal plants, the specific ingredient that causes a therapeutic effect is not known. Whole plant contains many ingredients and it is likely that they work together to produce the desired medicinal effect. The type of environment (climate, bugs, soil quality) in which a plant grew will affect its compounds, as well as and when it is harvested and processed (Patel *et al.,* 2012c).

This overreliance on antidiabetic medicinal plants has probably invoked scientists to bioassay these plants in an effort to elucidate more hypoglycemic medicinal plants. The antidiabetic potential of some medicinal plants extracts has been demonstrated in human and animal models of Type 2 Diabetes. However, more detailed research on the antidiabetic plants is inevitable to ameliorate the concerns of *in vivo* safety and efficacy (Piero *et al.,* 2015).

Amylase inhibitors also known as starch blockers prevent dietary starches from being digested and absorbed by the body. This could be useful for treating Diabetes Mellitus characterized by chronic hyperglycaemia resulting from defects in insulin secretion (Ali *et al*., 2006). Amylase inhibitors from medicinal plants are considerably safe and effective. Among about 1200 plants which possess hypoglycaemic property, only 30% of the anti-diabetic plants have been pharmacologically tested and investigated (Ayyanar *et al*., 2008).

In the present study, antidiabetic potential of alpha amylase inhibitors and antioxidants in selected edible plant sources namely *Momordica charantia* and *Trigonella foenum graecum* were studied through various experimental procedures in four phases.

Different Phases of the Study

PHASE I

3.1 *In vitro* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum*

3.1.1 Selection of the Plant Material

3.1.2 Alpha amylase Inhibitory Activity

3.1.3 Antioxidant Activity

PHASE II

3.2 *In vivo* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts on Streptozotocin–Nicotinamide administered Diabetes induced Rats

3.2.1 Experimental Animals

3.2.2 Evaluation of Antidiabetic Activity

3.2.3 Hematological Parameters

3.2.4 Histopathological Analysis

3.2.5 Statistical Analysis

PHASE III

3.3 Phytochemical Analysis and Characterization of Bioactive Compounds responsible for Alpha amylase Inhibition in *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

3.3.1 Qualitative Analysis and Quantification of Selected Phytochemicals

3.3.2 Characterization of Bioactive Compounds responsible for Alpha amylase Inhibition in *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

PHASE IV

3.4 *In silico* Molecular Docking Studies of the Selected Compounds Identified from *Momordica charantia* and *Trigonella foenum graecum* against Pancreatic Alpha amylase enzymes

3.4.1 Selection of Protein Targets

3.4.2 Protein Preparation

3.4.3 Ligand Preparation

3.4.4 Molecular Docking of the Selected Compounds

The methodology adopted in the present study "A Comparative Study on the Antidiabetic Potential of Alpha amylase Inhibitors in *Momordica charantia* and *Trigonella foenum graecum*" is discussed in this chapter.

Chemicals

Porcine pancreatic alpha amylase used in the present study was obtained from Sigma-Aldrich, India. All other chemicals, acids, bases, solvents and salts used were of analytical grade.

Phase I

3.1 *In vitro* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum*

3.1.1 Selection of the Plant Material

The plant materials used in the present study *Momordica charantia* and *Trigonella foenum graecum* (Plates1 and 2) were collected from local market identified and authenticated by botanist from Botanical Survey of India, Tamil Nadu Agricultural

University, Coimbatore, Tamil Nadu, India. The details of scientific classification of *Momordica charantia* and *Trigonella foenum graecum* and the plant parts used for the present study are shown in Tables 9 and10 respectively.

***Momordica charantia* flesh**

***Momordica charantia* seeds**

Plate 1

***Trigonella foenum graecum* leaves**

***Trigonella foenum graecum* seeds**

Plate 2

Table 9

Scientific classification of *Momordica charantia* and *Trigonella foenum graecum*

Classification	*Momordica charantia*	*Trigonella foenum graecum*
Kingdom	Plantae	Plantae
Subkingdom	Tracheobionta	Viridiplantae
Super division	Spermatophyta	Embryophyta
Division	Magnoliophyta	Tracheophyta
Class	Magnoliopsida	Magnoliopsida
Subclass	Dilleniidae	Rosidae
Order	Violales	Fabales
Family	Cucurbitaceae	Fabaceae
Genus	*Momordica*	*Trigonella*
Species	*M.charantia*	*T. foenum-graecum*
Common name	Bitter gourd	Fenugreek

Table 10

Plant parts used for the study

Plant	Plant parts selected for study
Momordica charantia	*Momordica charantia* flesh (MCF) *Momordica charantia* seeds (MCS)
Trigonella foenum graecum	*Trigonella foenum graecum* leaves (TGL) *Trigonella foenum graecum* seeds (TGS)

3.1.1.1 Preparation of the plant extracts and calculation of percentage yield

Momordica charantia fruits were washed and cut into small pieces. The outer flesh and the seeds were dried and used for the study. *Trigonella foenum graecum* leaves and seeds were washed and dried. The dried plant parts were finely powdered and stored in air tight containers at room temperature for further use.

Five grams of powdered plant parts were macerated with 50 ml of different solvents (petroleum ether, chloroform, ethyl acetate, ethanol, acetone and water) for 48 hours filtered and collected. The solution was evaporated in water bath shaker to get dry

extract and the yield percentage was calculated. The extracts were dissolved in distilled water and dimethyl sulphoxide based on their solubility and used for further analysis.

3.1.2 Alpha amylase Inhibitory Activity

Alpha amylase activity can be measured *in vitro* by hydrolysis of starch in presence of α-amylase enzyme. Screening of alpha amylase inhibitors in medicinal plants has received much attention. Plant extracts possess the inhibitory effect of these enzymes that may lead to reduction in post prandial hyperglycemia in diabetic condition. The *in vitro* anti-diabetic activity of *Momordica charantia* flesh and seeds, *Trigonella foenum graecum* leaves and seeds in all the six extracts (petroleum ether, chloroform, ethyl acetate, ethanol, acetone and water) were determined by estimating the inhibitory activity of the enzyme α-amylase which involves the breakdown of starch to produce maltose and then to glucose by modified method of Bernfeld (1955) (Appendix 1).

Among the six solvent extracts, ethyl acetate extracts of *Momordica charantia* flesh, seeds *Trigonella foenum graecum* seeds and ethanol extract of *Trigonella foenum graecum* leaves showed highest alpha amylase inhibition. Hence they were used for further studies.

3.1.2.1 Alpha - amylase inhibitory potential

The α-amylase inhibitory property of MCF, MCS, TGL and TGS was determined by the modified method of Bernfeld (1955) with varying concentrations ranging from 0.2 - 1.0 mg/ml in comparison with standard acarbose (20-100 µg) and IC_{50} values were calculated.

3.1.2.2 Mechanism of alpha - amylase inhibition

The mechanism of inhibition and enzyme kinetics of alpha amylase of MCF, MCS, TGL and TGS were studied by the method given by Dixon (1953) and Cornish-Bowden (1974) (Appendix 2).

3.1.3 Antioxidant Activity

Production of antioxidants from natural sources has been considered important way for treatment of human diseases (Mosquera *et al*., 2007). Modern physicians are increasing their use of pure natural antioxidants extracted from plants to treat many important common diseases due to their proven ability to restrain specific enzymes, to stimulate a number of hormones and neurotransmitters and to scavenge free radicals (Asif, 2015).

3.1.3.1 Total antioxidant activity- Ferric reducing antioxidant power (FRAP) assay

FRAP assay was used to estimate the reducing capacity of the sample, according to the method of Benzie and Strain (1996) (Appendix 3). The total antioxidant activity of the extracts of *Momordica charantia* flesh (MCF), *Momordica charantia* seeds (MCS) and *Trigonella foenum graecum* seeds (TGS) ethyl acetate extracts and *Trigonella foenum graecum* leaves (TGL) ethanol extracts were assayed by FRAP method (Ferric Reduction Antioxidant Power).

3.1.3.2 Free radical scavenging activity

A stock solution of 1mg/ml of *Momordica charantia* flesh (MCF), *Momordica charantia* seeds (MCS) and *Trigonella foenum graecum* seeds (TGS) ethyl acetate extracts and *Trigonella foenum graecum* leaves (TGL) ethanol extract were prepared. This was diluted to get various concentrations (200-1000 µg/ml) in the final volume of reaction mixture. The free radical scavenging activity of the extracts were analyzed by following the various standards *in vitro* radical generating model systems *viz.,*1,1-diphenyl-2-picryl hydrazyl (DPPH), nitric oxide (NO), hydroxyl (OH), superoxide anion (SO) and 2,2-azinobis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS). Inhibition of *in vitro* lipid peroxidation was studied using goat liver as a model system.

3.1.3.3 Estimation of DPPH radical scavenging activity

The antioxidant activity of the plant extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958) (Appendix 4).

3.1.3.4 Determination of nitric oxide radical scavenging activity

The nitric oxide scavenging activity of the plant extracts was measured according to the method of Sreejayan and Rao (1997) (Appendix 5).

3.1.3.5 Estimation of hydroxyl radical scavenging activity

The scavenging activity of the plant extracts on hydroxyl radical was measured according to the method of Klein *et al*. 1991 (Appendix 6).

3.1.3.6 Estimation of superoxide radical scavenging activity

The superoxide scavenging activity of the plant extracts was measured according to the modified method of Beauchamp and Fridovich (1971) (Appendix 7).

3.1.3.7 Estimation of free radical scavenging activity on ABTS

The antioxidant activity of the plant extracts was measured by ABTS radical cation decolorization assay according to the method of Re *et al*. (1999) (Appendix 8).

3.1.3.8 Determination of inhibition of lipid peroxidation

The lipid peroxidation inhibition ability of the plant extracts was carried out using a modified procedure of Ohkawa *et al.* (1979) (Appendix 9).

Phase II

3.2 *In vivo* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts on Streptozotocin - Nicotinamide administered Diabetes Induced Rats

3.2.1 Experimental Animals

Adult male albino Wistar rats (6 weeks), weighing 150 to 200 g were used for the present antidiabetic study. The animals were housed in clean polypropylene cages and maintained in a well-ventilated temperature controlled animal house with a constant 12 h light/dark schedule. The animals were fed with standard rat pelleted diet and clean drinking water was made available *ad libitum*. All animal procedures were performed after approval from the Ethical Committee Clearance No: 53 IAE1012/c/17/CPCSEA-2013 and in accordance with the recommendations for the proper care and use of laboratory animals.

3.2.1.1 Acute toxicity studies

Acute oral toxicity study of *Momordica charantia* seeds (MCS) extract and *Trigonella foenum graecum* seeds (TGS) extract was studied in healthy rats (n= 3) according to guidelines set by Organisation for Economic Co-operation and Development (OECD).The plant extract was evaluated for the pharmacological potential in normal rats weighing 150 to 200 g. The animals were kept fasting overnight providing water. The animals were treated with MCS and TGS starting dose of 200 mg/kg followed by 500,1000,1500,2000 mg/kg b.w and were evaluated for toxicity. The animals were observed for mortality for 24 hours. Since no mortality was observed in acute toxicity studies, $1/5^{th}$ and $1/10^{th}$ of the highest dose (2000mg/kg b.w) were chosen for performing oral glucose tolerance test (OGTT) in normal rats.

3.2.1.2 Oral glucose tolerance test

Overnight fasted rats were separated in 6 groups. Animals of all groups were administered with glucose (2g/kg) orally by means of gastric intubation. Animals in group 1 were given normal saline (0.9% w/v NaCl). Group 2 and 3 were treated orally with ethyl acetate extracts of MCS at a dose of 200 and 400 mg/kg and group 4 and 5 were treated with ethyl acetate extracts of TGS at a dose of 200 and 400 mg/kg. Group 6 received

standard drug glibenclamide 200 µg /kg b.w. Blood samples were collected by tail nipping of each animal just after oral glucose administration at 0, 60,120 and 180 min for the assay of glucose by Accu-chek glucometer.

3.2.1.3 Induction of Diabetes Mellitus

The animals were kept overnight fasting and the initial fasting blood glucose was checked from tip of rat tail vein. Streptozotocin was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Diabetes Mellitus was induced in overnight fasted rats by a single intraperitoneal injection of 60 mg/kg streptozotocin, 15 min after the i.p administration of 120 mg/kg of nicotinamide. Hyperglycemia was confirmed by the elevated levels of blood glucose determined after 72 hours. The animals with blood glucose concentration more than 250mg/dl were used for further study.

The vehicle (saline), standard drug glibenclamide and plant extracts were administered to the respective group animals for 21 days. Throughout the study period glibenclamide and plant extracts were freshly dispersed in normal saline and distilled water before the administration. The fasting blood glucose level was estimated on 1st, 7th, 14th and 21st day from the tip of rat tail vein.

3.2.2 Evaluation of Antidiabetic Activity

Treatment protocol

The animals were divided into seven groups of six animals each as follows. The experimental period was 21days.The experimental design for evaluation of antidiabetic activities is shown in Table 11.

Table 11

Experimental design for evaluation of antidiabetic activities

Groups	Treatment
Group 1	**Control** - Only normal saline (0.9% w/v NaCl)
Group 2	**STZ-NIC** - Diabetic control-Only Streptozotocin 60 mg/kg +Nicotinamide 120mg/kg b.w.
Group 3	**MCS-1** - Streptozotocin (60 mg/kg) +Nicotinamide 120mg/kg rats treated with MCS 200 mg/kg b.w.
Group 4	**MCS-2**- Streptozotocin (60 mg/kg) +Nicotinamide 120mg/kg rats treated with MCS 400 mg/kg b.w.
Group 5	**TGS-1**- Streptozotocin (60 mg/kg) +Nicotinamide 120mg/kg rats treated with TGS 200 mg/kg.
Group 6	**TGS-2**- Streptozotocin (60 mg/kg) +Nicotinamide 120mg/kg rats treated with TGS 400 mg/kg b.w.
Group 7	**Glib** - Streptozotocin (60 mg/kg) Nicotinamide 120mg/kg rats treated with Glibenclamide 200 µg /kg b.w.

3.2.2.1 Determination of body weight

Body weight of rats was determined initially and at weekly intervals during the treatment period.

3.2.2.2 Biochemical analysis

Estimation of blood glucose

Blood sample were collected from tip of rat tail vein and glucose levels were estimated using a glucose oxidase-peroxidase reactive strips and Accu-chek glucometer.

Sample collection

At the end of the experimental period rats were fasted over night and anaesthetized with diethyl ether (100ml/kg), blood samples were collected through retro-orbital sinus puncture with or without EDTA container for the estimation of selected biochemical and haematological parameters. The liver of the experimental rats were removed and a portion of each was stored at minus 40^0C for performing the assays involving enzymic, non enzymic antioxidants and selected enzymes of carbohydrate metabolism. Pancreas was obtained after the autopsy of rats by cervical dislocation for histopathological analysis.

Determination of proteins

In diabetic condition, alterations in hormonal and enzymatic activities can occur. Hence measurement of change in the total protein content would help in the management of Diabetes Mellitus. Total proteins were estimated by the method of Lowry *et al*. (1951) (Appendix 10).

Estimation of glycogen

Glycogen is the primary intracellular storage form of glucose and its level in liver is important in the management of Diabetes and hence liver glycogen was estimated using the anthrone reagent method of Seifter *et al*.(1950) (Appendix 11).

Lipid profile

Type 2 Diabetes is associated with a cluster of interrelated plasma lipid and lipoprotein abnormalities. The lipoprotein abnormalities commonly present in Type 2 Diabetes include an abnormally high level of triglycerides (TG), a high proportion of small dense low density lipoprotein cholesterol (LDL), low high density lipoprotein cholesterol (HDL) and postprandial lipemia. This pattern of lipid profile in Type 2 Diabetes Mellitus is termed diabetic dyslipidemia (Zeqollari *et al.*, 2014). Hence the efficacy of MCS and TGS

extracts on the levels of total cholesterol, triglycerides, HDL, LDL and VLDL in the experimental rats were estimated by the following methods:

Estimation of total cholesterol

Serum total cholesterol was measured according to Allain *et al*. (1974) (Appendix12).

Estimation of triglycerides

Serum triglyceride was measured according to Schettler and Nussel, (1975) (Appendix13).

Estimation of HDL cholesterol

Serum HDL Cholesterol was measured according to Grove (1979) (Appendix 14).

Estimation of LDL and VLDL cholesterol

The amount of LDL-cholesterol and VLDL-cholesterol were calculated by using the formula.

LDL = Total Cholesterol /1.19 + Triglycerides /1.9 – HDL/1.1 – 38 (mg/dl)

$$\text{VLDL} = \frac{\text{Triglycerides (mg/dl)}}{5}$$

Antioxidant activity

Free radicals are implicated in the pathogenesis of various diseases including metabolic disorders like Diabetes Mellitus. Use of antioxidants is considered as one of the therapeutic remedies for the prevention of diabetic complications (Xie *et al*., 2009). Plant based medicines have been found to possess excellent antioxidant activities (Yang *et al*., 2009).

Enzymic antioxidants

The activities of enzymic antioxidants namely superoxide dismutase, catalase and glutathione peroxidase were determined in the liver of the control and experimental rats in order to assess the protection rendered by MCS, TGS and glibenclamide.

Determination of the activity of superoxide dismutase

The activity of superoxide dismutase was assayed by the method of Kakkar *et al*. (1984) (Appendix 15).

Determination of the activity of catalase

The activity of catalase was assayed by the method of Luck (1974) (Appendix 16).

Determination of the activity of glutathione peroxidase

The activity of glutathione peroxidase was assayed by the method of Rotruck *et al.* (1973) (Appendix 17).

Non enzymic antioxidants

The activities of non enzymic antioxidants namely vitamin C, vitamin E and reduced glutathione were determined in the liver of the control and experimental rats in order to assess the protection rendered by MCS, TGS and glibenclamide.

Determination of vitamin C

Vitamin C, a water soluble antioxidant protects the biological system from oxidative stress. Vitamin C was estimated by the method of Roe and Kuether (1953)(Appendix 18).

Determination of vitamin E

Vitamin E was estimated by the method proposed by Rosenberg (1992) (Appendix19).

Estimation of reduced glutathione (GSH)

Reduced glutathione was measured by the method of Moron *et al.* (1979) (Appendix20).

Determination of lipid peroxidation

Hyperglycemia associated with hyperlipidemia could be the causative factor for the increased production of free radicals and lipid peroxides like malondialdehyde (MDA) (Kesavulu *et al.,* 2000). Hence lipid peroxidation in experimental rats was estimated by the method of Ohkawa *et al. (*1979*)* (Appendix 21).

Activity of carbohydrate metabolizing enzymes

In Diabetes Mellitus, enzymes of glucose metabolism are markedly altered. Glucose-6-phosphatase is a crucial enzyme of glucose homeostasis because it catalyses the ultimate biochemical reaction of both glycogenolysis and gluconeogenesis (Mithievre *et al*., 1996). Fructose 1, 6-diphosphatase is one of the key enzymes of gluconeogenic pathway (Pari and Murugan, 2005). Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway (Frederiks *et al.,* 2003*)*.This enzyme

plays a central role in cell metabolism and was found to play pathophysiologic roles in many diseases like diabetes, aldosterone-induced endothelial dysfunction and cancer (Stanton, 2012).

The activity of carbohydrate metabolic enzymes of liver namely glucose -6-phosphatase, fructose1, 6-diphosphatase and glucose -6-phosphate dehydrogenase were assayed after treatment with the plant extracts.

Estimation of glucose -6-phosphatase

Glucose-6-phosphatase activity in liver was assayed by the method of Koide and Oda (1959) (Appendix 22).

Estimation of fructose 1, 6-diphosphatase

Fructose1, 6-diphosphatase activity in liver was assayed by the method of Gancedo and Gancedo (1971) (Appendix 23).

Estimation of glucose -6-phosphate dehydrogenase

Glucose 6-phosphate dehydrogenase in liver was assayed by the method of Ellis and Kirkman (1961) (Appendix 24).

3.2.3 Hematological Parameters

Ingestion of medicinal plants or drugs can alter the normal hematological values (Ajagbonna *et al*., 1999). Therefore, hematological parameters could be an important tool in the assessment of deleterious effect of drugs, as well as medicinal plants (Yakubu *et al*., 2007). The changes in haematological parameters are well known to cause anaemic condition in humans (Balasubraimanian *et al.*, 2009). Hence the haematological parameters namely RBC s, WBC s and their indices were determined using autoanalyser in the experimental rats treated with MCS, TGS and glibenclamide.

3.2.4 Histopathological Analysis

Histopathological studies on animal models are useful techniques to evaluate the potential curative effect of the herbal drugs especially for Diabetes Mellitus. Damage caused to pancreas in Diabetes Mellitus and analysing the histopathology of pancreas treated with plant extracts would help to understand their potency in regenerating the pancreatic tissues. Histopathology of pancreas was performed by the method of Culling (1979) (Appendix 25).

3.2.5 Statistical Analysis

The data were statistically analyzed and statistical significance was determined by One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. 'p' value of 0.05 or less was considered as significant.

Phase III

3.3 Phytochemical Analysis and Characterization of Bioactive Compounds responsible for Alpha amylase Inhibition in *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

3.3.1 Qualitative Analysis and Quantification of Selected Phytochemicals

Phytochemicals are a large group of plant-derived compounds hypothesized to be responsible for much of the disease protection obtained from diets high in fruits, vegetables, beans, cereals and plant-based beverages (Arts and Hollman, 2005). Phytochemicals are the secondary metabolites present in smaller quantities in higher plants and they include the alkaloids, steroids, flavonoids, terpenoids, tannins and many others (Peteros, 2010). It is crucial to know the type of phytochemical constituent, thus knowing the type of biological activity which might be exhibited by the plant (Agbafor and Nwachukwu, 2011). The importance of medicinal plants and the contribution of phytomedicine to the well-being of people have attracted interest from diverse disciplines (Vasanthi *et al.,* 2014).

3.3.1.1 Qualitative analysis of selected phytochemicals

Phytochemical screening of the ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum* seeds were performed in order to characterize the classes of compounds which are present. Qualitative screening included the tests for flavonoids, phenols, tannins, terpenoids, steroids, saponins, alkaloids and glycosides which were performed by using standard reference methods (Trease and Evans 1989; Tiwari *et al.,* 2011) (Appendix 26).

3.3.1.2 Quantification of selected phytochemicals

Flavonoids, phenols, tannins and terpenoids present in medicinal plants are reported to possess antioxidant properties and can act as inhibitors of carbohydrate metabolizing enzymes (Mannan *et al*., 2014).Quantification of selected phytochemicals namely total flavonoids, phenols, tannins and terpenoids were assessed.

Estimation of total flavonoids

The total flavonoid content was determined by the use of a slightly modified colorimetry method described by Zhishen *et al*. (1999) (Appendix 27).

Estimation of total phenols and tannins

The total phenol content was determined according to the method described by Siddhuraju and Becker (2003).The total tannin content was determined according to the method described by Siddhuraju and Manian (2007) (Appendix 28).

Estimation of total terpenoids

The total terpenoid content was determined by the method described by Ferguson (1956) (Appendix 29).

3.3.2 Characterization of Bioactive Compounds responsible for Alpha amylase Inhibition in *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. Their separation still remains as a big challenge for the process of identification and characterization of them (Martin and Guiochon, 2005). The extraction of plant compounds is essential to isolate biologically active compounds and in understanding their role in disease prevention and treatment (Neeharika *et al*., 2012). Hence high performance thin layer chromatography (HPTLC) fingerprint analysis, thin layer chromatography (TLC) separation of compounds, Fourier transform infrared spectroscopic (FTIR) analysis and gas chromatography mass spectroscopy (GCMS) analysis of potent TLC fractions were performed to identify the phytocompounds which might be responsible for antidiabetic activity.

3.3.2.1 High Performance Thin Layer Chromatography (HPTLC) fingerprinting analysis of plant extracts for flavonoids and phenols

High performance thin layer chromatography (HPTLC) based methods could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. HPTLC analysis of MCS and TGS ethyl acetate extracts was performed by the method of Leena *et al.* (2014) with slight modifications (Appendix 30).

3.3.2.2 Separation of active compounds by Thin Layer Chromatography (TLC) and testing the fractions for alpha amylase inhibitory activity

Thin layer chromatography is a simple, quick and inexpensive process that can be used for the analysis of mixtures (Fried and Sharma, 1994). Separation of compounds in

MCS and TGS ethyl acetate extracts was performed by thin layer chromatography by the method of Roger *et al.* (1987) (Appendix 31).The fractions obtained by the separation of compounds by preparative TLC of MCS and TGS were tested for alpha amylase inhibitory activity by the method Bernfeld (1955) (Appendix 1).

3.3.2.3 Analysis of potent TLC fractions by Fourier Transform Infra Red spectroscopy (FT- IR)

FT-IR is one of the widely used methods to identify the chemical compounds and has been used as a requisite method to medicine in pharmacopeia of many countries (Liu *et al*., 2006). FT-IR analysis is used to identify the functional group of active compounds based on peak values in the region of infrared radiation (Coates, 2000). FT-IR analysis of MCS and TGS was performed by the method of Murugan *et al*. (2014) (Appendix 32).

3.3.2.4 Gas Chromatography Mass Spectroscopy (GCMS) analysis of potent TLC fractions

GC-MS analysis is a breakthrough in the analysis of phyto compounds and structure elucidation of these compounds as they have a sensitivity of detecting compounds as low as 1 ng (Liebler *et al*., 1996). GCMS analysis of potent TLC fractions of MCS and TGS was performed by the method of Mercy *et al*. (2013) (Appendix 33).

Phase IV

3.4 *In silico* Molecular Docking Studies of the Selected Compounds Identified from *Momordica charantia* and *Trigonella foenum graecum* against Pancreatic Alpha amylase enzymes

Computer-based methods are becoming increasingly important and complementary to wet laboratory experiments in studying the structure and function of biomolecules Jorgensen (2008) and Clark (2009). The integration of computational and experimental strategies has been of great value in the identification and development of novel promising compounds (Ferreira *et al.,* 2015). Docking studies are used at different stages of drug discovery such as to predict a ligand-receptor interaction and also to rank the compounds based on the binding energies or fitness score (Kitchen *et al.,* 2004). Molecular docking plays a significant role in structural based drug designing by predicting the binding orientation of small molecule drug candidates to their known 3D structures of the protein targets (Damayanthidevi,2015).The bioactive compounds identified from *Momordica charantia and Trigonella foenum graecum* seed extracts through GCMS were subjected to *in silico* studies to determine their binding affinities against the selected

target proteins human pancreatic alpha amylase and porcine pancreatic alpha amylase enzymes.

3.4.1 Selection of Protein Targets

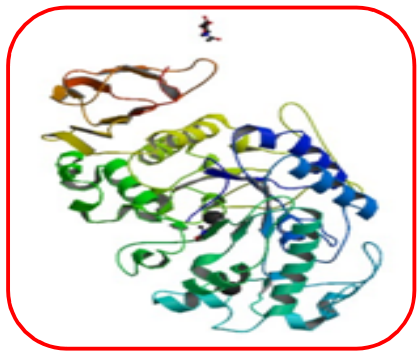
Pancreatic alpha amylases (EC 3.2.1.1) are the enzymes that catalyse the reaction which involves the hydrolysis of the alpha-1, 4 glycosidic linkages of the starch, amylopectin, amylose, glycogen and numerous maltodextrins and is responsible for starch digestion. A possible strategy to block dietary carbohydrate absorption is to use natural resources as carbohydrate digestive enzyme inhibitors as they have fewer side effects than synthetic drugs in the treatment of Type 2 Diabetes Mellitus (Agarwal and Gupta, 2016).

Human pancreatic α-amylase (HPA, α- 1, 4-glucan-4-glucanohydrolase) plays a pivotal role in DM. It catalyses the initial step in the hydrolysis of starch to maltose which is eventually degraded to glucose by α-glucosidases. Hence, retardation of starch digestion by HPA inhibition plays a key role in the control of post prandial hyperglycaemia in Type 2 Diabetes Mellitus (Sudha *et al.,* 2015).

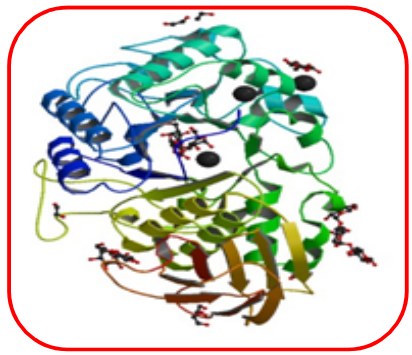
Porcine pancreatic α-amylase also performs similar function as that of human pancreatic alpha amylase and it is the enzyme used in *in vitro* alpha amylase inhibition studies. Brayer *et al*. (1995) have reported 97% primary sequence similarity between human salivary and human pancreatic α-amylases and a 70% structural similarity between porcine pancreatic alpha amylase (PPA) and human pancreatic alpha amylase (HPA) Thus, a high degree of similarity between PPA and HPA allows us to use it as the enzyme for screening inhibitors. Hence HPA and PPA were selected as target proteins in order to dock against the compounds obtained from GCMS analysis.

3.4.2 Protein Preparation

The protein data bank (PDB) is a repository that contains information about experimentally determined 3-D structural data of macromolecules (such as proteins and nucleic acids).The 3D structure of human pancreatic alpha amylase (PDB ID: 1XH1) and porcine pancreatic alpha amylase (PDB ID: 1UA3) were retrieved from Protein Data Bank (PDB) (http: /www.pdb.org/pdb/home/home.do) shown in Plate 3.



a. 1XH1- Human pancreatic alpha amylase



b.1UA3 - Porcine pancreatic alpha amylase

Three dimensional structures of target proteins

Plate 3

3.4.3 Ligand Preparation

The structures of selected compounds identified from *Momordica charantia* and *Trigonella foenum graecum* seed extracts through GCMS to be docked with target proteins were obtained from PubChem database of National Centre for Biotechnological Information (NCBI). The structures of compounds were prepared by converting from structure data format (SDF) format to Mol file format (MDL Molfile - is a file format for holding information about the atoms, bonds, connectivity and coordinates of a molecule) for the process of docking. The list of compounds used for docking with HPA and PPA are depicted in Table 12.

Table 12

Compounds used as ligands for docking

S.No	Name of the Compound
1	11-Methylspirostan-3, 11-diol
2	1-Phenanthrene carboxylicacid,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester (dehydroabietic acid)
3	2,6,10-Trimethyl- dodecane, (farnesane)
4	2,4-Di-tert-butylphenol
5	1-Nonadecene
6	1-Heptacosanol
7	1-Methyl-4-(1-methylethenyl) cyclohexene (limonene)
8	1,2 Benzene dicarboxylic acid
9	Nonahexacontanoic acid methyl ester

3.4.4 Molecular Docking of the Selected Compounds

Docking Server (https://www.dockingserver.com/web) was used for docking study. Docking server offers a web-based, easy to use interface that handles all aspects of molecular docking from ligand and protein set-up. Molecular docking of the compounds with human pancreatic alpha amylase and porcine pancreatic alpha amylase is done by the method as expressed in Appendix 34.