4

RESULTS AND DISCUSSION

Diabetes Mellitus has become a growing problem in the contemporary world. India has today become the diabetic capital of the world with over 20 million Diabetes and this number is likely to increase to 57 million by 2025 (Edwin *et al.,* 2008). Several epidemiological and clinical studies indicate a direct relationship between hyperglycemia and long term microvascular and macrovascular complications, which develop as the disease progresses, gradually decrease quality of life of diabetic patients. Therefore, it is essential to control blood glucose levels during the early stages of the disease (Mahesh *et al.,* 2016).

World Health Organization (WHO) estimates that about three-quarters of the population mainly in the countries of Africa, Asia and Latin America, confides on plant based preparations in their traditional medicinal system for primary healthcare (WHO, 2003). This dependence increased the knowledge gathering and exploration of novel and effective plant-derived compounds for commercialization. Herbal drugs have been widely used globally for diabetic treatment over thousands of years due to their traditional acceptability and lesser side effects. Therefore, screening for α-amylase inhibitors in medicinal plants has received much attention (Madhusudhan and Kirankumar, 2015).

Most of the commercially available amylase inhibitors are of microbial origin. Though these drugs help in maintaining constant level of glucose in blood by delaying the breakdown of starches, their usage has been limited due to their side-effects such as flatulence and diarrhoea (Mahmoud *et al*., 2010 ; Kim and Nho., 2004 ; Mioko *et al*., 2001). When compared to the microbial counterparts, amylase inhibitors from medicinal plants are considerably safe and effective (Ayyanar *et al*., 2008). Searching for a new class of compounds is essential to overcome diabetic problems. So, there is continuous search for alternative drugs (Syamsudin, 2010).

Momordica charantia and *Trigonella foenum graecum* are economically important medicinal plants used for the treatment of various ailments besides Diabetes**.** The present investigation was undertaken to compare the antidiabetic potential of alpha- amylase inhibitory activity of these two edible plant sources. The outcomes of the present study are presented in four phases under the following sub titles:

PHASE I

4.1 *In vitro* Antidiabetic and Antioxidant Potentials of *Momordica charantia* and *Trigonella foenum graecum*

4.1.1 Processing of the plant materials

4.1.2 Alpha amylase inhibitory activity

4.1.3 Anti oxidant activity

PHASE II

4.2 *In vivo* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts in Streptozotocin - Nicotinamide Administered Diabetes induced Rats

4.2.1 Acute toxicity

4.2.2 Results of Oral Glucose Tolerance Test (OGTT)

4.2.3 Antidiabetic activity of the selected plant parts

4.2.4 Hematological parameters

4.2.5 Histopathological Interpretations of sections of the pancreas

PHASE III

4.3 Bioactive Compounds with Alpha- amylase Inhibitory Properties from *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

4.3.1 Phytochemicals present in the seeds of *Momordica charantia* and *Trigonella foenum graecum*

4.3.2 High Performance Thin layer chromatography (HPTLC) of the ethyl acetate extracts of the seeds of *Momordica charantia* and *Trigonella foenum graecum*

4.3.3 Separation of active compounds by Thin Layer chromatography (TLC)

4.3.4 Fourier Transform Infra-Red (FT-IR) Spectroscopy analysis of potent TLC fractions of *Momordica charantia* and *Trigonella foenum graecum* seeds

4.3.5 Gas Chromatography Mass Spectroscopy (GCMS) profile of potent TLC fractions of *Momordica charantia* and *Trigonella foenum graecum* seeds

PHASE IV

4.4 *In silico* Molecular Docking of Selected Compounds Identified from *Momordica charantia* and *Trigonella foenum graecum* Seeds against Human Pancreatic Alpha-amylase (HPA) and Porcine Pancreatic Alpha-amylase (PPA) Enzymes

PHASE I

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

4.1.1 Processing of the plant materials

The plant materials *Momordica charantia* flesh (MCF), *Momordica charantia* seeds (MCS), *Trigonella foenum graecum* leaves (TGL) and *Trigonella foenum graecum* seeds (TGS) were extracted with non polar and polar solvents namely petroleum ether, chloroform, ethyl acetate, acetone, ethanol and water.

The percentage yield of *Momordica charantia* and *Trigonella foenum graecum* in various solvents has been presented in Table13. The yield percentage was higher in aqueous extracts of both *Momordica charantia* flesh and seeds (MCF- 12.8±0.78% and MCS-14.6±0.68 %) and *Trigonella foenum graecum* leaves and seeds (TGL-10.8±0.76% and TGS-11.6±0.10%) when compared to other solvent extracts followed by ethyl acetate and ethanol extracts. The yield % of *Momordica charantia* ethyl acetate extracts were (MCF- 8.9 ± 0.90 and MCS- 9.7±0.43).The yield % of *Trigonella foenum graecum* was high in ethanol extracts (TGL-9.8 ± 0.64 and TGS-8.7 ± 0.55) followed by ethyl acetate extracts (TGL- 6.2 ± 0.15 and TGS -7.9 ± 0.74)

Table 13

Percentage yield of *Momordica charantia* and *Trigonella foenum graecum* in different solvent extracts

Solvent extracts	MCF	MCS	TGL	TGS
Petroleum ether	2.7±0.25	3.1±0.72	4.7±0.62	3.8±0.55
Chloroform	1.2±0.50	2.6±0.65	2.3±0.58	3.6±0.82
Ethylacetate	8.9±0.90	9.7±0.43	6.2±0.15	7.9±0.74
Acetone	3.6±0.10	4.2±0.52	5.1±0.25	6.4±0.90
Ethanol	6.3±0.45	5.4±0.50	9.8±0.64	8.7±0.55
Water	12.8±0.78	14.6±0.68	10.8±0.76	11.6±0.10

Values are mean ±SD of triplicates

MCF- *Momordica charantia* flesh TGL- *Trigonella foenum graecum* leaves

MCS - *Momordica charantia* seeds TGS- *Trigonella foenum graecum* seeds

The extraction yield is a measure of the solvent's efficiency to extract specific components from the original material and it is defined as the amount of extract recovered in mass compared with the initial amount of whole plant. It is presented in percentage. The plant parts were extracted with non polar and polar solvents namely petroleum ether, chloroform, ethyl acetate, acetone, ethanol and water. Different solvents were used as bioactive compounds responsible for medicinal properties may be soluble in solvents of same polarity but in different proportions. Successful determination of biologically active compounds from plant materials is largely dependent on the type of solvent used in the extraction procedure (Eloff, 1998).

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical compounds from the plant materials for further separation and characterization (Fabricant and Farnsworth, 2001).

Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive or inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilise compounds with similar polarity (Ncube *et a*l., 2008). The products obtained after extraction from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts (Remington and Remington, 2005).

The use of medicinal plants in the industrialised societies has been traced to the extraction and development of several drugs from plants as well as traditionally used folk medicine (Shrikumar and Ravi, 2007). Crude extracts from medicinal plants are more biologically active than isolated compounds due to their synergistic effects (Jana and Shekhawat, 2010).Different solvent extractions provide different types of compounds because of their varied chemical nature and sensitivity toward extraction or hydrolysis methods (Jahanban *et al.*, 2010).

4.1.2 Alpha amylase inhibitory activity

Diabetes Mellitus is a common metabolic disorder which may eventually lead to multiple organ damage and syndromes (Rahimi *et al., 2005)* The most important digestive enzyme is pancreatic alpha-amylase, a calcium metalloenzyme that catalyzes the

hydrolysis of the alpha-1, 4 glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins and is responsible of most of starch digestion in humans (Tundis *et al*., 2010). Inhibitors of saccharide hydrolyzing enzymes (α-amylase and α-glucosidase) have been useful as oral hypoglycemic drugs for the control of hyperglycemia especially in patients with Type 2 Diabetes Mellitus (Gin and Rigalleau, 2000).

4.1.2.1 Alpha-amylase inhibitory activity of *Momordica charantia* and *Trigonella foenum-graecum* in different solvent extracts

Inhibition of alpha amylase by various solvent extracts of *Momordica charantia* and *Trigonella foenum-graecum* were studied and the results are presented in Table 14. Among the six solvent extracts used, ethyl acetate extract showed maximum inhibition of 94.2±2.55 % by *Momordica charantia* seed and 92.6±2.32 % by *Momordica charantia* flesh followed by ethanol, aqueous, petroleum ether and acetone. In *Trigonella foenum-graecum,* maximum inhibition was found in ethyl acetate extracts of seeds with 90.8±1.20 % and 80.3±2.5 % ethanol extracts.

Table 14

Alpha- amylase inhibitory activity in different solvent extracts of the selected plant parts (% Inhibition)

Solvent extracts	MCF	MCS	TGL	TGS
Petroleum ether	44.3 ± 2.54	52.9±1.45	10.4±1.25	82.5 ±2.65
Ethylacetate	92.6±2.32	94.2±2.55	50.9±0.55	90.8±1.20
Chloroform	21.4±1.48	23.5±1.20	14.2±0.40	16±0.58
Ethanol	58.6±1.60	79.4 ± 2.12	80.3±2.5	50.4±1.73
Acetone	10.6±0.35	12.3±0.36	51.4±1.58	11.4±2.24
Water	40.9±0.94	32±0.80	75.4±1.75	67.99±1.55

Values are mean ±SD of triplicates

MCF- *Momordica charantia* flesh TGL- *Trigonella foenum graecum* leaves

MCS - *Momordica charantia* seeds TGS- *Trigonella foenum graecum* seeds

The efficiency of alpha amylase inhibition might depend upon the solvents used for extraction. The polar solvents namely ethyl acetate extracts of *Momordica charantia* flesh and seeds and *Trigonella foenum-graecum* leaf ethanol extracts and seed ethyl acetate extracts showed maximum inhibition when compared to other non polar solvent extracts. This might be due the active principles present in the solvent extracts.

Sangeetha and Vedasree (2012) had reported that out of the four extracts used in studying alpha-amylase inhibitory activity of the leaves of *Thespesia populnea*, ethyl acetate and methanol extracts were effective than petroleum ether and chloroform extracts. Findings of Gad *et al.* (2006) suggested that the hypoglycemic effect of fenugreek and balanites extracts is mediated through Inhibition of the intestinal glucosidase activity as demonstrated by *in vitro* inhibition of *α*-amylase activity.

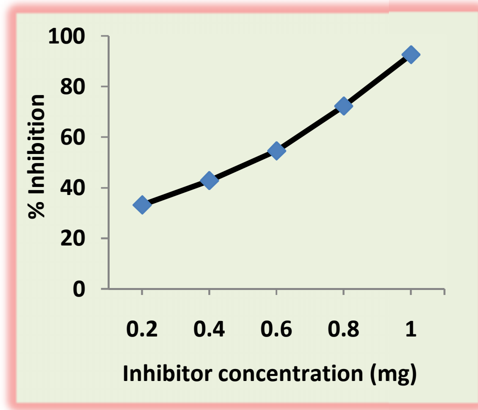
Kumar and Lalitha (2014) evaluated *in vitro* inhibitory effects of various extracts (petroleum ether, chloroform, ethyl acetate, acetone, ethanol, and water) of *Anacyclus pyrethrum* root on porcine pancreatic amylase activity and revealed that ethanol extracts showed 88.26% significant α-amylase inhibitory effect than the other extracts. Higher α-amylase inhibitory effect of about 77.53% was shown by callus of *Costus pictus* commonly known as 'Spiral ginger' 'Step ladder' or 'Insulin' plant in ethyl acetate and ethanol extracts (Amanpreet *et al.,* 2014). Ethanol extracts of *Oxalis corniculata* Linn. leaves revealed better α- amylase inhibitory activity than petroleum ether and ethyl acetate extracts (Das and Himaja, 2015).

Among the extracts selected in the present study, ethyl acetate extracts of MCF, MCS, TGS and ethanol extract of TGL showed maximum alpha-amylase inhibitory activity when compared to other solvent extracts. Hence, they were selected for further studies.

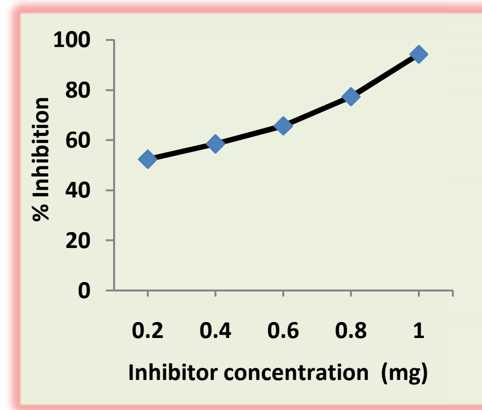
4.1.2.2 Alpha- amylase inhibitory potential of the selected plant extracts

Alpha amylase inhibitory potential of the selected plant extracts were studied with different inhibitor concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg). The alpha- amylase inhibitory potential of MCF, MCS, TGL, TGS and standard acarbose are shown in Figures 7a, b, c, d and e respectively.

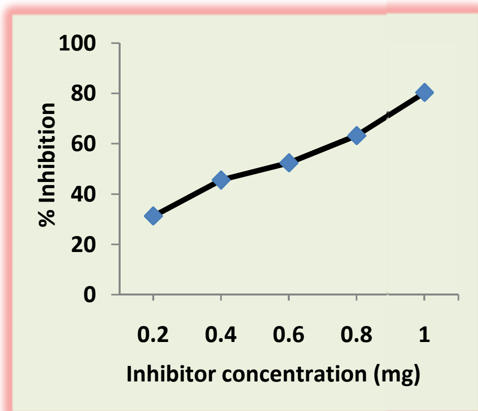
There was an increased inhibition by MCF, MCS, TGL and TGS extracts in dose dependant manner. As the concentration of the inhibitor increased, the amylase enzyme activity was decreased. Inhibition was found to be dose dependant by standard acarbose drug which is a commercially available alpha-amylase inhibitor for the treatment of Diabetes The IC_{50} values for alpha-amylase inhibition of MCF, MCS, TGL, TGS and standard acarbose were found to be 480, 220, 520, 260 and 26.8 µg respectively as shown in Table15.



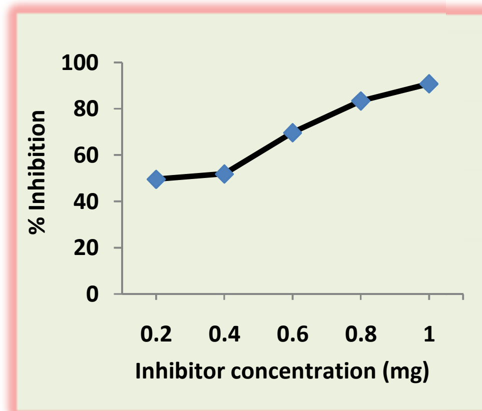
a. *Momordica charantia* flesh



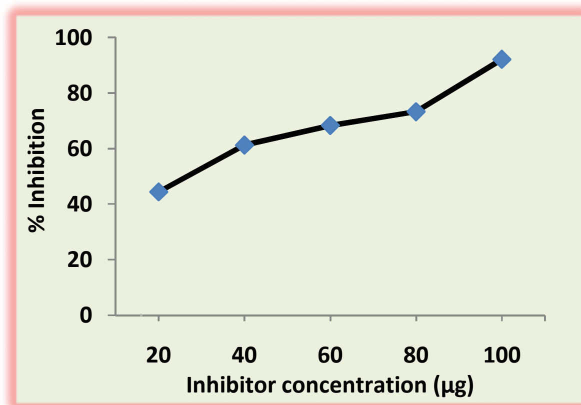
b. *Momordica charantia* seeds



c. *Trigonella foenum-graecum* leaves



d. *Trigonella foenum-graecum* seeds



e. Acarbose

Values are mean of triplicates

Figure 7

Alpha- amylase inhibitory potential of the selected plant extracts and the standard drug acarbose

Table 15

IC $_{50}$ values for alpha- amylase inhibition

Plant extract/Standard	IC $_{50}$ (µg)
***Momordica charantia* flesh**	480
***Momordica charantia* seeds**	220
Trigonella foenum-graecum leaves	520
***Trigonella foenum-graecum* seeds**	260
Acarbose	26.8

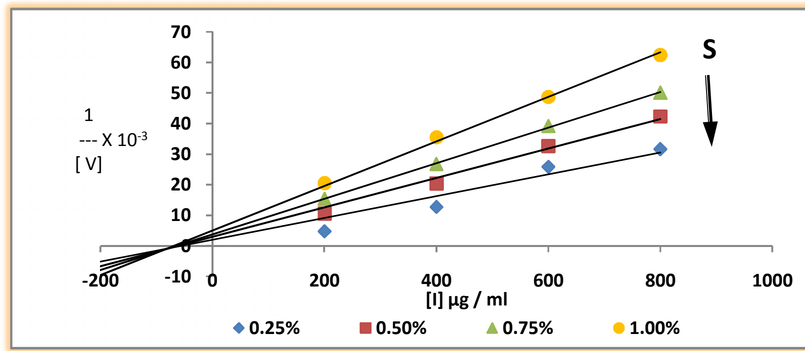
Values are mean of triplicates

The results of the present study are supported by Hasan *et al.* (2014) who had reported that ethyl acetate extracts of *Mallotus repandus* stem significantly inhibited α-amylase activity in a dose dependent manner like acarbose and also suggested that the bioactive compounds present in the ethyl acetate stem extract may be responsible for multifaceted medicinal property. Pavithra *et al.* (2014) had reported that ethyl acetate extracts of nine endophytic fungi isolated from anti-diabetic plants *Momordica charantia* and *Trigonella foenum-graceum* were found to be positive for alpha-amylase and alpha-glucosidase inhibitors.

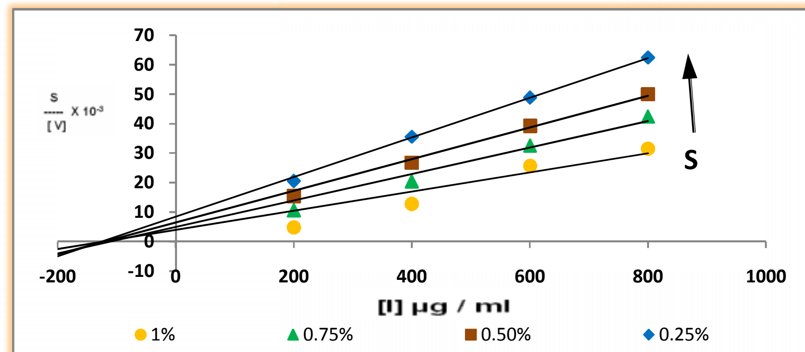
Talukdar *et al.* (2016) reported that the ethyl acetate extracts of the bark of *Elaeocarpus ganitrus* showed significant inhibitory effect on alpha-amylase activity in vitro in a dose dependent manner. Swathi *et al.* (2015) showed that aqueous, ethanol, ethyl acetate, chloroform and petroleum ether extracts of *Areva lanata* whole plant extracts were found to possess dose dependent increase in percentage inhibitory activity against alpha-amylase enzyme.

4.1.2.3 Mechanism of alpha-amylase Inhibition

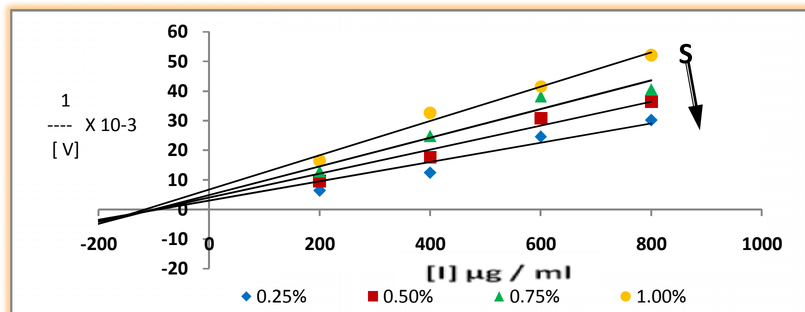
Mechanism of alpha-amylase inhibition of *Momordica charantia* and *Trigonella foenum-graecum* was studied by Dixon and Cornish- Bowden plot as shown in Figures 8 and 9. Results of Dixon and Cornish- Bowden plot showed that the type of inhibition in *Momordica charantia* was non-competitive type in both the MCF and MCS extracts whereas in *Trigonella foenum-graecum* the mechanism was found to be competitive type in TGL and mixed inhibition in TGS.



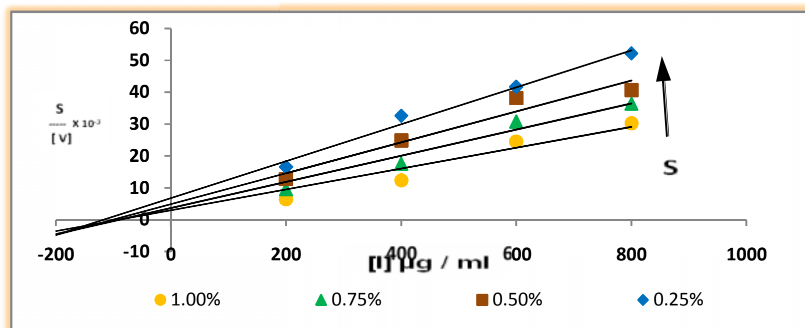
MCF Dixon Plot (Non- Competitive inhibition)



MCF Cornish-Bowden Plot (Non - Competitive inhibition)



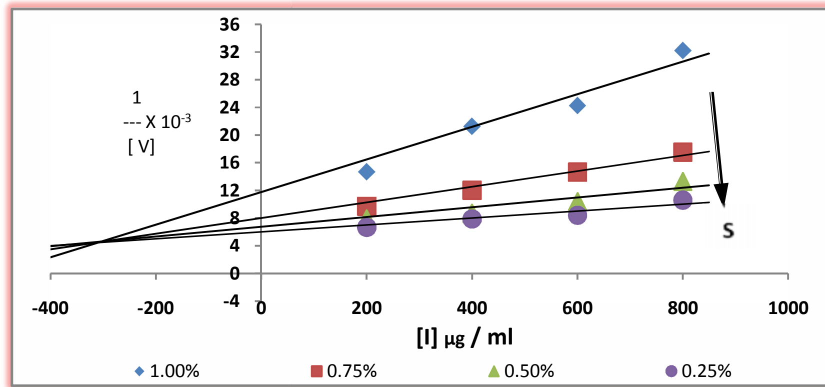
MCS Dixon Plot (Non - Competitive inhibition)



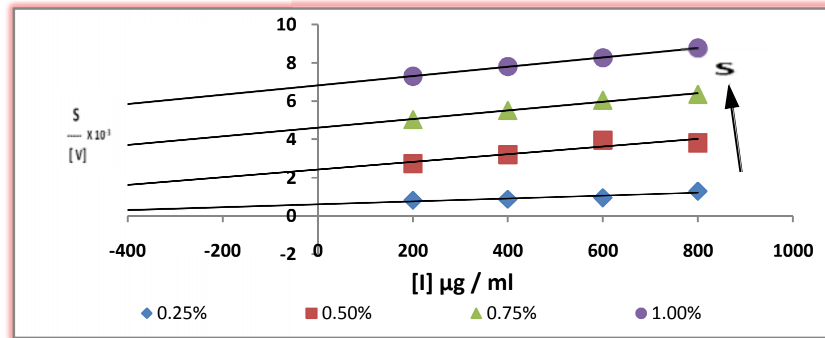
MCS Cornish-Bowden Plot (Non- Competitive inhibition)

Figure 8

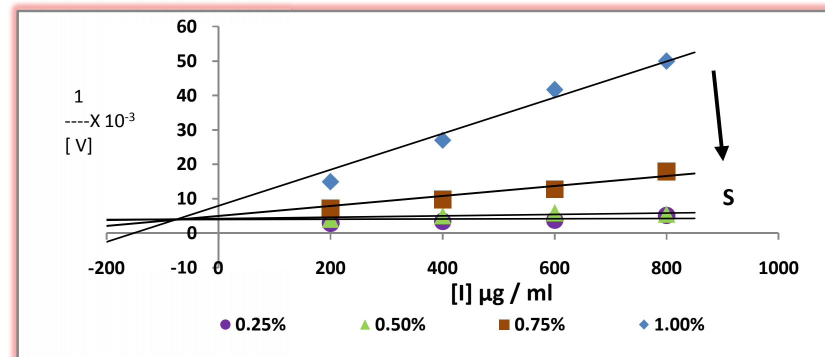
Mechanism of alpha- amylase inhibition by MCF and MCS extracts



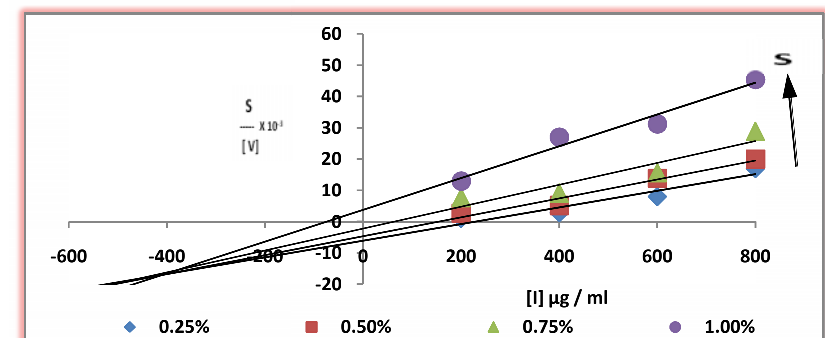
TGL Dixon Plot (Competitive inhibition)



TGL Cornish-Bowden Plot (Competitive inhibition)



TGS Dixon Plot (Mixed inhibition)



TGS Cornish-Bowden Plot (Mixed inhibition)

Figure 9

Mechanism of alpha-amylase inhibition by TGL and TGS extracts

In non-competitive inhibition, a molecule binds to an enzyme somewhere other than the active site. This changes the three-dimensional structure of the enzyme so that its active site can still bind substrate with the usual affinity, but is no longer in the optimal arrangement to stabilize the transition state and catalyze the reaction. This type of inhibition reduces the maximum rate of a chemical reaction without changing the apparent binding affinity of the catalyst for the substrate. This suggests that the active component of the extract binds to a site other than the active site of the enzyme and combines with either free enzyme or enzyme substrate complex possibly interfering with the action of both (Mayur *et al*., 2010)

Kang *et al.* (2009) had reported that α-glucosidase inhibitors from ethyl acetate extract of *Luculia pinciana* showed strongest inhibitory activity. Out of five active compounds isolated and identified, four of them showed non competitive type of alpha-glucosidase inhibition. Kazeem *et al.* (2013) had reported non-competitive mode of alpha-amylase inhibition by *Picralima nitida* leaf extracts.

Competitive inhibition is a form of enzyme inhibition where binding of the inhibitor to the active site on the enzyme prevents binding of the substrate and *vice versa.* Competitive inhibitors bind in the same binding site as the substrate, but same-site binding is not a requirement. A competitive inhibitor could bind to an allosteric site of the free enzyme and prevent substrate binding, as long as it does not bind to the allosteric site when the substrate is bound (Dick, 2011).

Competiive inhibition shown by TGL extracts are similar to that of Rahimzadeh *et al.* (2014) who had reported that *Urtica dioica* and *Juglans regia* extracts inhibited porcine pancreatic alpha amylase through competitive mechanism as shown by Dixon plot. Probably, there was a compound in the extract that could compete with the substrate for binding to the active site of the enzyme. This type of mechanism is usually seen for the competitive type of inhibitor.

Mixed inhibition is a type of enzyme inhibition in which the inhibitor may bind to the enzyme whether or not the enzyme has already bound the substrate but has a greater affinity for one state or the other. Mixed inhibitors bind in a way that is reflective of some of the properties of a competitive and a non-competitive, or a non-competitive and an uncompetitive inhibitor. Most commonly, these are inhibitors that bind at a site distant from the active site, but in binding, they influence substrate binding as well as turnover.

Mixed type of inhibition shown by TGS might be due to binding of bioactive compounds to either in active site or other sites to bring about the inhibition of alpha amylase enzyme**.** Sun *et al.* (2016) studied the kinetics of inhibition of green, oolong (partially oxidized tea) and black tea extracts, through Dixon, Cornish-Bowden and Lineweaver–Burk plots and had reported that epigallocatechin gallate, theaflavin-3, 3′-digallate and tannic acid were competitive inhibitors of porcine pancreatic alpha amylase, whereas epicatechin gallate, theaflavin-3′-gallate and theaflavin were mixed-type inhibitors with both competitive and uncompetitive inhibitory characteristics.

4.1.3 Antioxidant activity

4.1.3.1 Total Antioxidant activity

Antioxidants are substances that protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants are capable of stabilizing or deactivating free radicals before they attack cells (Arockiamary *et al.,* 2014). The FRAP assay (ferric reducing ability of plasma), a simple test of the total antioxidant power has been chosen to assess the effects of the *Momordica charantia* and *Trigonella foenum-graecum* extracts. The ability of the plant extracts to reduce ferric ions was determined using the FRAP assay. The FRAP values of MCF, MCS and TGS ethyl acetate extracts and TGL ethanol extracts are presented in Table 16.

Table 16

Total antioxidant activity of the plant extracts

Plant extracts/ Standard	FRAP (mmol (Fe(II) /g sample)
***Momordica charantia* flesh**	49.05±1.10
***Momordica charantia* seeds**	78.71±0.59
***Trigonella foenum-graecum* leaves**	27.56±4.57
***Trigonella foenum-graecum* seeds**	57.92±1.13
Ascorbic acid	195.3±18.37

Values are mean ±SD of triplicates

The ethyl acetate extracts of *Momordica charantia* seeds showed highest ferric ion reducing activity of about 78.71±0.59 mmol Fe(II)/g among all the plant extracts studied, followed by TGS 57.92±1.13 mmol Fe(II)/g and then MCF 49.05±1.10 mmol Fe(II)/g and in TGL ethanol extracts the activity was about 27.56±4.57 mmol Fe(II)/g) when compared to standard ascorbic acid with maximum of 195.3±18.37 mmol Fe(II)/g

sample. *In vitro* antioxidant assay by Kumaravel *et al.* (2013) revealed that the free radical scavenging activity of antioxidants is present in ethyl acetate extracts of leaf of *Pterocarpus marsupium*.

Fidrianny *et a*l. (2014) in their studies had reported that ethyl acetate extracts of *Sechium edule* leaves belonging to Cucurbitaceae species had the highest FRAP capacity of 4.54 %. Solvents play a vital role in the extraction of the plant compounds (Bukhari *et al.*, 2008).

El-Shora *et al.* (2015) had reported that FRAP scavenging activity of fenugreek extract showed the values close to the standard L-ascorbate. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain through donating an H-atom. The results of the present study on reducing power demonstrated the electron donor properties of the plant extracts, thereby neutralizing free radicals by forming stable products. The antioxidant property of ethyl acetate extracts might be due to the active compounds present in the solvent extracts.

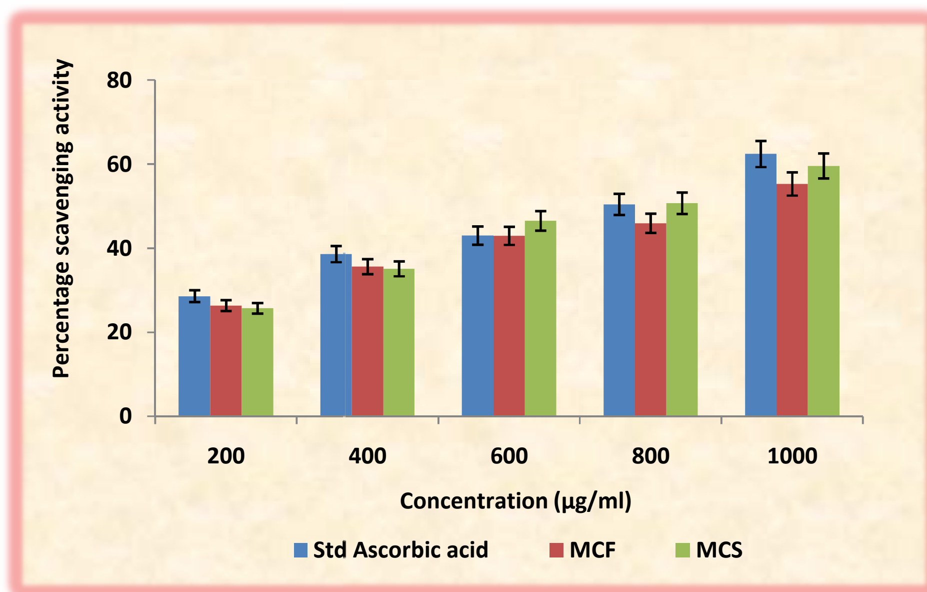
4.1.3.2 Free radical scavenging activity

Free radicals are highly reactive unstable molecules that have an unpaired electron in their outer shell (Awah *et al.,* 2010). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. The most common ROS include superoxide anion, hydrogen peroxide (H_2O_2), peroxyl (ROO__) radicals and reactive hydroxyl (OH__) radicals and the nitrogen derived free radicals are nitric oxide and peroxynitrite anion (ONOO__) (Pavithra and Vadivukkarasi, 2015).

Antioxidant compounds of the plant material act as radical scavengers and help in converting the radicals to less reactive species. A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea (Sulekha *et al.,* 2009).Free radical scavenging assays and lipid peroxidation of *Momordica charantia* and *Trigonella foenum-graecum* extracts were performed with standard antioxidants, ascorbic acid and quercetin.

4.1.3.3 DPPH radical scavenging activity

The free radical scavenging capacity of MCF, MCS, TGL and TGS was tested by their ability to bleach the stable DPPH. The DPPH (2, 2, diphenyl-1-hydrazyl) radical scavenging activity of the plant extracts was found to be dose dependant and the results are shown in Figures 10 a and 10 b.

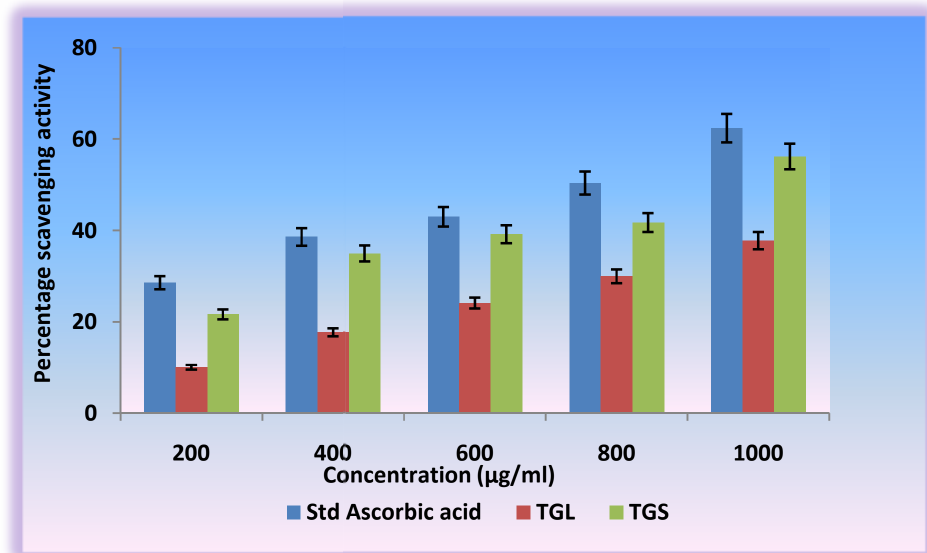


Values are mean ±SEM of triplicates

MCF- *Momordica charantia* flesh   MCS - *Momordica charantia* seeds

Figure 10 a

DPPH radical scavenging activity of *Momordica charantia*



Values are mean ±SEM of triplicates

TGL-*Trigonella foenum graecum* leaves TGS-*Trigonella foenum graecum* seeds

Figure 10 b

DPPH radical scavenging activity of *Trigonella foenum-graecum*

The scavenging activity of DPPH by plants was found to be higher in MCS with 59%, TGS 56%, MCF 55% ethyl acetate extracts and ethanol extract of TGL showed about 38% at 1000 µg /ml .Standard ascorbic acid exhibited scavenging of 62% at 1000 µg /ml. The IC_{50} values of the plant extracts for DPPH were found to be 777 µg for MCF, 830 µg for MCS 1368 µg for TGL, 895 µg for TGS µg and 734 µg for standard ascorbic acid as shown in Table 17 on page 84.

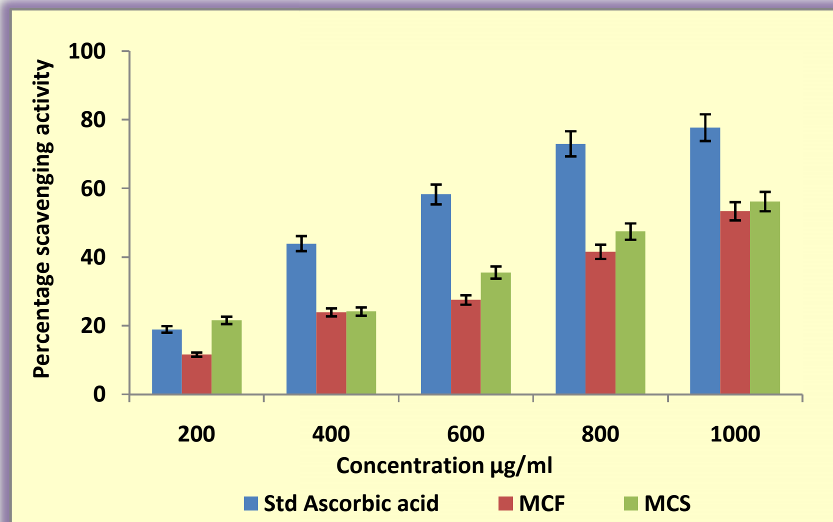
Kumaravel *et al.* (2013) had reported that DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. The bleaching effect of DPPH molecules can be correlated with the number of available hydroxyl groups and suggested that the antioxidant activity of plant extracts might be probably due to the presence of compounds with an available hydroxyl group.

The DPPH assay of *Pimpenella tirupatensis* leaf extracts revealed that both the methanol and ethyl acetate extracts exhibited significant antioxidant activity as reported by Ranjit and Archana (2016). Ganie *et al*. (2014) had reported that ethyl acetate extracts of *Arnebia benthamii* exhibited considerably higher DPPH radical scavenging activity. The free radical scavenging activities of different extracts decreased in the order of ethyl acetate extract > ethanol extract > aqueous extract. Babu *et al.* (2016) had reported that DPPH activity of crude methanolic, ethanolic and ethyl acetate extracts of *Elytraria acualis*.

DPPH radical scavenging activities of the extracts depend not only on plant type but also upon the extraction solvent (Soni and Sosa, 2013). MCF, MCS and TGS ethyl acetate extracts showed good scavenging activity when compared to ethanol extracts of TGL. This may be due to the active compounds present in ethyl acetate extracts which might be responsible for scavenging properties. The present study results are supported by Talukder *et al.* (2013) who had reported the scavenging effect of ethanol and ethyl acetate extracts of the whole fruit of *Momordica charantia* by DPPH radical scavenging assay.

4.1.3.4 Nitric oxide radical scavenging activity

The Nitric oxide radical scavenging activity of *Momordica charantia* and *Trigonella foenum-graecum* are shown in Figures 11a and 11b.

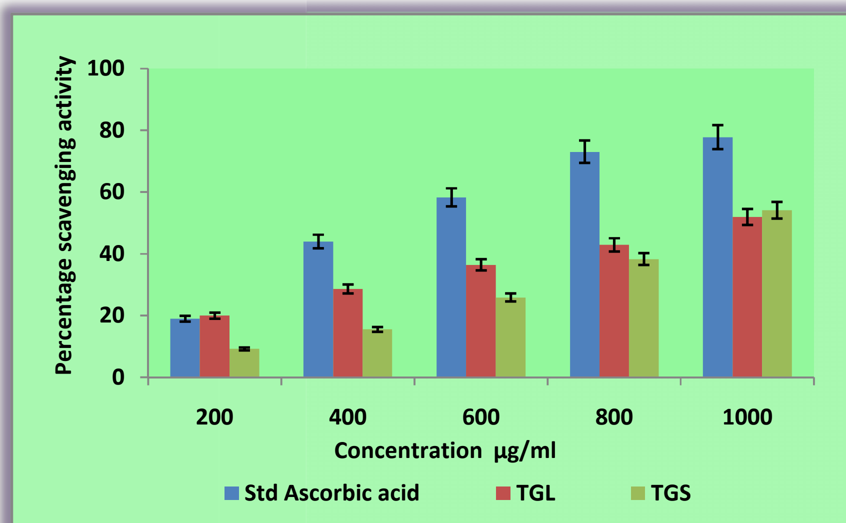


Values are mean ±SEM of triplicates

MCF- *Momordica charantia* flesh MCS - *Momordica charantia* seeds

Figure 11 a

Nitric oxide radical scavenging activity of *Momordica charantia*



Values are mean ±SEM of triplicates

TGL-*Trigonella foenum graecum* leaves TGS-*Trigonella foenum graecum* seeds

Figure 11 b

Nitric oxide radical scavenging activity of *Trigonella foenum-graecum*

Nitric oxide scavenging activity of all the extracts was found to be moderate with MCF 53% MCS 56% TGL 52% and TGS 54% when compared to ascorbic acid standard with 77% scavenging effect. The IC_{50} values of MCF - 964 µg, MCS - 881 µg, TGL - 960 µg, TGS - 981 µg and for standard ascorbic acid was 540 µg as shown in Table 17.

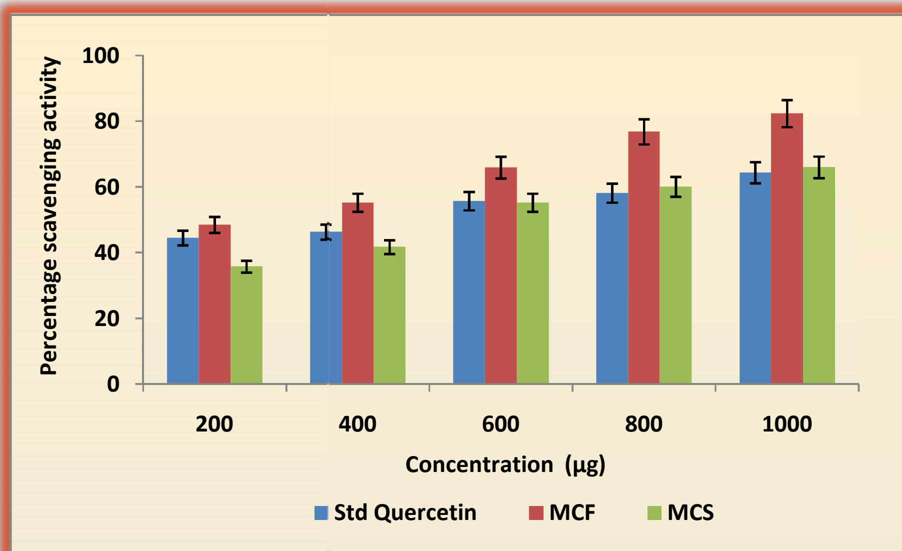
Nitric oxide is an unstable free radical involved in many biological processes which is associated with several diseases. It reacts with oxygen to produce stable product nitrate and nitrite through intermediates and high concentration of nitric oxide can be toxic and inhibition of over production is an important goal (Wang *et al.,* 2005). Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent (Chittama *et al.,* 2016).

Ethyl acetate extract of *Calycopteris floribunda* showed moderate nitric oxide radical scavenging activity when compared with standard ascorbate (Santharam *et al*., 2015). Thenmozhi and Rajan (2015) had reported that *Psidium guajava* leaf aqueous and alcoholic extract also moderately inhibited nitric oxide in dose dependent manner. Amudha and Rani (2016) showed the presence of the NO scavenging capacity of the ethanol and ethyl acetate extract of aerial parts of *Cordia retusa*. Boora *et al.* (2014) showed that ethanol and water extracts of *Combretum zeyheri, Combretum platypetalum* and *Parinari curatellifolia* scavenged the nitrite radical showing that they are potent antioxidants.

From the present study, we can find that the selected plant extracts exhibited moderate nitric oxide scavenging activity in a dose dependant manner. The scavenging effect might be due to the antioxidant compounds from plant extracts which may compete with the O_2 to react with the NO and thus inhibits the generation of nitrite.

4.1.3.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of *Momordica charantia* and *Trigonella foenum-graecum* are shown in Figures 12a and 12b. The peroxyl radical scavengineg activity was determined and the results were compared with standard quercetin. The hydroxyl radical scavenging activity of MCF, MCS, TGL and TGS were found to be 82%, 66%, 61% and 56%.Among the four extracts, MCF showed higher hydroxyl radical scavenging activity than the standard quercetin which showed a maximum of 64%. The IC_{50} values of MCF, MCS, TGL, TGS and standard quercetin were found to be 246 µg, 555 µg, 789 µg, 867 µg and 453 µg as shown in Table 17 respectively.

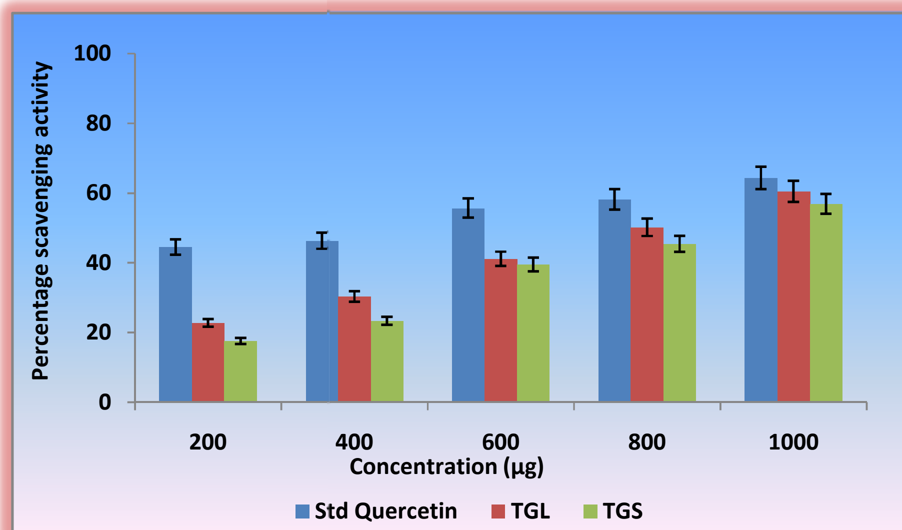


Values are mean ± SEM of triplicates

MCF- *Momordica charantia* flesh MCS - *Momordica charantia* seeds

Figure 12 a

Hydroxyl radical scavenging activity of *Momordica charantia*



Values are mean ± SEM of triplicates

TGL- *Trigonella foenum graecum* leavesTGS- *Trigonella foenum graecum* seeds

Figure 12 b

Hydroxyl radical scavenging activity of *Trigonella foenum-graecum*

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The Fenton reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. Extent of hydroxyl radical scavenged was determined by the decrease in intensity of pink coloured chromophore and determined at 532 nm. Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects. Thus, removing hydrogen peroxide as well as O_2^- is very important for protection of food systems (Kumaravel *et al*., 2013).

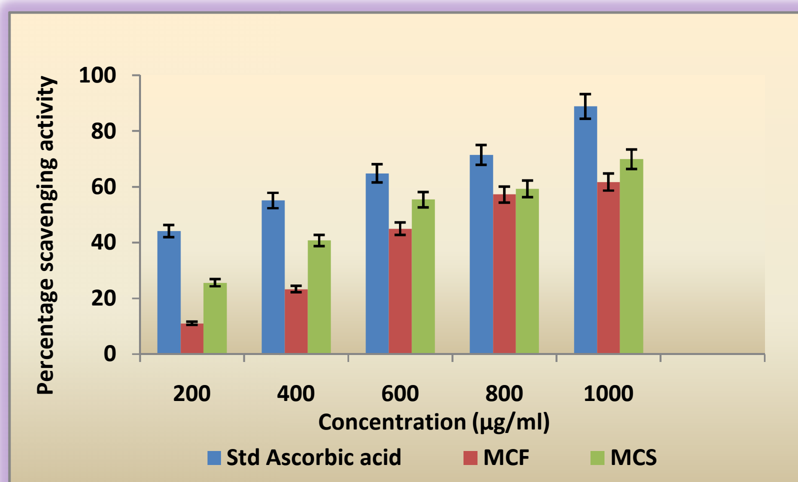
Raja *et al*. (2012) had reported that *Juglans regia L* was capable of reducing oxidative deoxyribose damage in dose dependent manner. Santharam *et al*. (2015) had reported that ethyl acetate extract of *Calycopteris floribunda* exhibited a maximum hydroxyl radical scavenging activity. The ethyl acetate extract of *C. floribunda* was found to be more effective than petroleum ether and methanolic extract.

Govindan and Suriyavathana (2013) reported the ability of the ethanolic extract of *Boerhavia erecta* to quench hydroxyl radicals which seemed to be directly related to the prevention of propagation of the process of lipid peroxidation and seem to be good scavenger of active oxygen species.

In the present study, hydroxyl radical scavenging activity was found to be higher in MCF than MCS, TGL and TGS extracts when compared to standard quercetin. This might be due to the presence of active compounds in the plant extracts.

4.1.3.6 Superoxide radical scavenging activity

The superoxide radical scavenging activity of *Momordica charantia* and *Trigonella foenum-graecum* are shown in Figures 13a and 13b. The superoxide radical scavenging activity was determined and the results were compared with standard ascorbic acid. The superoxide radical scavenging activity of MCF, MCS, TGL and TGS and standard ascorbic acid were found to be 70%, 62%, 55 %, 63% and 89%. The IC_{50} values of MCF, MCS, TGL, TGS and standard ascorbic acid were found to be 752 µg, 597 µg, 837 µg, 703 µg and 319 µg as shown in Table 17 respectively. Superoxide radical scavenging activity was found highest in MCS extracts followed by TGS, MCF and TGL.

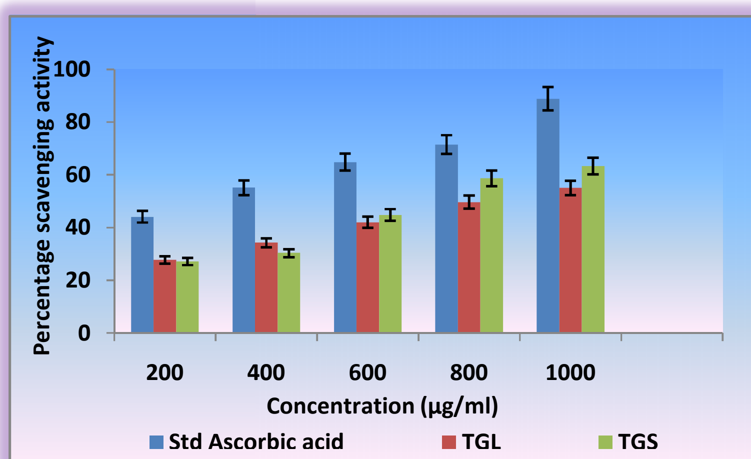


Values are mean±SEM of triplicates

MCF- *Momordica charantia* flesh   MCS - *Momordica charantia* seeds

Figure 13 a

Superoxide radical scavenging activity of *Momordica charantia*



Values are mean ±SEM of triplicates

TGL-*Trigonella foenum graecum* leaves TGS-*Trigonella foenum graecum* seeds

Figure 13 b

Superoxide radical scavenging activity of *Trigonella foenum graecum*

Superoxide anion radical is a precursor to active free radicals that have the potential of reacting with biological macromolecules and there by inducing tissue damage (Pardini, 1995). Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as via non enzymatic reactions such as auto-oxidation by catecholamines. Superoxide is biologically important since it can be decomposed to form

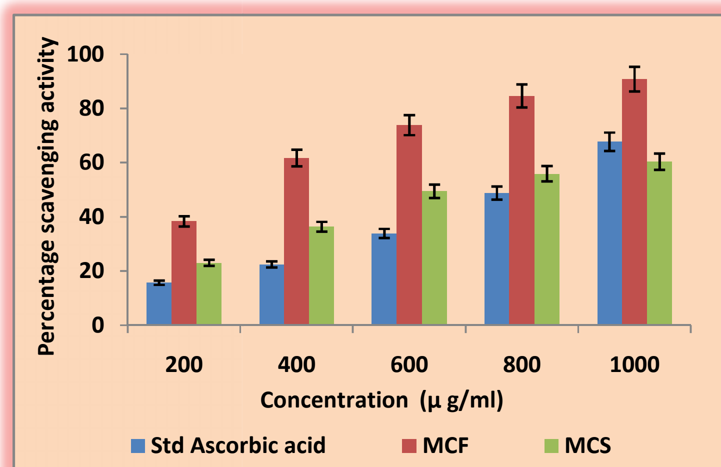
stronger oxidative species such as singlet oxygen and hydroxyl radicals (Vijayarajan and Pandian, 2016) The ethanolic mixture extracts of rhizome were found to be efficient scavengers of superoxide radical generated in PMS–NADH–NBTsystem *in vitro* and their activities were incomparable to that of ascorbic acid as reported by Mohanasundari and Suja (2016).

Hilaria *et al.* (2016) had reported that ethyl acetate extracts of *Uvaria rufa* Blume leaves had lower IC_{50} value compared to n - hexane extracts so that the ethyl acetate extract has anti-oxidant properties which is higher compared to the hexane extracts.

The present study findings are supported by Talukder *et al*. (2013) who had reported that the ethanol and ethyl acetate extracts of *Momordica charantia* fruits displayed a dose dependent activity in inhibiting the superoxide radicals against reference agent curcumin. This finding demonstrates that *Momordica charantia* fruit extract is capable of non-enzymatically inhibiting the superoxide radical, produced in biological system, which is a precursor of many ROS shown to be harmful for various cellular components.

4.1.3.7 ABTS free radical scavenging activity

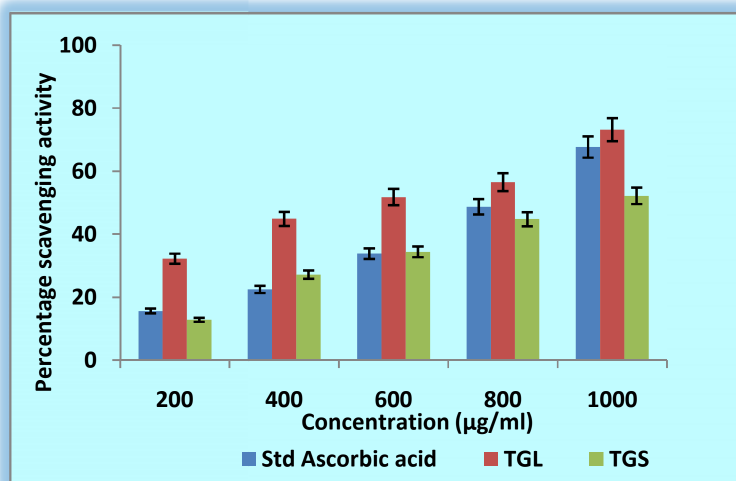
To determine the ABTS [2,2--azino-bis (3-ethyl-benzothiazoline-6-sulphonic acid)] radical scavenging activity of *Momordica charantia* and *Trigonella foenum-graecum* cationic ABTS radical decolorization was carried out. The results are shown in Figures 14a and 14b.



Values are mean ±SEM of triplicates
MCF- *Momordica charantia* flesh MCS - *Momordica charantia* seeds

Figure 14 a

ABTS radical scavenging activity of *Momordica charantia*



Values are mean ±SEM of triplicates

TGL-*Trigonella foenum graecum* leaves TGS-*Trigonella foenum graecum* seeds

Figure 14 b

ABTS radical scavenging activity of *Trigonella foenum-graecum*

The ABTS radical scavenging activity of MCF, MCS, TGL and TGS and standard ascorbic acid were found to be 91%, 60 %, 73 %, 52 % and 68 %. The IC_{50} values of MCF, MCS, TGL, TGS and standard ascorbic acid were found to be 290 µg, 707 µg, 564 µg, 926 µg, and 790 µg respectively as shown in Table 17. ABTS radical scavenging activity was highest in MCF followed by TGL, MCS and TGS when compared to standard ascorbic acid. The scavenging activity was dose dependant in all the extracts.

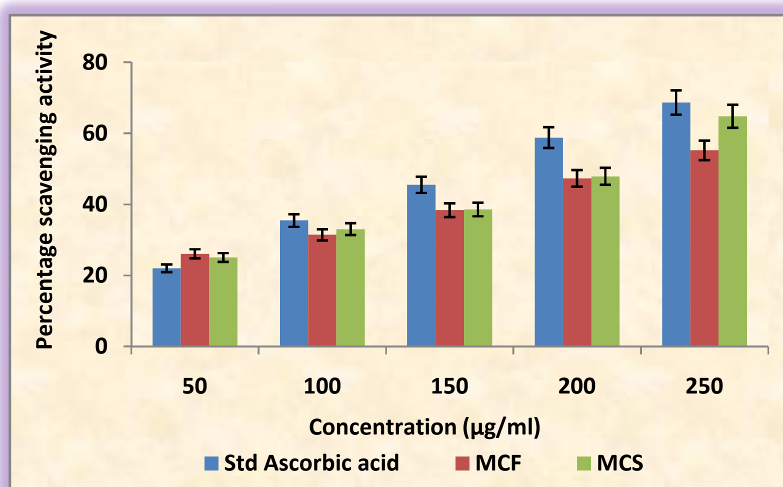
The ABTS method depends on the inhibition of the absorbance of radical cation ABTS, which has a feature wavelength at 734 nm. Decolorization of ABTS reflects the capacity of the antioxidant species to donate electrons or hydrogen atoms to inactivate these radical actions. In the presence of antioxidant reductant, the colored radical is converted back to colorless ABTS (Sreejayan and Rao, 1996).

ABTS radical scavenging activity is often used for screening complex antioxidant mixtures such as plant extracts and involves a more drastic radical, chemically produced (Govindan and Suriyavathana , 2013). ABTS radical is relatively stable but readily reduced by antioxidants. The scavenging activity against cationic ABTS radical indicates the ability of fractions to act as electron donors or hydrogen donors in free radical reactions (Prior *et al*., 2005).

The present study results are supported by the findings of Bouaziz *et al*. (2014) who had reported that *Crataegus azarolus* leaf ethyl acetate extracts exhibited a strongest antioxidant activity in ABTS method and also suggested that the effect of ethyl acetate extracts might be probably attributed to their high phenolic compounds and flavonoids. Mohan *et al.* (2014) had observed that ethyl acetate extracts of *Jatropha maheshwarii* tuber exhibited potent ABTS radical cation scavenging activity in concentration dependent manner and ethanol extract had least scavenging activity.

4.1.3.8 Assay of inhibition of lipid peroxidation

Inhibition of lipid peroxidation by *Momordica charantia* and *Trigonella foenum-graecum* was studied and the results are shown in Figures 15a and 15b. Inhibition of lipid peroxidation of MCF, MCS, TGL and TGS and standard ascorbic acid were found to be 55%, 65%, 50%, 59% and 69%. The IC_{50} values of MCF, MCS, TGL, TGS and standard ascorbic acid were found to be 220 µg, 193 µg, 252 µg, 189 µg and 167 µg respectively as shown in Table 17. Among the four extracts studied, inhibition of lipid peroxidation was high in the ethyl acetate seed extracts of *Momordica charantia* and *Trigonella foenum-graecum.*

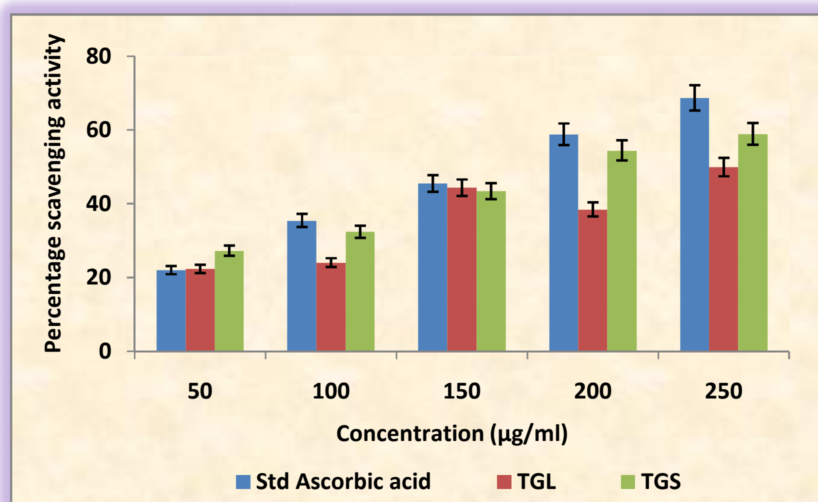


Values are mean ± SEM of triplicates

MCF- *Momordica charantia* flesh   MCS - *Momordica charantia* seeds

Figure 15 a

Inhibition of lipid peroxidation activity of *Momordica charantia*



Values are mean ± SEM of triplicates

TGL- *Trigonella foenum graecum* leaves TGS- *Trigonella foenum graecum* seeds

Figure 15 b

Inhibition of lipid peroxidation activity of *Trigonella foenum-graecum*

Lipid peroxidation is a natural phenomenon and occurs on its exposure to oxygen. Recently, free radicals-induced lipid peroxidation has gained much importance because of its involvement in several pathological conditions such as aging, wound healing, oxygen toxicity, liver disorders, inflammations etc. The ethyl acetate extracts of *Basella alba* was found to inhibit lipid peroxidation maximally compared to the chloroform extract (Adedosu *et a*l., 2013).

Initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to stabilize by a molecular rearrangement to produce a conjugated diene, which then readily reacts with oxygen molecule to give a peroxy radical. Peroxy radicals can abstract a hydrogen atom from another molecule to give lipid hydroperoxide, R-OOH. Ethyl acetate extract fractions of *Acacia nilotica* inhibited lipid peroxidation in rat liver homogenate, induced by the $FeCl_2$–H_2O_2 system (Rajbir *et al.*, 2009). Lipid peroxidation inhibiting assay revealed that the extracts might prevent reactive radical species from damaging biomolecules such as lipoprotein, DNA, amino acids, sugar, proteins and poly unsaturated fatty acids in biological and food systems (Adithya *et al.*, 2013).

Dhivya and Sivakumar (2014) had reported that ethanol and ethyl acetate extracts of *Cordia monoica* (Roxb) leaves exhibited dose dependant activity when compared to gallic acid standard. Raja *et al.* (2012) had reported that ethanol extract of nuts of *Juglan*

regia L. showed better dose-dependent prevention towards generation of lipid peroxides in the *in vitro* lipid peroxidation induced in goat liver by using Fe SO_4 and ascorbic acid.

Table 17

IC_{50} values of radical scavenging activity and *in vitro* lipid peroxidation

Plant extract/ Standard	MCF (µg)	MCS (µg)	TGL (µg)	TGS (µg)	Ascorbic acid (µg)	Quercetin (µg)
DPPH	777	830	1368	895	734	-
NO	964	881	960	981	540	-
OH	246	555	789	867	-	453
SO	752	597	837	703	319	-
ABTS	290	707	564	926	790	-
Lipid Peroxidation	220	193	252	189	167	-

Values are mean of triplicates

MCF- *Momordica charantia* flesh TGL- *Trigonella foenum graecum* leaves

MCS - *Momordica charantia* seeds TGS- *Trigonella foenum graecum* seeds

The results of the present study demonstrated that seeds extracts of *Momordica charantia* and *Trigonella foenum-graecum* had potent alpha-amylase inhibitory and antioxidant activities compared to *Momordica charantia* flesh and *Trigonella foenum – graecum* leaves extract. Hence, further studies were carried out with extracts of *Momordica charantia* and *Trigonella foenum-graecum* seeds extract to substantiate the *in vitro* results by employing *in vivo* models for their effective utilization as therapeutic agents in the treatment of Diabetes.

PHASE II

4.2 *In vivo* Antidiabetic and Antioxidant Potential of *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts in Nicotinamide-Streptozotocin Administered Diabetes Induced Rats

4.2.1. Acute toxicity

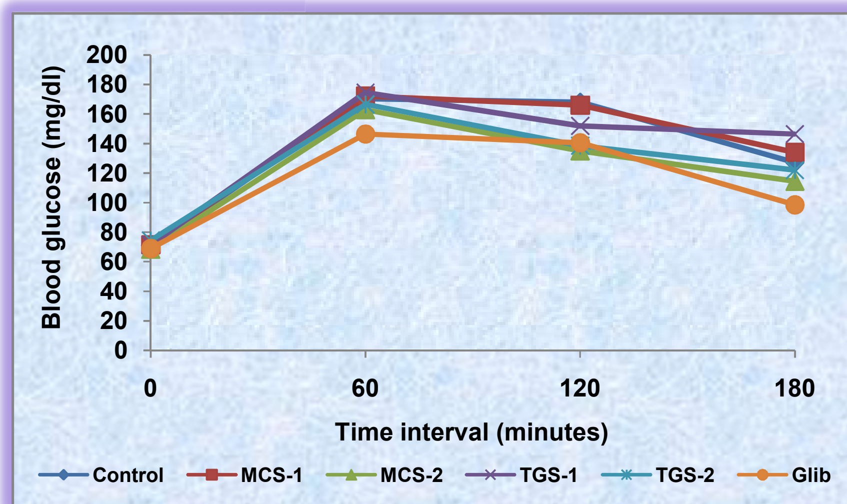
The results of acute toxicity study of ethyl acetate extracts of *Momordica charantia* seeds and *Trigonella foenum graecum* seeds given by oral route were safe up to a maximum dose of 2000 mg/kg body weight. There were no changes in the normal behaviour pattern and no signs and symptoms of toxicity and mortality observed as per Organization for Economic Co-operation and Development (OECD) guidelines. Since no

mortality was observed 1/10th (200 mg/kg b,w) and 1/5th (400 mg/kg b,w) of the highest dose (2000mg/kg b.w) were chosen for further studies.

4.2.2 Results of Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test of *Momordica charantia* seeds extract and *Trigonella foenum graecum* seeds extract was done on fasting normoglycaemic rats and the results are presented in Figure 16.

OGTT is the only form of glucose tolerance recommended for the diagnosis of Diabetes. The changes in blood glucose concentration, which result from an oral carbohydrate load is theoretically dependent on the rate at which carbohydrate enters the small intestine, the rate of digestion and intestinal absorption of glucose and the rate of insulin-driven metabolism (Shrinivasan and Karundevi, 2005).



Values are expressed as mean (n=6)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 16

Blood glucose level in normal rats administered with *Momordica charantia* seeds extract and *Trigonella foenum graecum* seeds extract and glibenclamide during OGTT

OGTT determines how quickly glucose is cleared from the blood after a given oral glucose dose. In the present study, a dose-dependent effect was observed. In glucose-fed rats treated with normal saline, there was a significant increase in blood glucose levels after 60 minutes following administration of glucose. The maximum increase in blood glucose was observed 60 minutes after administration of glucose. The ethyl acetate

extracts of *Momordica charantia* seeds, *Trigonella foenum graecum* seeds and glibenclamide significantly prevented a rise of the blood glucose level after 60 and 180 minutes compared to the control group. Treatment of the rats with glibenclamide produced a maximum reduction in blood glucose after 60 to 180 min of glucose administration. Higher doses 400mg/kg b.w of both MCS and TGS prevented increase in blood glucose after 60 minutes and it was similar to the action of standard drug glibenclamide. This shows that both the extracts possess glucose lowering effect indicating a better glucose utilization capacity. This could be due to tissue glucose uptake and reduced hepatic glucose output, there by producing an antihyperglycemic effect.

The results are similar to those of Hasan *et al*. (2014) who had reported that the oral glucose test performed with the ethyl acetate extracts of *Mallotus repandus* showed good hypoglycemic activity like glibenclamide each hour after administration. Arokiyaraj *et al*. (2011) had reported that *Hypericum perforatum* ethyl acetate extract was administered to fasting normal rats and hypoglycemia was observed after 30 min. The decline in blood sugar level reached its maximum after 2 hours.

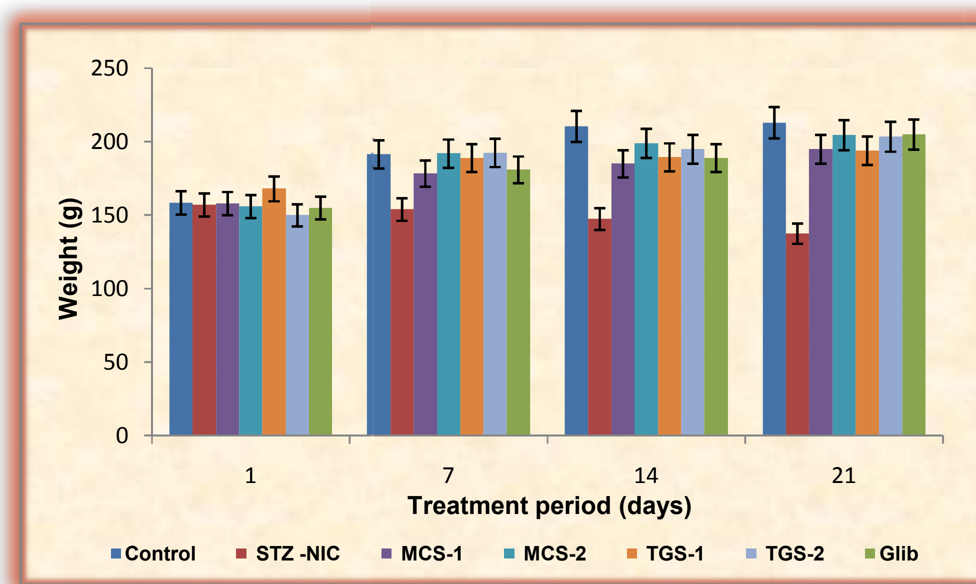
4.2.3 Antidiabetic activity of the selected plant parts

The experimental rats selected for the studies were kept fasting overnight and the initial fasting blood glucose and body weights were noted. Diabetes Mellitus was induced by intraperitoneal injection of nicotinamide and streptozotocin. Hyperglycemia was confirmed by the elevated levels of blood glucose determined after 72 hours. The rats with blood glucose concentration more than 250mg/dl were used for further study. The experimental rats were divided into seven groups of six rats in each group. Group1 served as control, Group 2 diabetic control (STZ-NIC), Group 3 treated with MCS 200 mg/kg b.w, Group 4 treated with MCS 400 mg/kg b.w, Group 5 treated with TGS 200 mg/kg b.w, Group 6 treated with TGS 400 mg/kg b.w and Group 7 treated with standard drug glibenclamide 200 µ g/kg b.w.

4.2.3.1 Body weight

The body weight of rat is an excellent physical interpretation of the effect of drugs or foods on the biochemical profile of the animal (Aja *et al*., 2015). Thus, the body weight of experimental rats during 21 days of treatment with ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum* and standard drug glibenclamide were recorded initially before induction of Diabetes and at weekly intervals during the treatment period. The mean weights of the rats are presented in Figure 17.

The effect of MCS, TGS extracts and standard drug glibenclamide on the body weight was measured from 1st to 21st day of post induction. The result showed initial reduction in mean body weight of all the groups after induction except in the healthy control. The control group showed significant weight gain throughout the treatment period. There was significant weight reduction in STZ-NIC induced diabetic rats. After treatment with MCS, TGS and glibenclamide, the mean body weight of the treated groups was restored to near that of the healthy control group. This shows that as the glucose level decreases the body weight improves.



Values are mean ± SEM (n= 6)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 17

Weight of the experimental rats during the treatment period

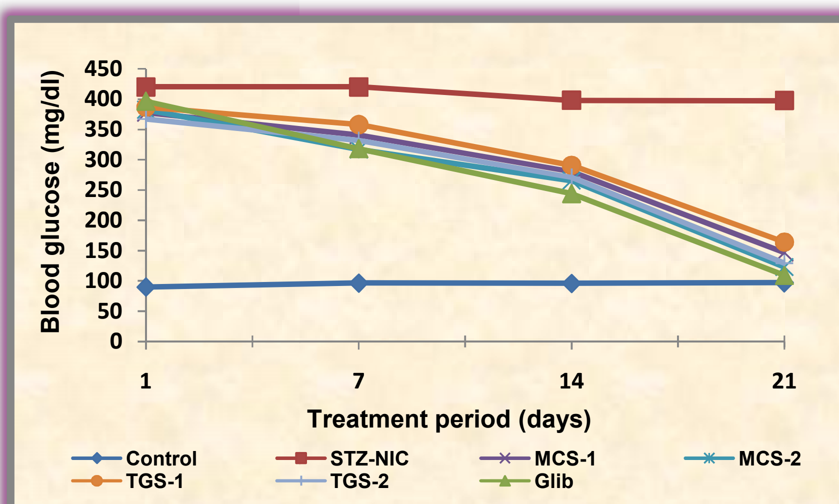
Decrease in body weight of diabetic rats might be attributed to the intensive catabolism of fats and structural proteins which are used as energy source due to unavailability of carbohydrates **(**Mohamed *et al*., 2016).

Diabetic rats treated with plant extracts seemed to restore the body weight. This might be due to improved insulin secretion by the plant extracts which reduces the hyperglycemia by peripheral utilization of glucose by the cells that ultimately improved the body weight. The plant extracts with dose of 400mg/kg b.w seemed to increase the body weight in groups 4 and 6 which might indicate that MCS and TGS have beneficial effect in maintaining the body weight which was similar to that of drug glibenclamide.

4.2.3.2 Biochemical analysis

(i) Blood glucose

The initial blood glucose level among the groups showed no significant differences. But after induction of Diabetes, the blood glucose levels effectively showed hyperglycaemia which was followed with the administration ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum* seeds and standard drug glibenclamide. The fasting blood glucose levels of control, Diabetes induced and treated rats during the experimental period is depicted in Figure 18.



Values are expressed as mean (n=6)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w, Glib: 200μg/kg b.w.

Figure 18

Fasting blood glucose levels in the experimental rats during treatment period

After 7 days of treatment, there was a significant decrease in fasting blood glucose levels in MCS, TGS and glibenclamide treated groups respectively. The fasting blood glucose level decreased gradually on 14th and 21st days in MCS, TGS and glibenclamide treated groups as compared to onset of the study. The effect of plant extracts was found to be time dependant up to 21st day of the study. The results indicate that both low (200 mg/kg b.w) and high (400 mg/kg b.w) doses lowered blood glucose levels in diabetic rats. The diabetic rats treated with high dose of 400 mg mg/kg b.w of MCS and TGS showed similar reduction as that of glibenclamide. This difference in reduction of blood glucose observed between groups might be dose dependant.

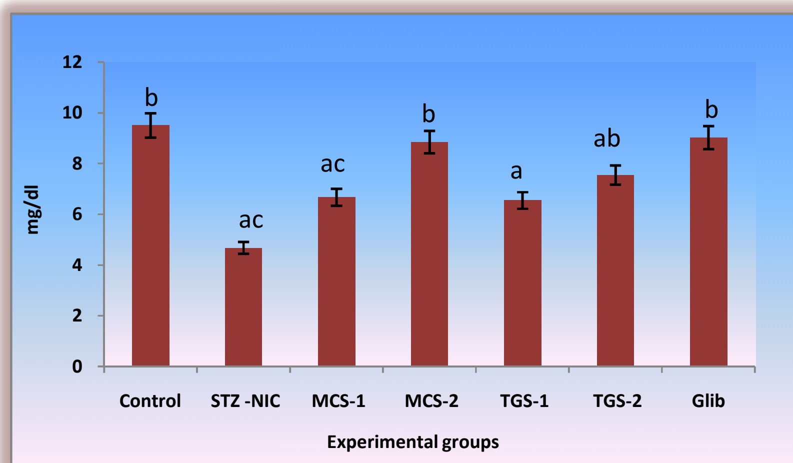
The results are similar to those of Malarkodi *et al*. (2011) who had reported that repeated administration of ethyl acetate and ethanolic extract of *Scindapsus officinalis*

fruits (once a day for 21 days) as well as glibenclamide caused significant reduction in the blood glucose level as compared to diabetic control group. Nizam *et al*. (2014) had reported that *Citrus macroptera* extracts were found to decrease the activity of α-amylase in the digestive canal, improve the metabolism of glucose and increase insulin secretion by stimulating beta cells and also suggested that the bioactive compounds present in the fruit extract might be responsible for multifaceted effects.

The present study results are supported by the observation of Ramya and Lakshmidevi (2015) who had recorded that administration of *Andrographis paniculata* ethanol leaf extract to streptozotocin induced diabetic rats for four weeks produced a significant reduction in blood glucose. Among the two doses 200mg/kg bw and 500 mg/kg bw of the extracts used for treatment, as per their observation 500 mg/kg bw of the extract showed greater reduction in blood glucose level which was comparable to glibenclamide as per their observation.

(ii) Serum proteins

The effect of ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum* seeds and standard drug glibenclamide on total serum proteins of the experimental rats was studied at the end of the treatment period and results are depicted in Figure 19.



Values are mean± SEM (n= 6)
a-p <0.05 compared with control group
b-p <0.05 compared with STZ –NIC group
c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400 mg/kg b.w.

Figure 19

Total protein levels in the serum of the experimental rats

The serum total protein levels of diabetic control was significantly reduced ($p<0.05$), whereas the rats treated with plant extracts and glibenclamide showed a significant increase ($p<0.05$) in the total protein content. Ethyl acetate seed extracts of MCS and TGS treatment were found to increase the content of protein. The increase in serum proteins was observed to be dose dependant. Higher dose of 400 mg/kg b.w of MCS and TGS showed better results when compared to 200 mg/kg b.w. Restoration of protein content by MCS was more than by TGS extracts.

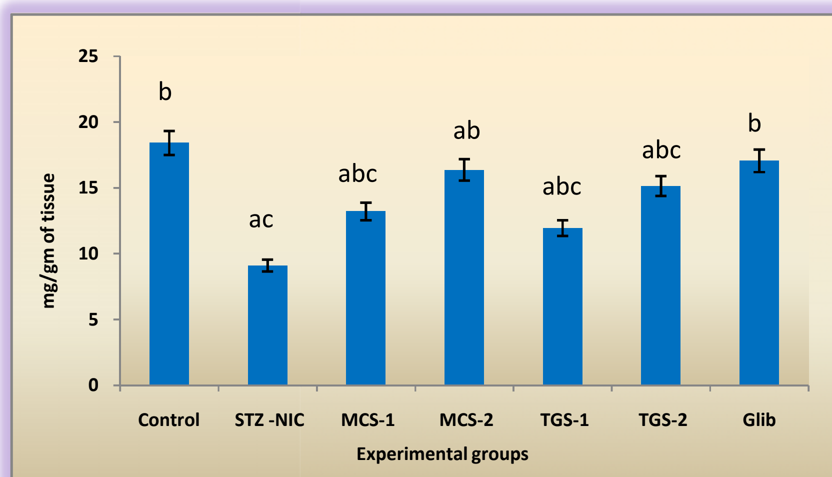
There is increased protein catabolism with the flow of amino acids into the liver, which feeds gluconeogenesis as a result of insulin deficiency during uncontrolled Diabetes Mellitus (Senthilkumar and Subramanian, 2008). This might account for the decrease in serum total protein content in STZ induced diabetic control rats (Narendhirakannan *et al*., 2006).

Kondeti *et al.* (2010) had reported that STZ induced diabetic rats seemed to account for the observed decrease in the total protein content. The progressive restoration of total protein in the serum of STZ diabetic rats treated with plant extracts and glibenclamide might be due to inhibition of proteolytic activity as a result of increasing insulin secretion and proper utilization of blood glucose. Balasubramanian *et al.* (2014) reported that daily administration of ethyl acetate fraction of *Stereospermum suavelolens* for 14 days caused a significant elevation in serum total protein levels in diabetic rats when compared to diabetic control.

The results of the present study showed that the treatment of rats with MCS and TGS ethyl acetate extracts caused a significant increase in serum total protein. This might be attributed to an improvement in glycemic control and insulin secretion that might increase protein synthesis or decrease protein degradation as reported by Gao *et al.* (2009).

(iii) Glycogen

The results of liver glycogen in control and experimental rats are shown in Figure 20. The levels of hepatic glycogen were observed to be significantly reduced ($p<0.05$) in STZ-NIC induced diabetic rats. In rats treated with plant extracts and glibenclamide, there was a significant increase ($p<0.05$) in liver glycogen. The increase in glycogen content was found to be dose dependant. The increase in glycogen content of MCS 400 mg/kg bw treated rats was similar to that of glibenclamide treated rats.



Values are mean± SEM (n= 6)
a-p <0.05 compared with control group
b-p <0.05 compared with STZ –NIC group
c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 20

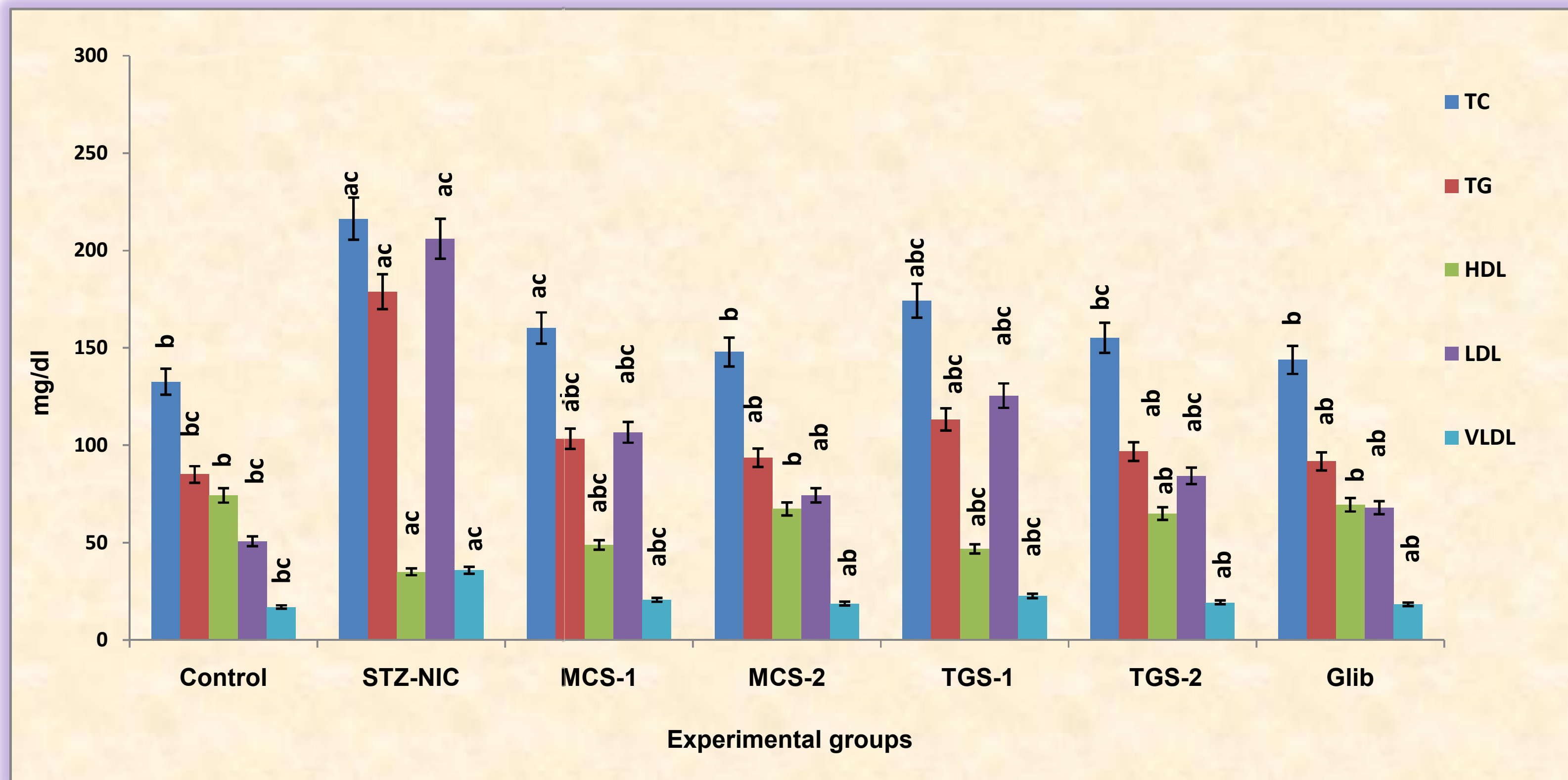
Glycogen content in the liver of experimental rats

Grover *et al*. (2002) had reported that glycogenesis in muscle and liver is mainly regulated by serum insulin level. The regulation of glycogen metabolism *in vivo* that occurs by the multifunctional enzyme glycogen synthase and glycogen phosphorylase plays a major role in the glycogen metabolism. The reduced glycogen stored in diabetic rats has been attributed to reduced activity of glycogen synthase and increased activity of glycogen phosphorylase as reported by Kamalakkannan and Balakrishnan (2015).The significant increase in the glycogen content of the treated groups might be due to reactivation of the glycogen synthase enzyme. Hence, improvement of glycogenesis could be another probable way of anti-diabetic action as suggested by Maiti *et al*. (2004).

The decrease in hepatic glycogen reported in the present study might be due to low level of serum insulin in diabetic rats, which could have inactivated the glycogen synthesis system. Treatment with MCS and TGS extracts for 21 days in experimental rats was found to result in increase in liver glycogen levels. This might highlight one possible way of antidiabetogenic action of the plant extracts.

(iv) Lipid Profile

The results of lipid profile tests performed on control and experimental rats are depicted in Figure 21.



Values are mean± SEM (n= 6)
a-p <0.05 compared with control group; b-p <0.05 compared with STZ –NIC group; c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 21

Lipid profile in the serum of experimental rats

Levels of total cholesterol 216mg/dl, triglycerides 178 mg/dl, low density lipoprotein (LDL) 200 mg/dl and very low density lipoprotein (VLDL) 35.29 mg/dl were found to be significantly increased ($p<0.05$) and high density lipoprotein (HDL) levels (34.83 mg/dl) levels were significantly reduced ($p<0.05$) in STZ-NIC rats compared to normal control rats.

Rats treated with glibenclamide, MCS and TGS ethyl acetate extracts showed a significant decrease ($p<0.05$) in the levels of total cholesterol, triglycerides, LDL, VLDL and a significant increase ($p<0.05$) in HDL when compared to STZ-NIC rats. There was a significant difference ($p<0.05$) in the lipid profile of diabetic rats treated with plant extracts. High dose of 400 mg/kg b.w was found to be more effective when compared to 200 mg/kg b.w of ethyl acetate extracts. This shows that the activity is dose dependant.

Lipids play a vital role in the pathogenesis of Diabetes Mellitus. The most common lipid abnormalities in Diabetes are hypertriglyceridemia and hypercholesterolemia. The levels of increased serum lipids in Diabetes represent risk factor for coronary heart disease (Lavie *et al*, 2013). Under normal circumstances, insulin activates lipoprotein lipase and hydrolyzes triglycerides (Anupama *et al*., 2012). Giribabu et *al*. (2014) had reported that HDL-cholesterol is an antiatherogenic lipoprotein. It transports cholesterol from peripheral tissues into the liver and thereby acts as a protective factor against coronary heart disease.

Nyunai *et al.* (2015) had reported that the level of HDL-cholesterol that was increased after *Ageratum conyzoides* administration might be due to an increase in the activity of lecithin cholesterol acyl transferase, which may contribute to the regulation of blood lipids. Administration of *Ageratum conyzoides* seemed to lower triglycerides and to a lesser extent LDL cholesterol level and increase the serum HDL-cholesterol in diabetic rats.

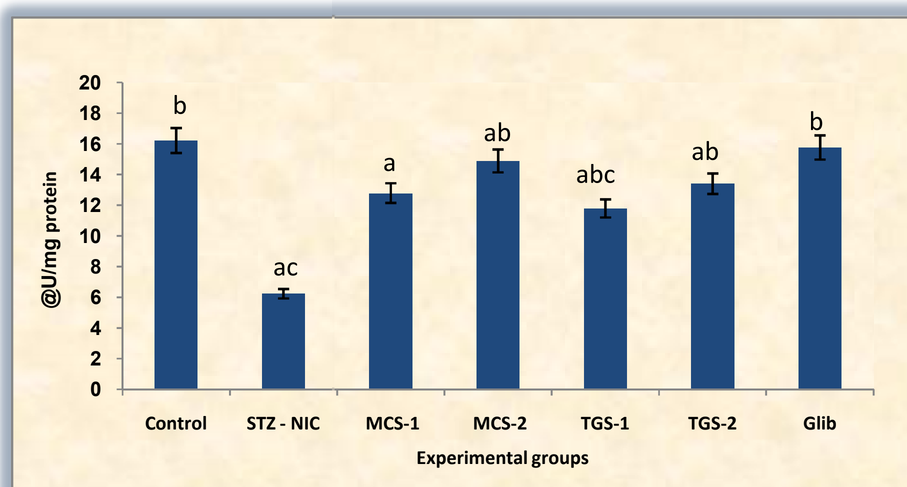
MCS and TGS in reversing the elevation of total cholesterol, triglycerides, LDL,VLDL and decreased HDL in experimental rats agrees with the findings of Adaramoye *et al.*(2005) who had reported the anti-atherogenic effect of kolaviron (a Garcinia kola seed extract) in hypercholesterolaemic rats. Amaechi *et al*. (2015) had reported that the action of plant extracts in reducing the plasma concentration of total cholesterol could be due to the ability of one or more phytochemicals in the plant extracts to activate the functioning of enzymes in rats responsible for cholesterol absorption.

(v) Antioxidant activities

There is increasing evidence in both experimental and clinical studies which suggests that Diabetes is associated with oxidative stress, leading to an increased production of reactive oxygen species, including superoxide radical, hydrogen peroxide and hydroxyl radical (Gokce and Haznedaroglu, 2008). Streptozotocin induced hyperglycemia induces free radical generation which thereby leads to DNA damage, protein degradation, lipid peroxidation and finally culminating into damage to various organs of the body namely liver, kidney, brain and eyes (Yazdanparast *et al*., 2007).

Enzymic antioxidants

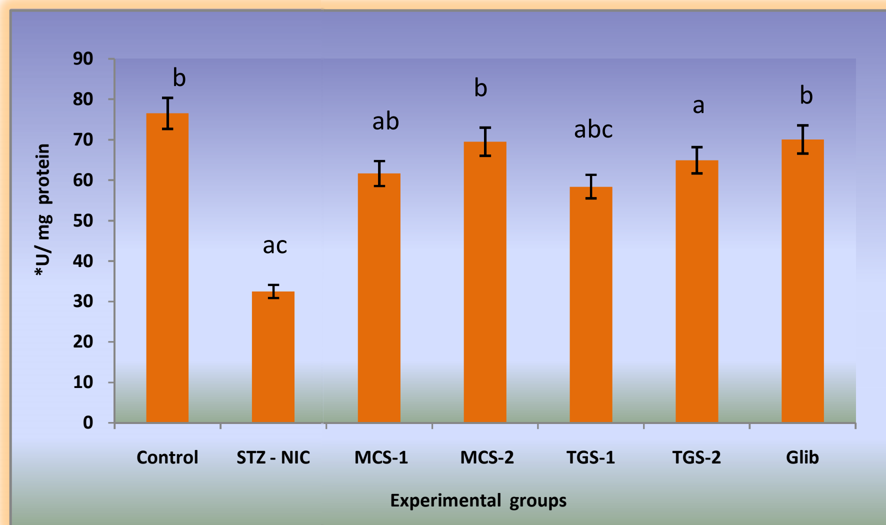
An imbalance of oxidant and antioxidant defence systems result in alterations in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx). In the present study, the activity of superoxide dismutase, catalase and glutathione peroxidase in normal and Diabetes induced rats were evaluated. The results of antioxidant activity of enzymes on control and experimental rats are depicted in Figures 22 a, b and c.



Values are mean± SEM (n= 6)
@ 1 Unit**:** Amount of enzyme that causes 50% reduction in NBT oxidation
a-p <0.05 compared with control group
b-p <0.05 compared with STZ –NIC group
c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 22 a

Activity of hepatic superoxide dismutase in the experimental rats



Values are mean± SEM (n= 6)
*1 Unit: Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units
a-p <0.05 compared with control group
b-p <0.05 compared with STZ –NIC group
c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 22 b

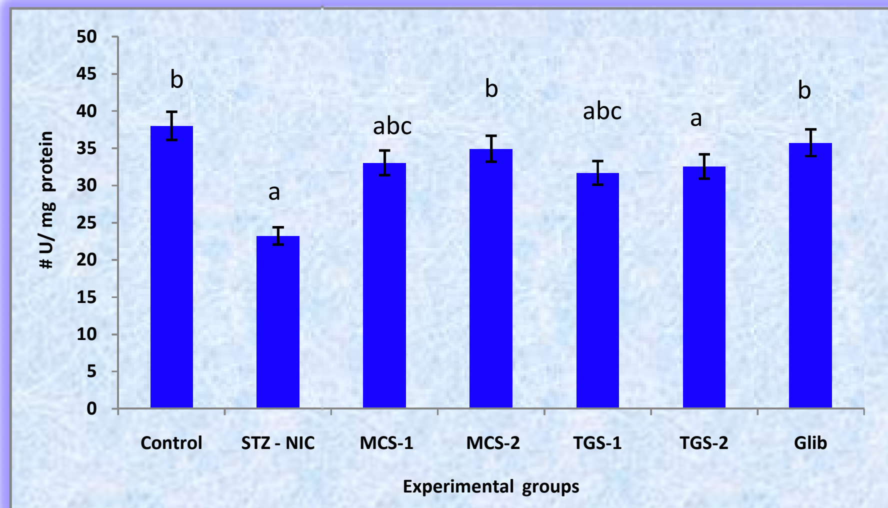
Activity of hepatic catalase in the experimental rats

There was a significant decrease ($p<0.05$) in the activity of enzymic antioxidants namely superoxide dismutase, catalase and glutathione peroxidise in the liver of diabetic control rats. In diabetic rats treated with glibenclamide and plant extracts, there was a significant improvement ($p<0.05$) in the activity of these enzymes. The activity of these enzymes in rats treated with highest dose of 400mg/kg b.w of MCS was comparable to the activity of enzymes in glibenclamide treated rats.

Oxidative stress is a condition of reduction in anti oxidative enzyme activities of SOD, CAT and GPx. The antioxidant enzymes SOD and CAT play an important role in reducing cellular stress. SOD scavenges the superoxide radical by converting it to hydrogen peroxide and molecular oxygen, while CAT brings about the reduction of hydrogen peroxides and protects higher tissues from the highly reactive hydroxyl radicals (Ragini *et al.,* 2011).

The decreased activities of CAT and SOD may be response for increased production of H_2O_2 and O_2 by the auto-oxidation of glucose. These enzymes play an important role in maintaining physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating organic peroxides and

hydroperoxides generated from inadvertent exposure to STZ (Pari and Latha, 2004).Treatment with MCS and TGS seemed to increase the activity of these enzymes and might help to control free radicals when compared to diabetic rats.



Values are mean± SEM (n= 6)

1 Unit: μ moles of GSH consumed/minute//mg liver protein.

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200μg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Fig 22 c

Activity of hepatic glutathione peroxidase in the experimental rats

Glutathione peroxidase enzyme is relatively stable, but it has been reported that is disabled in severe oxidative stress conditions (Condell and Tappel, 1983).Oseni *et al.* (2015) had reported that *Citrullus lanatus* (watermelon) treated diabetic rats showed an increase in the activity of Gpx status which was almost close to the control level and this is remarkable as this implies that the juice could have an ameliorating effect on the altered antioxidant status of diabetic rats.

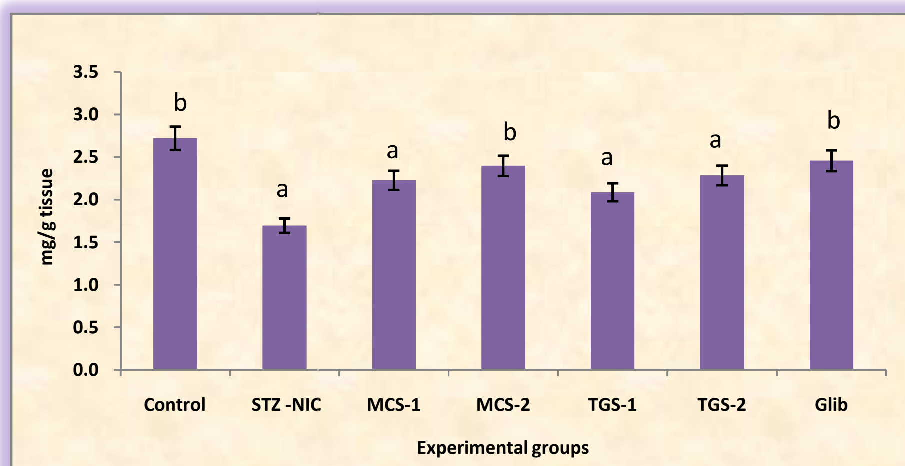
Balasubramanian *et al.* (2014) had reported that the activities of antioxidant enzymes SOD and CAT were significantly increased after the treatment of ethyl acetate fraction of ethanol extract of *Stereospermum suaveolens* in STZ-induced diabetic rats indicating the free radical scavenging activity and their protective effect against diabetic kidney cellular damage.

The present study results are also supported by the observation of Shilpa *et al.* (2012) who had reported that treatment with root extracts of *Premna corymbosa* (Rottl.) increased the activity of antioxidant enzymes SOD, CAT and GPx when compared to diabetic rats. The effect produced by plant extract was comparable with that of standard drug glibenclamide.

Non enzymic antioxidants

The changes in the levels of non-enzymic antioxidants namely vitamin C, vitamin E and reduced glutathione (GSH) are important in cellular system in curtailing reactive oxygen species. The levels of these non – enzymic antioxidants in control, diabetic and treated rats were assessed and the results are depicted in Figures 23 a, b and c.

There was a significant reduction ($p<0.05$) in the nonenzymatic antioxidants namely vitamins C, E and reduced glutathione (GSH) in diabetic rats when compared to control rats. The levels of these antioxidants were significantly increased ($p<0.05$) in rats by treating with glibenclamide, MCS and TGS ethyl acetate extracts. The levels of vitamin C, E and reduced glutathione were found to be increased significantly ($p<0.05$) on treatment with 400mg /kg b.w.



Values are mean± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

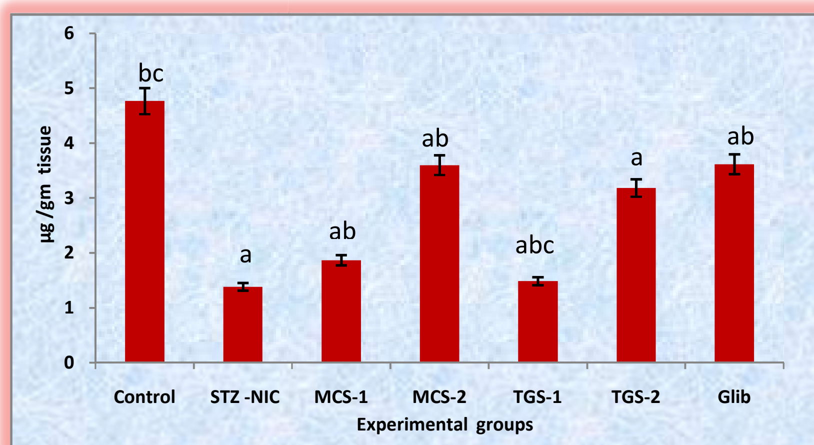
c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett’s multiple Comparison test)

MCS -1:200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 23 a

Levels of hepatic vitamin C in the experimental rats



Values are mean± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg) treated group

(One way ANOVA followed by Dunnett’s multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 23b

Levels of hepatic vitamin E in the experimental rats

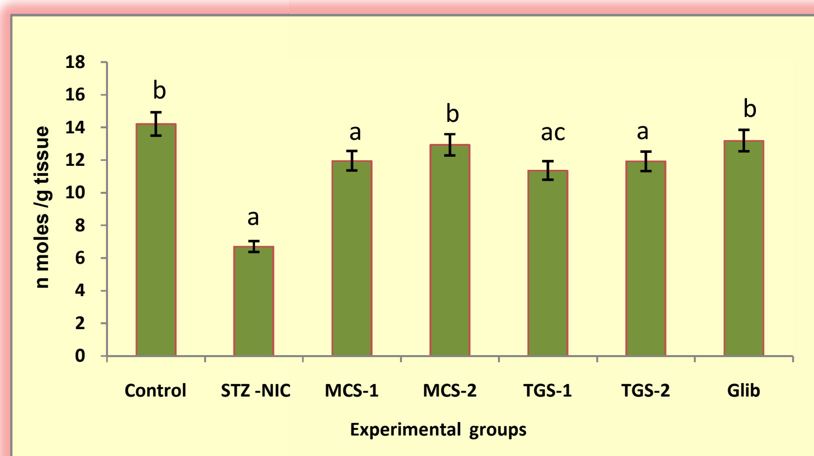
Ambali *et al*. (2007) reported that vitamin C is an effective antioxidant in various biological systems. Vitamin C plays a central role in the antioxidant protective system, protecting all lipids undergoing oxidation and diminishing the number of apoptotic cells (Sadi *et al.,* 2008). Vitamin E acts as a non-enzymatic antioxidant and reduces chain reactions of lipid peroxidation (Punithavatki *et al.,* 2008).

Vitamin E reduces lipid hydroperoxides generated during the process of peroxidation and protects cell structures against damages. The decreased level of vitamin E found in the liver of diabetic rats as compared with control rats could be due to increased oxidative stress, which accompanies the decrease in the level of antioxidant and might be related to the cause of Diabetes Mellitus (Halliwell and Gutteridge, 1984 and Annamalai *et al*., 2003) Enhanced level of vitamin E or tocopherols in plant extract treated groups is based on their ability to donate phenolic hydrogens to lipid radicals. Vitamin E protects poly unsaturated fatty acids from being oxidized (Sharma, 2000).

Shilpa *et al*. (2012) had reported decreased levels of nonenzymatic antioxidant vitamin C and E in diabetic rats, when compared to those of control rats. The levels of these antioxidants were significantly increased in different organs (liver, kidney, brain,

heart and pancreas) of diabetic rats by treatment with root extracts of *Premna corymbosa* (Rottl) as per their reports.

GSH has a multifaceted role in anti-oxidant defence. It is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by glutathione peroxidase (Kaleem *et al*., 2006). Hyperglycemia is found to be an indirect cause of GSH depletion. As GSH is an important antioxidant molecule, its depletion leads to an increase of oxidative stress (Nandhini and Victor, 2014)



Values are mean± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS 2:400mg/kg b.w.

Figure 23c

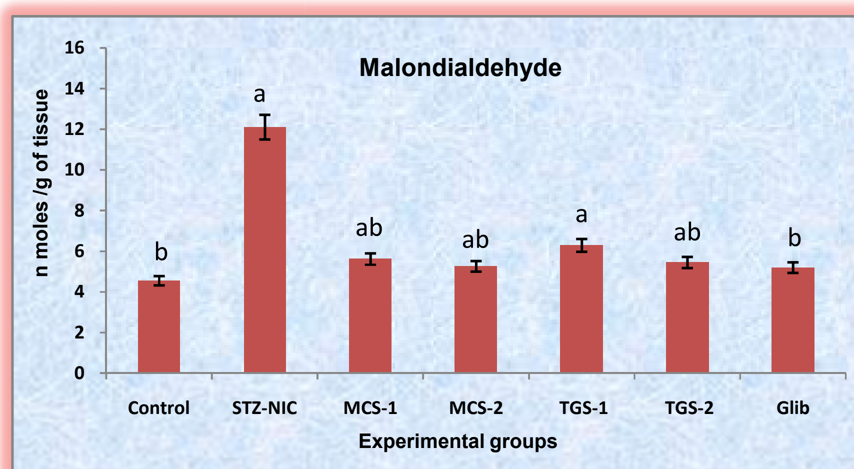
Levels of hepatic reduced glutathione in the experimental rats

Anusooriya *et al*. (2014) had reported that oral administration of aqueous fruit extract of *Passiflora ligularis* for 30 days showed significant elevation in all the non enzymatic antioxidants values and reached near normal values. This can reduce the oxidative stress leading to less degradation of GSH due to less production of ROS in diabetic stage.

In the present study, there was an increased level ($p<0.05$) of reduced glutathione in MCS and TGS treated groups which imply that the plant extracts might have an enhanced amount of GSH activity which plays a role in coordinating the body's antioxidant defense processes. Reduced glutathione, synthesized mainly in the liver is an important non- enzymic antioxidant in the antioxidant defense system.

(vi) Lipid peroxidation

The status of lipid peroxidation of control and experimental rats were studied and the results are depicted in Figure 24. Lipid peroxidation was increased significantly ($p<0.05$) in diabetic rats as compared to that of control rats. The rats treated with glibenclamide, MCS and TGS showed significant reduction ($p<0.05$) in lipid peroxidation. The diabetic rats treated with highest dose of 400mg/kg b.w showed significant improvement ($p<0.05$) in antioxidant activity and the reduction in malondialdehyde production was comparable to glibenclamide treated rats.



Values are mean± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/ kgb.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 24

Lipid peroxidation in the liver of experimental rats

Davey *et al*. (2000) had reported that lipid peroxidation is an autocatalytic free radical process formed by oxidative damage of cells. ROS produced in tissues results in lipid peroxidation and subsequently enhances the levels of malondialdehyde which is the major end product and index of lipid peroxidation.

Lipid peroxidation is a free radical mediated process leading to oxidative deterioration of polyunsaturated lipids. The increase in oxygen free radicals in Diabetes could be primarily due to increase in blood glucose levels, which upon auto-oxidation generates free radicals (Malini *et al.,* 2011).

Gopalakrishnan and Dhanapal (2014) had reported that *Coleus vettiveroides* Jacob extracts possess potent antioxidant and lipid peroxidation activities and can be employed in protecting tissue from the oxidative stress, which might be responsible for its hypoglycemic property.

Balasubramanian *et al.* (2014) had reported that STZ-induced diabetic rats showed an increase in TBARS (Thiobarbituric Acid Reactive Substances) level in kidney as compared to nondiabetic rats and treatment with ethyl acetate fractions of *Stereospermum suavelolens* significantly decreased TBARS in diabetic rats as compared to the diabetic control rats.

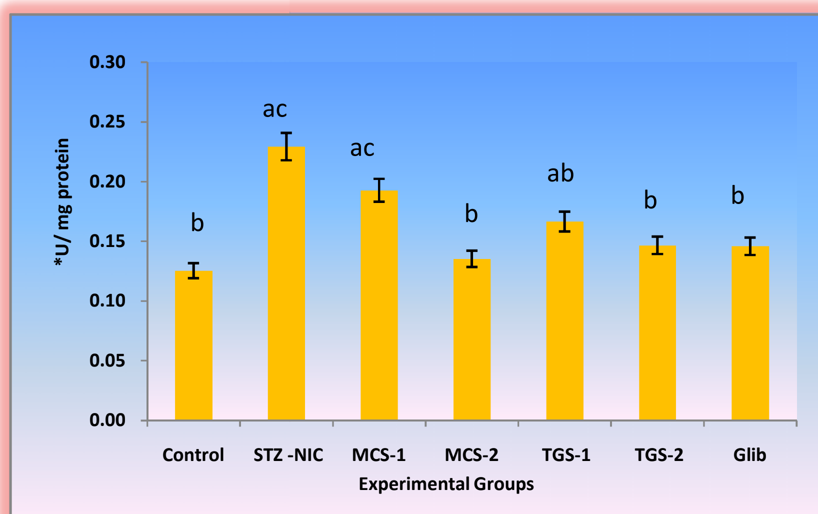
In the present study, increased lipid peroxidation in STZ-induced diabetic rats might be due to an increase in the generation of free radicals by STZ. The ability of MCS and TGS extracts to quench hydroxyl radicals seems to be directly related to inhibiting the process of lipid peroxidation. After oral administration of the plant extracts for 21 days the elevated values restored back to near normal level. The treated groups showed significant decrease in lipid peroxidation, suggesting its role in protection against lipid peroxidation.

(vii) Activities of carbohydrate metabolizing enzymes

In Diabetes, enzymes of glucose metabolism are markedly altered. The activities of carbohydrate metabolic enzymes of liver namely, glucose -6-phosphatase, fructose 1, 6-diphosphatase and glucose -6-phosphate dehydrogenase were assayed after treatment with glibenclamide and plant extracts.

(a) Glucose -6-phosphatase

The results of the activity of glucose -6-phosphatase in control and experimental rats are depicted in Figure 25 a. The activity of hepatic glucose -6-phosphatase was found to be increased significantly ($p<0.05$) in STZ-NIC induced diabetic rats when compared to control group. Administration of plant extracts and glibenclamide resulted in significant reduction ($p<0.05$) in the activity of the enzyme. Higher concentrations of 400mg/kg b.w showed better results in decreasing the activity of the enzyme. Also there was no significant difference observed in the activities of the enzymes in the liver of rats treated with low dose of MCS/TGS (200 mg/kg b.w)



Values are mean± SEM (n= 6)
* Glucose -6-phosphatase units: µ moles of Pi liberated/min/mg protein
a-p <0.05 compared with control group
b-p <0.05 compared with STZ –NIC group
c-p <0.05 compared with Glib (200µg/kg b.w) treated group
(One way ANOVA followed by Dunnett's multiple Comparison test)
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 25 a

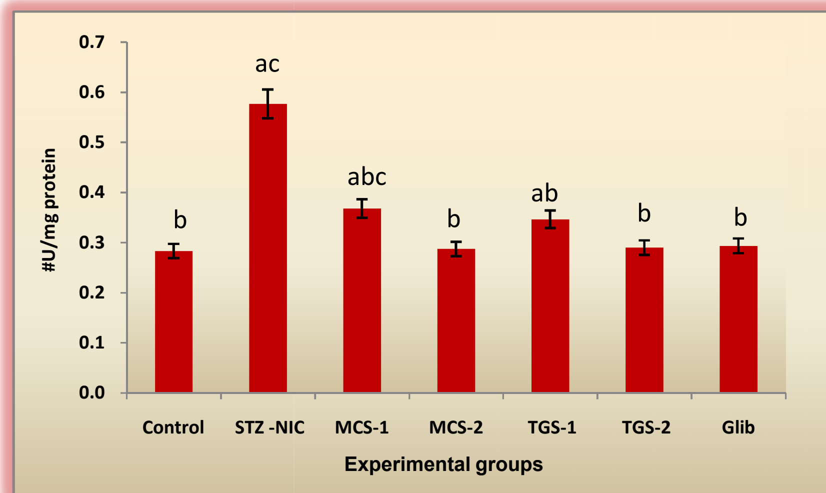
Activity of glucose - 6 - phosphatase in the experimental rats

Glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose concentration, is expressed mainly in the liver and kidney and is critical in providing glucose to other organs during Diabetes, prolonged fasting or starvation (Marella *et al*., 2016 and Bouche *et al*., 2004). It catalyzes the dephosphorylation of glucose-6-phosphate to free glucose as the terminal step in gluconeogenesis and glycogenolysis (Prabakaran and Ashokkumar, 2012). Gluconeogenic enzyme activation is due to the state of insulin impairment as under normal conditions, insulin functions as a suppressor of gluconeogenic enzymes (Pari and Murugan, 2005).

Enhanced hepatic glucose production and impaired hepatic glucose utilization in STZ prompted diabetic rats might be mediated by dysregulation of hepatic glucose-6-phosphatase activity (Alemzadeh *et al*., 2002).Diabetic rats administered with MCS, TGS extracts and glibenclamide modified the levels of glucose -6-phosphatase to near normal, which might be due to higher insulin secretion. Similar results were reported by Arokiyaraj *et al.* (2011) in their studies on *Hypericum perforatum* ethyl acetate extract which showed a decrease in the activity of glucose-6-phosphatase when compared to diabetic control rats and thereby decreased gluconeogenesis.

(b) **Fructose 1, 6-diphosphatase**

The results of the activity of fructose 1, 6-diphosphatase in control and experimental rats are depicted in Figure 25 b.The activity of fructose 1,6-diphosphatase was significantly elevated ($p<0.05$) in STZ-NIC induced diabetic rats when compared to control group. Administration of plant extracts and glibenclamide resulted in significant reduction ($p<0.05$) in the activity of the enzyme.



Values are mean± SEM (n= 6)

Fructose 1, 6-diphosphatase units: µ moles of Pi liberated/min/mg protein

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 25 b

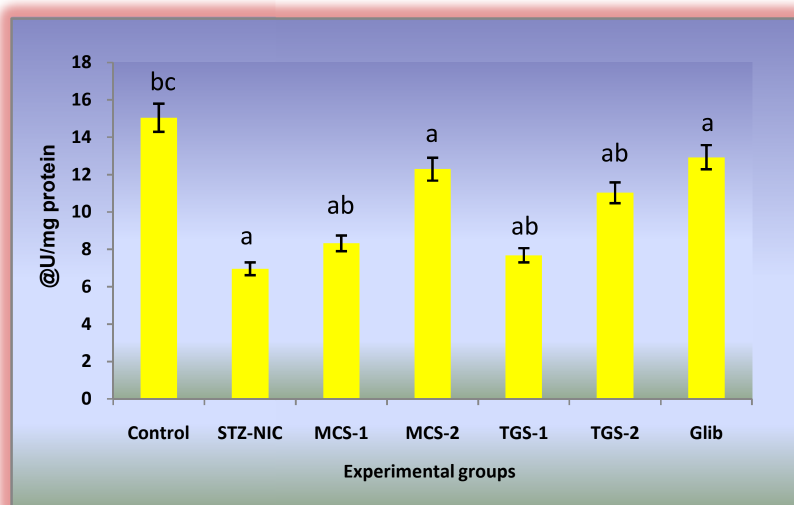
Activity of fructose 1, 6-di phosphatase in the experimental rats

Fructose1, 6 diphosphatase is one of the key enzymes of gluconeogenic pathway. Hepatic glucose production is raised in diabetic state and is associated with the impaired suppression of the gluconeogenic enzyme fructose 1, 6 diphosphatase (Pari and Murugan, 2005). It catalyzes the rate limiting step of fructose 1, 6-diphosphate to fructose-6-phosphate (Baquer *et al*., 1998). Hepatic glucose production is raised in diabetic state and is associated with the impaired suppression of the gluconeogenic enzyme fructose1,6 diphosphatase (Minnassian and Mitheux, 1994). Devaki *et al*. (2016) had reported that the increased activities of these gluconeogenic enzymes in diabetic rats were decreased to near-normal levels after the administration of *Erythrina variegata* L. bark extracts.

Diabetic rats administered with *Chloroxylon swietenia* bark extracts and glibenclamide modified the levels of glucose -6-phosphatase and fructose 1, 6 diphosphatase to near normal, which may be due to higher insulin secretion (Jayaprasad *et al*., 2016). *Euryale ferox salisb* seeds extract administration significantly decreased the activity of gluconeogenic enzymes in diabetic rats (Ahmed *et al*., 2015).

(c) Glucose -6-phosphate dehydrogenase

The results of the activity of glucose -6-phosphate dehydrogenase (G6PDH) in the normal and treated rats are depicted in Figure 25 c.



Values are mean± SEM (n= 6)

@ Glucose -6-phosphate dehydrogenase units: µ moles of NADH oxidized/min/mg protein

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Figure 25 c

Activity of glucose -6-phosphate dehydrogenase in the experimental rats

The activity of glucose -6-phosphate dehydrogenase was found to be significantly decreased ($p<0.05$) in the STZ-NIC induced diabetic rats when compared to normal control rats. Oral administration of MCS and TGS significantly increased the activity of this hepatic enzyme which was similar to glibenclamide treated rats. The increase in the activity was found to be significantly higher ($p<0.05$) in rats treated with MCS 400mg/kg b.w than treated with TGS.

Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway. It is required for the antioxidant defense because it produces NADPH, the main cellular reductant and the fuel for glutathione recycling within the cells (Frederiks *et al*., 2003). Reduced activity of this enzyme in diabetic rats shows that glucose does not enter into pentose phosphate pathway to a greater extent. G6PDH activity and NADPH/NADP ratio vary inversely in relation to blood glucose concentration. Studies of Díaz-Flores *et al*. (2006) indicated that decrease in hepatic G6PDH activity is dependent on the severity of hyperglycemia. Lowered hepatic G6PDH activity was observed in STZ-diabetic rats.

Gonçalves *et al.* (2015) had reported that green tea normalized the activity of glucose 6-phosphate dehydrogenase, a key enzyme of an important metabolic route pentose monophosphate pathway. Bhat *et al.* (2011) had reported that *Bougainvillea spectabilis* extracts showed significant increase in glucose-6-phosphate dehydrogenase activity and hepatic, skeletal muscle glycogen content.

Ugochukwu and Babady (2002) had reported that there was a decrease in the activity of glucose-6- phosphate dehydrogenase in diabetic rats and on treatment with ethyl acetate extracts of plants the activity of the enzyme was increased. This might be due to increased secretion of insulin which increases the influxes of glucose into pentose monophosphate shunt in an attempt to reduce high blood glucose levels.

4.2.4 Hematological parameters

Several hematological changes affecting the red blood cells, white blood cells and the coagulation factors are shown to be directly associated with Diabetes Mellitus (Mbata *et al* ., 2015). The effect of *Momordica charantia* and *Trigonella foenum graecum* seed extracts in control and STZ-NIC administered Diabetes rats on haematological parameters namely red blood corpuscles (RBC), platelets and indices in experimental rats are shown in Table 18.

Haematological evaluations revealed significant alterations in the levels of RBCs, platelets, haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) in STZ-NIC induced diabetic rats when compared to control rats. However upon treatment with glibenclamide, MCS and TGS it was found that there was significant increase ($p<0.05$) in all these parameters.

Table 18

RBCs, platelets and hematological indices in the experimental rats

Groups	RBCs $X10^6$ µL	Platelets $X\ 10^5\ mm^3$	Hb g/ dL	PCV %	MCV (fL)	MCH (pg)	MCHC %
Control	6.3 ± 0.92^{b}	841 ± 8.67^{bc}	15.7 ±0.60 ^{bc}	46 ± 2.11^{b}	28 ±1.13^{bc}	82 ± 1.16b	33 ± 0.58b
STZ - NIC	4.4 ± 0.79^{a}	566 ± 5.75^{ac}	8.6 ±1.08 ^{ac}	27 ± 1.34^{ac}	20 ±0.79^{ac}	64 ± 2.70^{ac}	27 ± 1.02^{ac}
MCS-1	5.1 ± 0.78	749 ± 6.19^{ab}	12.7±0.63 ^{ab}	38±1.05^{abc}	21±1.24^{abc}	71 ± 1.00^{abc}	31 ± 0.85^{ab}
MCS-2	5.5 ± 0.37	780±7.31^{ab}	14.9± 0.60 ^{b}	43± 1.01^{b}	25±0.46^{abc}	74 ± 0.83^{ab}	32 ± 0.76^{b}
TGS-1	4.9 ± 1.13	726 ±7.42^{bc}	12.4± 0.72^{abc}	33 1.15^{abc}	22 ±0.43^{ac}	68 ± 0.92^{abc}	29 ± 0.77^{ab}
TGS-2	5.1 ± 0.32	765 ± 7.70^{ab}	14.1± 0.56 ^{ab}	41 ± 0.75^{bc}	24±0.56^{abc}	73 ± 1.32^{ab}	31 ± 1.00^{b}
Glib	5.7 ± 0.33	772.±12.27^{ab}	14.1±0.66 ^{ab}	43 ± 0.86^{b}	26 ± 0.88^{ab}	77 ± 1.29^{b}	30 ± 1.60^{b}

Values are mean ± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

Regular monitoring of variable hematological parameters is important to be considered since it may be a predictor of some diabetic complications (Nahla, 2016). The assessment of haematological parameters could be used to reveal the deleterious effect of foreign compounds including plant extracts on the blood constituents of animals. They are also used to determine possible alterations in the levels of biomolecules such as enzymes, metabolic products, haematology, normal functioning and histomorphology of the organs (Magalhaes *et al*., 2008). Hematological indices are indicators and reflection of the effects of dietary treatments on animals in terms of the quality of feed ingested and nutrients available to the animal to meet their physiological requirements (Parameswari *et al.,* 2016).

Akindele *et al.* (2012) and Saliu *et al.* (2012) had reported that in Diabetes Mellitus, there is the development of anemia, particularly, the hypochromic type, due to fall in the iron content of the body resulting from oxidation stress associated with the condition. Ohlsson and Aher, (2012) had indicated that flavonoids might stimulate the formation or secretion of erythropoietin, which in turn stimulates stem cells in the bone marrow to produce red blood cells. Abu-Zaition, (2010) had reported that the stimulation of erythropoietin hormone enhanced rapid synthesis of RBC supported by the improved level of MCH and mean corpuscular hemoglobin concentration.

Plant extracts increased the values of haematological parameters in the experimental rats after 21 days of treatment. The increase in RBC count in glibenclamide, MCS and TGS treated rats might be due to the lowered lipid peroxide level in RBC membrane leading to a decreased susceptibility of RBC to hemolysis. Therefore, from the present study results, it may be suggested that the plant extracts and glibenclamide might have stimulated the synthesis (erythropoiesis) of RBCs in anemic diabetic rats.

In the present study, platelet count seemed to be significantly decreased in diabetic rats when compared to normal and treated rats. This reduction in diabetic rats indicates suppression of haemopoesis as a result of STZ application and hyperglycemia. But it is shown that there was significant increase in glibenclamide, MCS and TGS treated groups. Results of the present study are supported by Hala *et al.* (2011) who had reported that cinnamon prevented platelet aggregation during hyperlipidemia and hyperglycemia.

Odoh *et al.* (2016) had reported that administration of *Ceiba pentandra* extracts appreciably improved the levels of RBC and its differential counts especially at 400 and 800 mg/kg doses of the extract. This gives an indication that the plant extract may contain

some phytochemicals that can stimulate the formation or secretion of erythropoietin in the stem cells of the animals.

The values of hemoglobin (Hb), an iron-containing conjugated protein that performs the physiological function of transporting oxygen and carbon dioxide, showed significant changes in diabetic groups compared to the control group and diabetic group treated with glibenclamide and plant extracts. This might suggest that the oxygen-carrying capacity of the blood of the animals might be affected in diabetic groups. Low Hb concentration is strongly associated with diabetic profiles (Kwon and Ahn, 2012) as it contributes to the development of cardiovascular disease in patients with Diabetes (Stevens, 2012).

The values of packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were found to be improved significantly on administration of *Momordica charantia* and *Trigonella foenum graecum* seed extracts. The present findings are similar to observations of Parameshwari *et al*. (2016) who had reported that oral administration of zingerone, improved the Diabetes-induced disturbances of hematological parameters namely RBC's, MCV, MCH and MCHC in STZ-induced diabetic rats and also suggested that daily treatment with Zingerone markedly improved hematological status of rats with STZ-induced Diabetes.

The results of white blood cell (WBC) count and their differential count are depicted in Table 19. WBC s showed a significant increase ($p<0.05$) in diabetic group when compared to the control group. There was no significant difference in the percentage of monocytes, eosinophils and polymorphs. However there was a significant decrease ($p<0.05$) in lymphocyte percentage of diabetic rats when compared to normal control and treated groups.

In the present study, the total WBCs count in diabetic group showed a significant increase. This was similar to the reports of Svenyl *et al.* (1990) who had observed that increase in total WBCs count might be due to the increased hemopoitic activity as a result of hemolysis of RBCs in diabetic rats. In the diabetic group, lymphocytes were decreased in number which might be due to stressful condition after antigen injection. ElFeki *et a*l. (1997) had suggested that it might be due to the production of specific or non specific antibodies against different antigens, since lymphocytes are responsible for achieving the defense mechanism in the body.

Table 19

White blood corpuscles and differential count in the experimental rats

Groups	WBCs X 10^3 µL	Lymphocytes%	Monocytes %	Eosinophils %	Polymorphs %
Control	8.7±0.59 ^{b}	92 ±1.87^{b}	3.2 ± 0.75	3.2±0.98	4.3 ±1.37
STZ - NIC	15.1±0.77^{ac}	64 ±3.60^{ac}	2.2± 0.41	2.3±0.52	6.2 ±1.47
MCS-1	12.0±0.58^{abc}	83 ±3.54^{ab}	2.2±0.41	2.5±0.55	4.8 ±1.17
MCS-2	10.7±0.95^{ab}	84 ±3.13^{ab}	2.8± 0.41	2.7±0.52	4.5 ±0.55
TGS-1	12.3±0.92^{abc}	78 ±2.26^{abc}	2.5±0.55	2.3 ±.82	5.5 ±0.55
TGS-2	11.2±0.82^{ab}	82 ±2.58^{ab}	2.7±0.52	2.5 ± 0.55	4.7 ± 0.52
Glib	9.5±0.71^{b}	86 ±2.34^{b}	2.5± 0.55	2.8±0.75	4.5 ± 0.84

Values are mean ± SEM (n= 6)

a-p <0.05 compared with control group

b-p <0.05 compared with STZ –NIC group

c-p <0.05 compared with Glib (200µg/kg b.w) treated group

(One way ANOVA followed by Dunnett's multiple Comparison test)

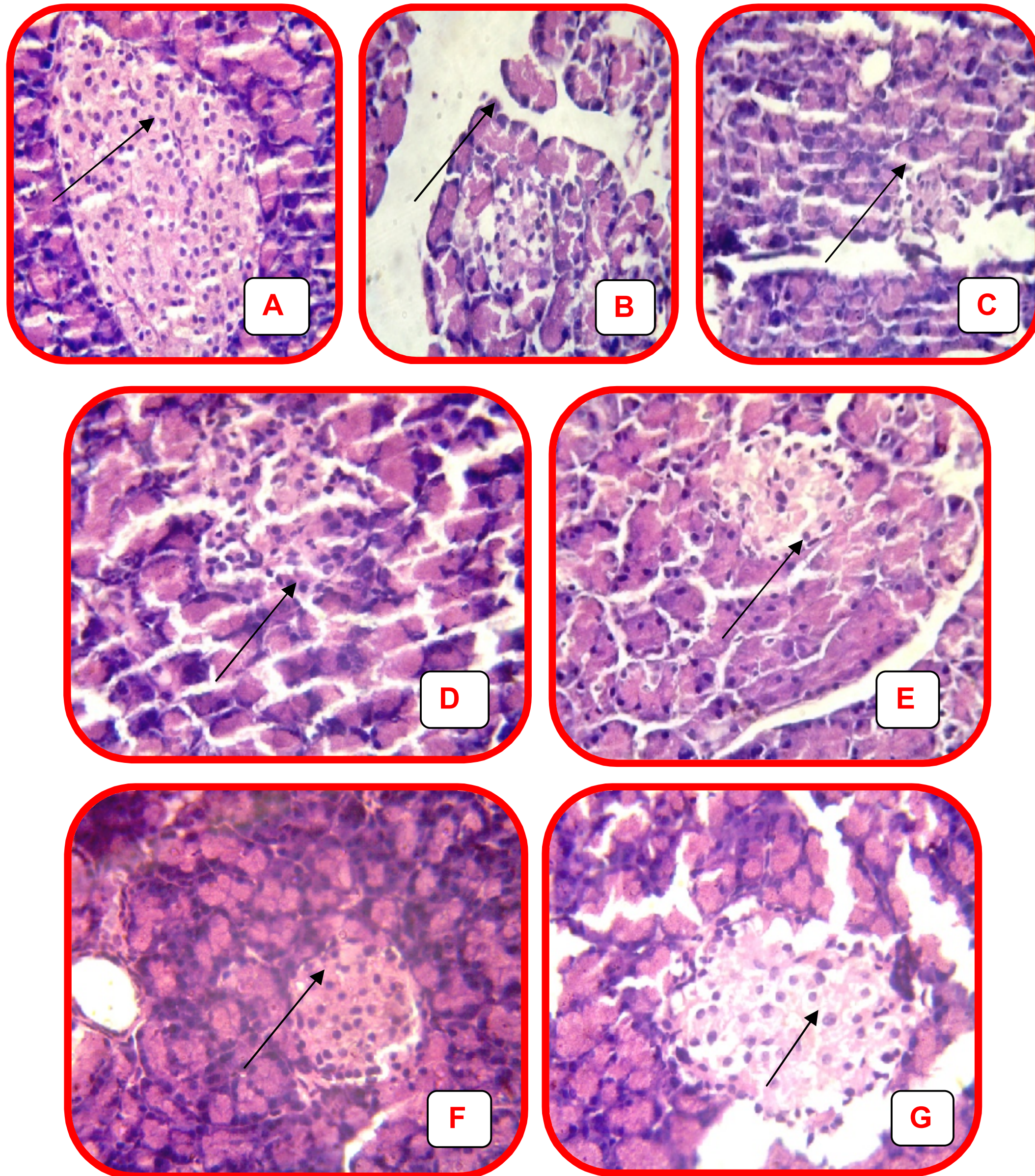
MCS -1: 200mg/kg b.w, MCS-2:400mg/kg b.w, TGS -1: 200mg/kg b.w, TGS -2:400mg/kg b.w.

The results of the present study are similar to the observations of Akomas *et al*. (2014) who had recorded significant increase in WBC count in diabetic rats which lowered after treatment with *Ficus sur* leaves extract. The profile of WBC count reflects the balance between the rate of granulocyte production and that of WBC. Peripheral WBC count has been shown to be associated with insulin resistance in Type 2 Diabetes (Ohshita *et al.,* 2004). Kozlov *et al.* (1995) had reported that Diabetes in mice was accompanied by moderate neutrophilic leukocytosis and prolonged circulation times of neutrophils, monocytes and a shortened circulation time of lymphocytes, which increases the susceptibility to infection. The raised leukocyte count may also reflect low-grade inflammation. High white blood cell count, a marker of inflammation, predicts a worsening of insulin action, insulin secretory function and the development of Type 2 Diabetes as suggested by Vozarova *et al*. (2002).

Lymphocytes are the main effector cells of the immune system. An increase in the lymphocyte count in rats on vegetable diet may be an indication of immunostimulation as opined by Saliu *et al*. (2012). Sakuljaitrong *et al.* (2012) had reported that lymphocytes from diabetic controls were significantly less than those from normal controls.

4.2.5 Histopathological interpretations of sections of the pancreas

The histopathological sections of pancreas of normal, Diabetes induced and treated rats are presented in Plate 6. Histological examination of normal control rats exhibited normal acini with normal cellular population in the islets in slide (A). Many rounded normal proportions of islet of Langerhans were found all around the pancreatic acini. Prominent nuclei with well arranged lobules with surrounding islet cells were found among normal control rats. In diabetic control rats extensive damage to the islet of Langerhans and reduced dimensions of islets were visible which demonstrated cellular damage to the pancreatic acini and islets, showing pancreatic β-cell damage and degeneration with asymmetrical vacuoles in slide (B). Treatment with glibenclamide showed increased number of β cells and size of islets in diabetic rats in slide (G). MCS slides (C, D) and TGS slides (E, F) treated STZ induced-diabetic rats showed marked improvement of the cellular injury, as evident from the partial restoration of islet cells, reduced β-cell damage, more symmetrical vacuoles and an increase in number of islet cells.



A. Control rat showing normal pancreatic acini with islets in normal number and size.
B. Diabetic control rat showing necrosis, cytoplasmic vacuolation of pancreatic acini and reduced number of islets and size.
C. Diabetic rat treated with MCS (200 mg/kg) showing normal pancreatic acini and reduced number of islets and cytoplasmic vacuolations.
D. Diabetic rat treated with MCS (400 mg/kg) showing normal pancreatic acini, islets and normal cytoplasmic vacuolations.
E. Diabetic rat treated with TGS (200 mg/kg) showing normal pancreatic acini with few islets decreased in size.
F. Diabetic rat treated with TGS (400 mg/kg) showing normal pancreatic acini improved number of islets and normal cytoplasmic vacuolations.
G. Diabetic rat treated with glibenclamide shows normal pancreas with regenerated islets with few cytoplasmic vacuolations.

Plate 4

Histopathological sections of the pancreas of experimental and control rats

Streptozotocin (STZ) is an antibiotic produced by *Streptomyces achromogenes*. It has been widely used for inducing experimental Diabetes Mellitus in a variety of animals; it stimulates the naturally occurring metabolic disorder Diabetes Mellitus by causing degeneration of pancreatic β cells (Merzouk *et al.,* 2000 and Coskunm *et al.,* 2005). The selective β cell toxicity of STZ is related to the glucose moiety in its chemical structure, which enables STZ to enter the cell via the low affinity glucose transporter Glut2 in the plasma membrane (Elsner *et al*., 2000).

Gandhi and Sasikumar (2012) had reported that the diabetic rats treated with *Merremia emarginata* resulted in normalizing the pancreatic histoarchitecture quite appreciably. An increase in the number of beta cells in the islets showed that they were regenerated. Also, the increase in secretory granules in the cells indicates that the cells were stimulated for insulin synthesis. Ayesha *et al.* (2008) also reported that pancreas and liver sections of diabetic rats fed with *Aloe vera* were normal when compared to diabetic control rats.

Shirwaikar *et al*. (2006) reported on histopathology studies in which STZ was suspected of partially destroying the pancreas. Diabetic rats showed reduced islet cells, which were restored to near normal upon treatment with the extract. Similarly, Yin *et al*. (2006) observed that high-dose STZ in Diabetes indicates β cell destruction. After 120 days of normoglycemia, a statistically significant 3.7- fold increase in β cell mass was observed. This study supports the possibility of regeneration as a means for generating new β cells, even in severely diabetic rats or individuals with significantly reduced β cell mass.

As other possible mechanisms, the extracts from MCS and TGS might sensitize the insulin receptor to insulin, or stimulate the stem cells of the islets of Langerhans in the pancreas of STZ-induced diabetic rats.

The recovery of damaged islets and an improvement in the number of β cells after treatment with the plant extracts were therefore demonstrated by the histopathological observations of the pancreas in the rats. It can thus be assumed that *Momordica charantia* and *Trigonella foenum graecum* seed extracts has a therapeutic effect that alleviates Diabetes Mellitus.

Phase III

4.3 Bioactive Compounds with Alpha- amylase Inhibitory Properties from *Momordica charantia* and *Trigonella foenum graecum* Seed Extracts

Results of *in vitro* (Phase I) and *in vivo* studies (Phase II) on antidiabetic and antioxidant potentials have shown that ethyl acetate extracts of *Momordica charantia* seeds (MCS) and *Trigonella foenum graecum* seeds (TGS) were more potent than *Momordica charantia* flesh and *Trigonella foenum graecum* leaves. Hence in Phase III, further studies were carried out in order to find the active compounds present in ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum* seeds responsible for antidiabetic activities. Phytochemical analysis, high performance thin layer chromatography (HPTLC) analysis, thin layer chromatography (TLC) separation of components, fourier transform infra –red (FT-IR) spectroscopic analysis and gas chromatography mass spectroscopy (GCMS) analysis of potent TLC fractions were performed to identify the bioactive compounds which might be responsible for antidiabetic activity.

4.3.1 Phytochemicals present in the seeds of *Momordica charantia* and *Trigonella foenum graecum*

Qualitative analysis of phytochemicals namely flavonoids, phenols, tannins, terpenoids, steroids, saponins, alkaloids and glycosides in ethyl acetate extracts of seeds of *Momordica charantia* (MCS) and *Trigonella foenum graecum* (TGS) were performed and the results are presented in Table 20.

Table 20

Phytochemicals in the seeds of *Momordica charantia* and *Trigonella foenum-graecum*

Phytochemicals	*Momordica charantia*	*Trigonella foenum graecum*
Flavonoids	+	+
Phenols	+	+
Tannins	+	+
Terpenoids	+	+
Steroids	+	+
Saponins	+	+
Alkaloids	-	-
Glycosides	-	-

+ Presence - Absence

The bioactive compounds in the plant extract vary considerably with the solvent extraction. The present study results reveal the presence of phytocompounds namely flavonoids, phenols, tannins, terpenoids, steroids and saponins in both the plant extracts. Alkaloids and glycosides were absent in ethylacetate extracts of both the seed extracts.

Phytochemicals are bio- active chemicals of plant origin. They are regarded as secondary metabolites because the plant that manufactures them may have little need for them. They are naturally synthesized in all parts of the plant body; bark, leaves stem, root, flower, fruits and seeds i.e. any part of the plant body may contain active components (Tiwari *et al.,* 2011). Phytochemical screening is a simple, quick and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and is an important tool in bioactive compound analysis (Sasidharan *et al.,* 2011). The plant screened for phytochemical compounds seemed to have the potential to act as a source of useful drugs (Mir *et al*., 2013).

Ghosh *et al.* (2014) in their phytochemical screening showed the presence of alkaloids, carbohydrates, proteins, saponin glycosides triterpenoids and flavonoids in *Momordica charantia* aqueous extracts. Kannan *et al*. (2011) had reported the presence of alkaloids, carbohydrates, flavonoids, fatty acids, saponins, steroids and terpenoids in ethanol and ethyl acetate extracts of *Momordica charantia.* Solvent plays a major role in the extraction of plant constituents. Ethanol is a highly polar solvent which showed the presence of more flavonoid content when compared to hexane and ethyl acetate (Thiyagarajan *et al.,* 2016). The present study findings are supported by the phytochemical tests conducted on ethyl acetate extracts of *Trigonella foenum-graecum* seeds by Seasotiya *et al.* (2014) and had reported the presence of various phytocompounds namely alkaloids, saponins, flavonoids, phenols and tannins.

4.3.1.1 Quantification of the selected phytochemicals

In the present study, total flavonoids, phenols, tannins and terpenoid contents of *Momordica charantia* and *Trigonella foenum-graecum* extracts were estimated and the results are given in Table 21. Total flavonoid contents of MCS and TGS were estimated and found to be 0.99 ± 0.36 and 0.65 ± 0.68 mg rutin equivalents. Grape seed and tea extracts are easily available sources of flavonoids, many of which have shown efficacy in controlling the symptoms of Diabetes (Yilmazer-Musa *et al.,* 2012). Polyphenols and flavonoids are natural antidiabetic agents, which interfere in the production of free radicals, reduce oxidative stress and inhibit digestive enzyme, thus lowering postprandial

glucose. Studies have shown that some flavonoids have more antioxidant activity than vitamins C and E (Anil, 2016).

Table 21

Levels of selected phytochemicals in seeds of *Momordica charantia* and *Trigonella foenum-graecum*

Plant sample	Total flavonoid content (mg RE /g)	Total phenol content (mg TAE/g)	Total tannin content (mg TAE/ g)	Total terpenoid content (mg / g)
MCS	0.99 ± 0.36	60 ± 0.4	50 ± 0.72	38 ± 0.46
TGS	0.65 ± 0.68	86 ± 0.64	80 ± 0.21	31± 0.18

Values are ± SD of triplicates

RE- Rutin equivalent TAE-Tannic acid equivalent

MCS - *Momordica charantia* seeds TGS- *Trigonella foenum graecum* seeds

The total phenol content of MCS and TGS were found to be 60 ± 0.4 and 86 ± 0.64 mg tannic acid equivalents. The seed extracts of *Trigonella foenum-graecum* had higher phenol content when compared to *Momordica charantia* seed extracts.

Several phenolic compounds have the potential to serve as a remedy against hyperglycemia-induced chronic diseases. The inhibition of α-amylase and α-glucosidase is mediated by different phenolics found in varieties of raspberry. Maltase inhibitory activities of chebulagic acid and chebulinic acid from fruits of *Terminalia chebula* are comparable to that of acarbose (Asgar, 2013). High polyphenolic content of *Heracleum persicum* and *Ziziphus jujuba* implied the potent capacity of the plants in the clearing of oxidants as reported by Afrisham *et al.* (2015).

Total tannin contents of MCS and TGS were estimated and found to be 50 ± 0.72 and 80 ± 0.21 mg tannic acid equivalents. Tannin contents of seed extracts were found to be higher. This may be responsible for alpha-amylase inhibitory and antioxidant activities. Tannins are considered as a food product in plant vegetable. They decrease the bacterial proliferation by blocking key enzymes of microbial metabolism as reported by Geidam *et al.* (2007) and Sharma *et al.* (2013).

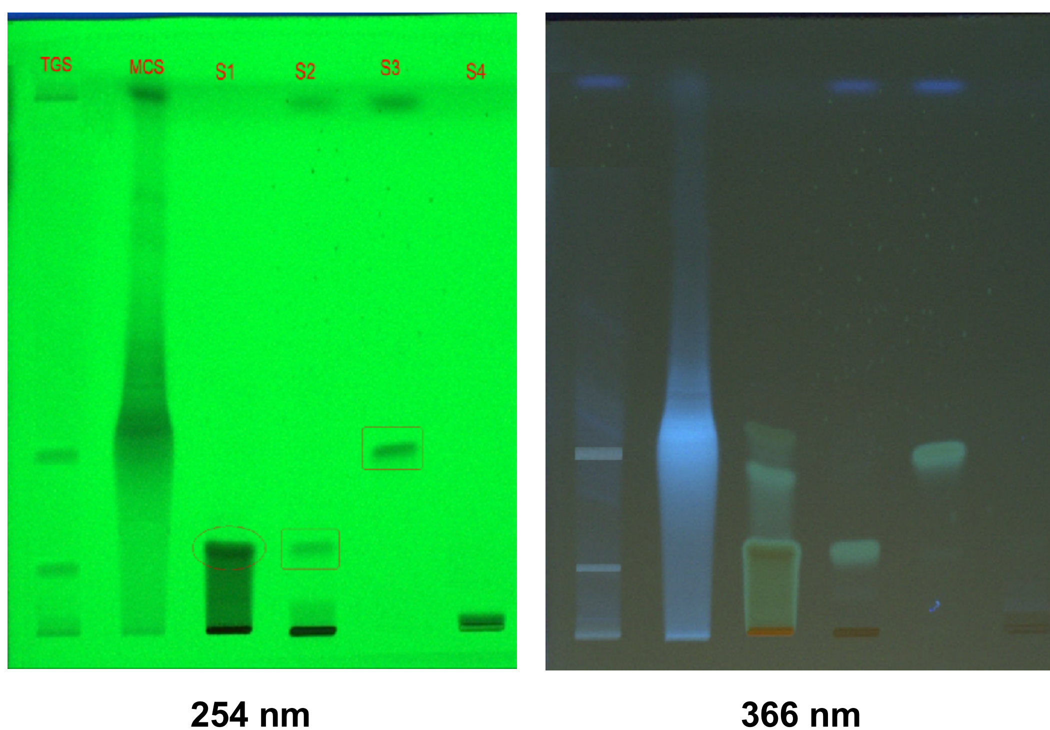
Total terpenoid contents of MCS and TGS were estimated and found to be 38 ± 0.46 and 31 ± 0.18 mg/g extract. Terpenoid contents were found to be higher in MCS when compared to TGS extracts. Pancreatic α-amylase inhibitors offer an effective strategy to

lower the levels of post-prandial hyperglycemia via control of starch breakdown. Regarding the enzyme inhibition phenomenon, some constituents like tannins, alkaloids, terpenoids and flavonoids could be responsible for inhibiting the effective alpha amylase (Hakkim *et al*., 2007; Myung- Hee *et al*., 2010).

4.3.2 High Performance Thin Layer chromatography (HPTLC) of the ethyl acetate extracts of the seeds of *Momordica charantia* and *Trigonella foenum graecum*

4.3.2.1 HPTLC profile of flavonoids

The flavonoids present in the ethyl acetate extracts of *Momordica charantia and Trigonella foenum graecum* seeds were analysed using quercetin, rutin, kaempferol and catechin as reference standards. Fluorescence bands in the UV region at 366nm showed the presence of flavonoids. Fluorescence bands were observed in the standard tracks corresponding to quercetin, rutin and kaempferol. Similar type of bands was observed in MCS and TGS tracks (Plate 5).

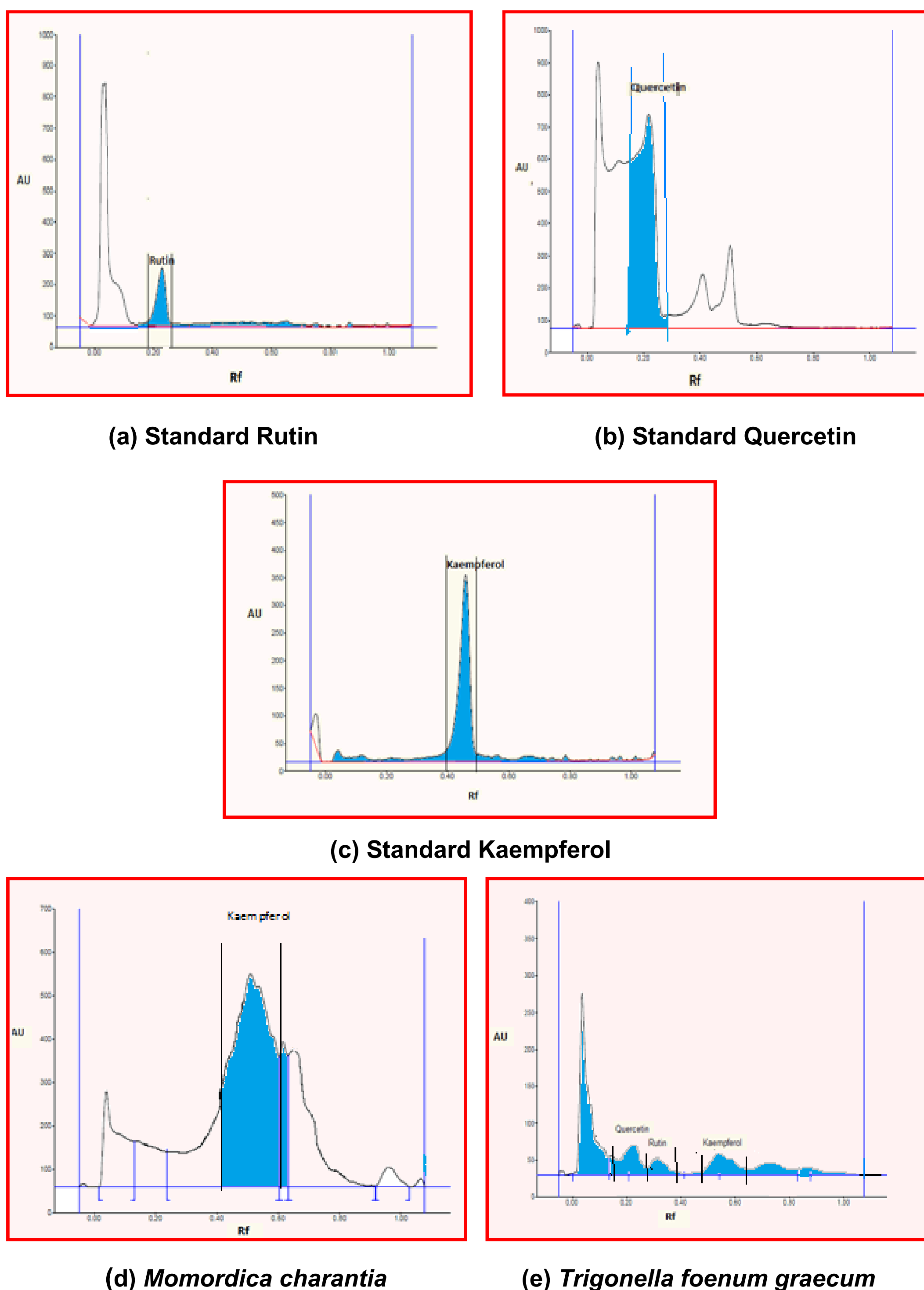


TGS: ***Trigonella foenum graecum*** **seed; MCS:** ***Momordica charantia*** **seed**

S 1: Quercetin; S 2: Rutin; S 3: Kaempferol; S 4: Catechin

Plate 5

HPTLC profile of the flavonoid fractions of ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum - graecum*



(a) Standard Rutin

(b) Standard Quercetin

(c) Standard Kaempferol

(d) *Momordica charantia*

(e) *Trigonella foenum graecum*

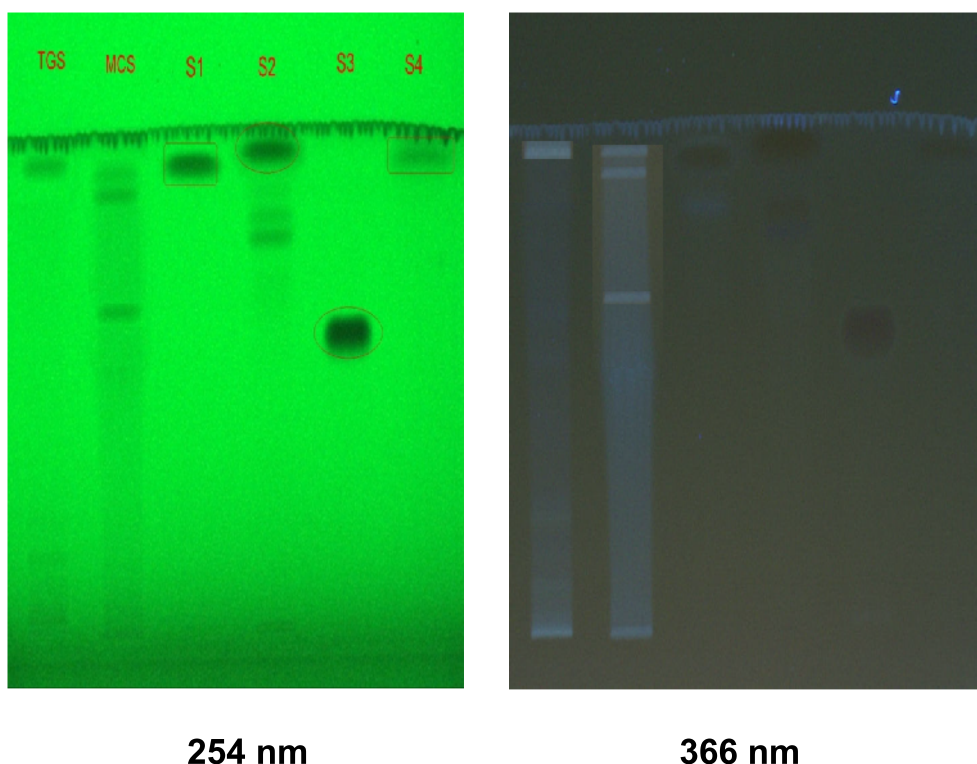
Figure 26

HPTLC peak densitogram of the flavonoid standards rutin, quercetin, kaempferol and ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum- graecum*

Bands corresponding to catechin standard were not observed in the tracks of MCS and TGS. The Rf value of the peaks observed in MCS (0.51), TGS (0.16 and 0.52) was found to be closer to the Rf values of standards quercetin (0.22), rutin (0.24) and kaempferol (0.46). This might reveal the presence of flavonoids and its derivatives in the ethyl acetate extracts of *Momordica charantia and Trigonella foenum graecum* seeds. The peak densitogram of flavonoid standards, *Momordica charantia and Trigonella foenum* are shown in Figures 26 a, b, c, d and e.

4.3.2.2 HPTLC profile of phenols

The phenols present in the ethyl acetate extracts of *Momordica charantia and Trigonella foenum graecum* seeds were analysed using resorcinol, catechol, gallic acid and hydroquinone as reference standards and the results are shown in Plate 6.



254 nm **366 nm**

TGS: *Trigonella foenum graecum* seed; MCS: *Momordica charantia* seed
S 1: Resorcinol; S 2: Catechol; S 3: Gallic Acid; S 4: Hydroquinone

Plate 6

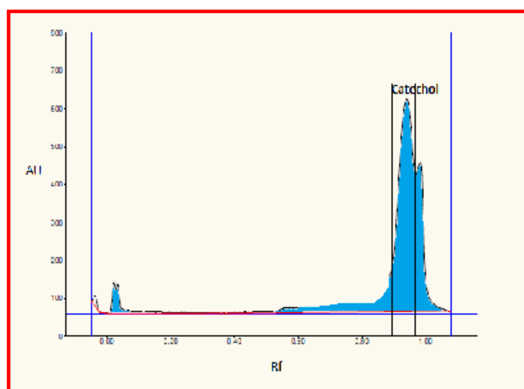
HPTLC profile of the phenol fractions of ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum -graecum*

Phenols were identified as blue grey coloured spots.This might be due to the presence of phenolic compounds in *Momordica charantia and Trigonella foenum graecum.* Bands were observed in the tracks of MCS, TGS, hydroquinone and catechol. The Rf values of the peaks observed in MCS (0.63, 0.82 and 0.95) and for TGS (0.96) are closer to Rf value of standard hydroquinone (0.97) Rf value for cathechol (0.94) and Rf value for gallic acid (0.52) No bands were observed corresponding to resorcinol standards in MCS and TGS tracks. Peak densitogram of phenolic standards and plant extracts are shown in Figures 27 a, b, c, d and e.

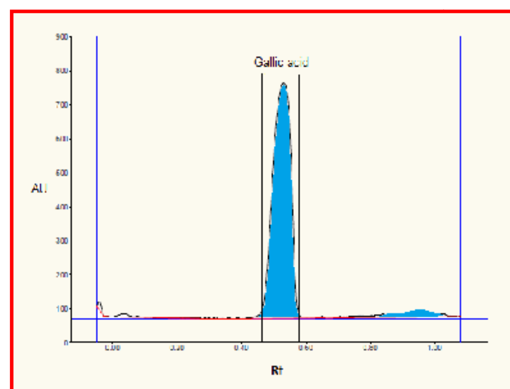
High performance thin layer chromatography is one of the chromatographic techniques used to identify active principles present in plant extracts. The present study results could be supported by HPTLC analysis of Mythili and Sathiavelu (2012) who had reported that methanol extract of *Cassytha filiformis* showed the presence of various phytochemicals such as phenols and flavonoids. The methanolic extract of *Cassytha filiformis* displayed the presence of 13 types of phenolic substances with 13 different Rf values ranging from 0.01 to 0.96. Phenols and flavonoids are strong antioxidants and are associated with many useful biological effects as reported by Kim *et al.* (2012).

Joshi *et al.* (2012) had reported the presence of quercetin and kaempferol in leaves of *Centella asiatica* by HPTLC method. Nickavar and Gholamreza (2011) had reported that antidiabetic effects observed from *Vaccinium arctostaphylos* leaves might be due to the inhibition of *α*-amylase by the flavonoid quercetin. (Yadao *et al*. (2015) had identified five types of polyphenols and hydroquinone from methanol extracts of *Cassia auriculata* flowers by HPTLC analysis and suggested that these compounds might be responsible for antidiabetic and antioxidant properties.

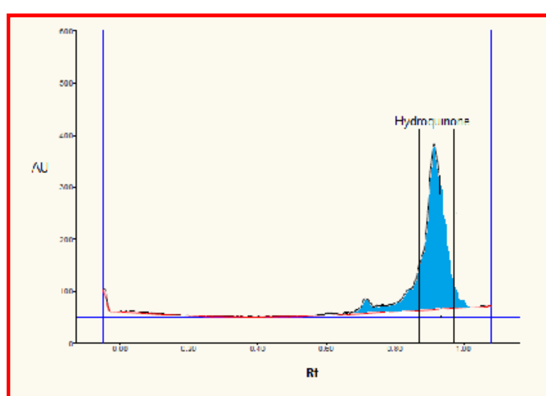
HPTLC analysis of the ethyl acetate extracts of seeds of *Momordica charantia and Trigonella foenum graecum* showed the presence of phenols and flavonoids and these compounds might be responsible for antidiabetic and anti oxidant properties.



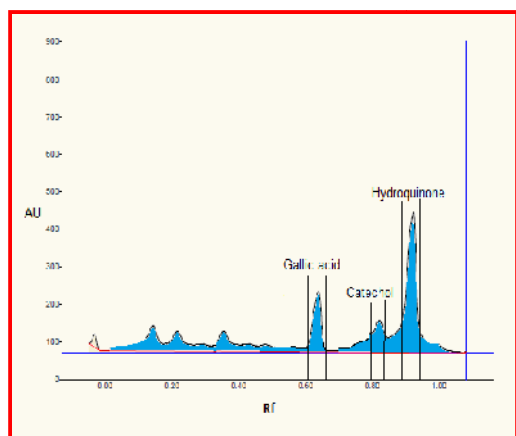
(a) Standard Catechol



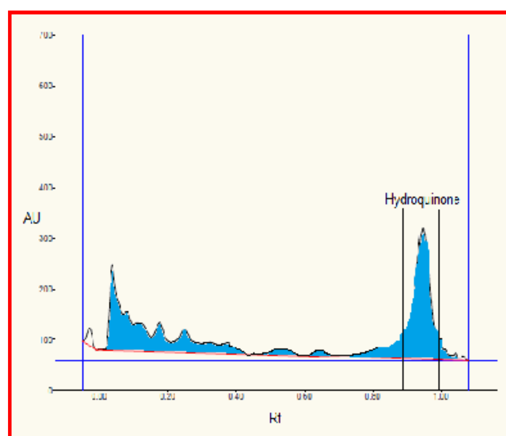
(b) Standard Gallic acid



(c) Standard Hydroquinone



(d) *Momordica charantia*



(e) *Trigonella foenum graecum*

Figure 27

HPTLC peak densitogram of the phenol standards catechol, gallic acid, hydroquinone and ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum -graecum*

4.3.3 Separation of active compounds by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) patterns of ethyl acetate extracts of seeds of *Momordica charantia and Trigonella foenum graecum* are shown in plates 7 and 8.

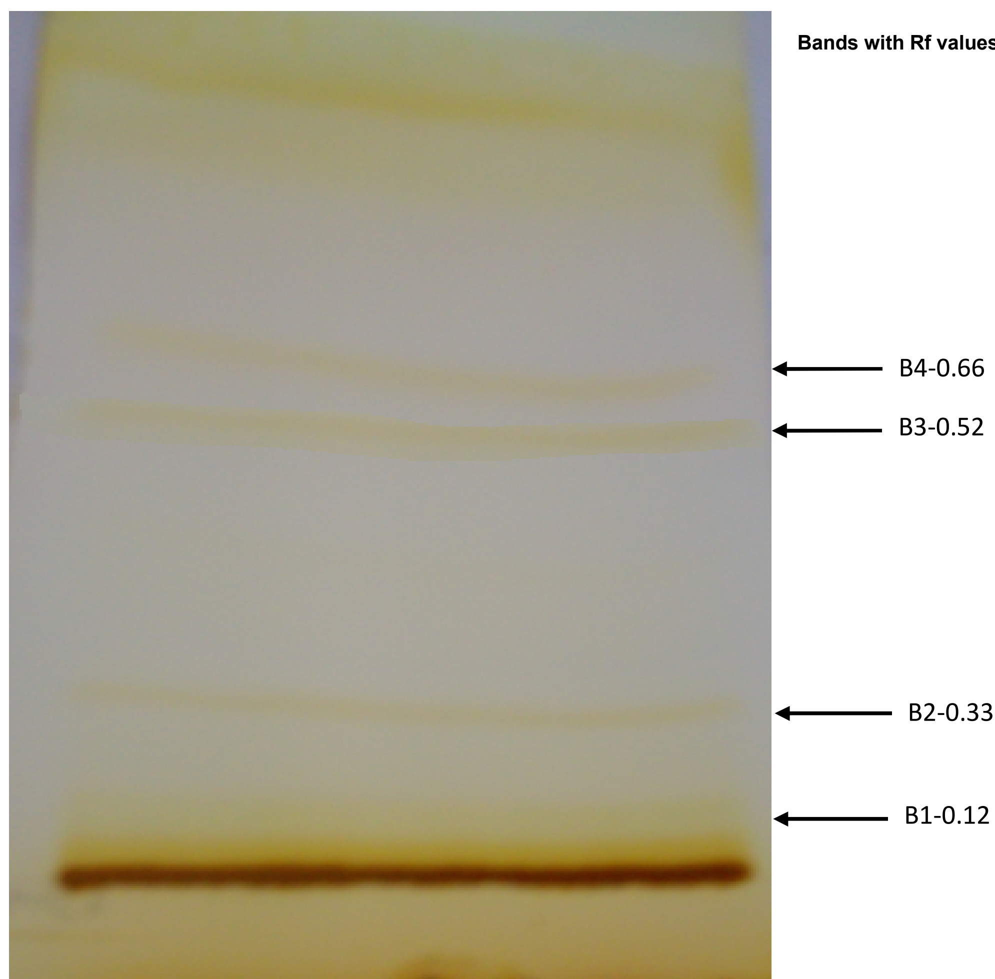


Plate 7

TLC profile of ethyl acetate extracts of *Momordica charantia* seeds

TLC profile of *Momordica charantia* showed four visible bands with Rf values 0.12, 0.33, 0.52 and 0.66 respectively. In TLC profile of *Trigonella foenum graecum* five bands with Rf values 0.21, 0.42, 0.55, 0.66 and 0.81 were observed. Preparative thin layer chromatography was performed and the bands obtained after development were scrapped out and eluted with methanol. The eluted fractions were tested for alpha-amylase inhibitor activity.

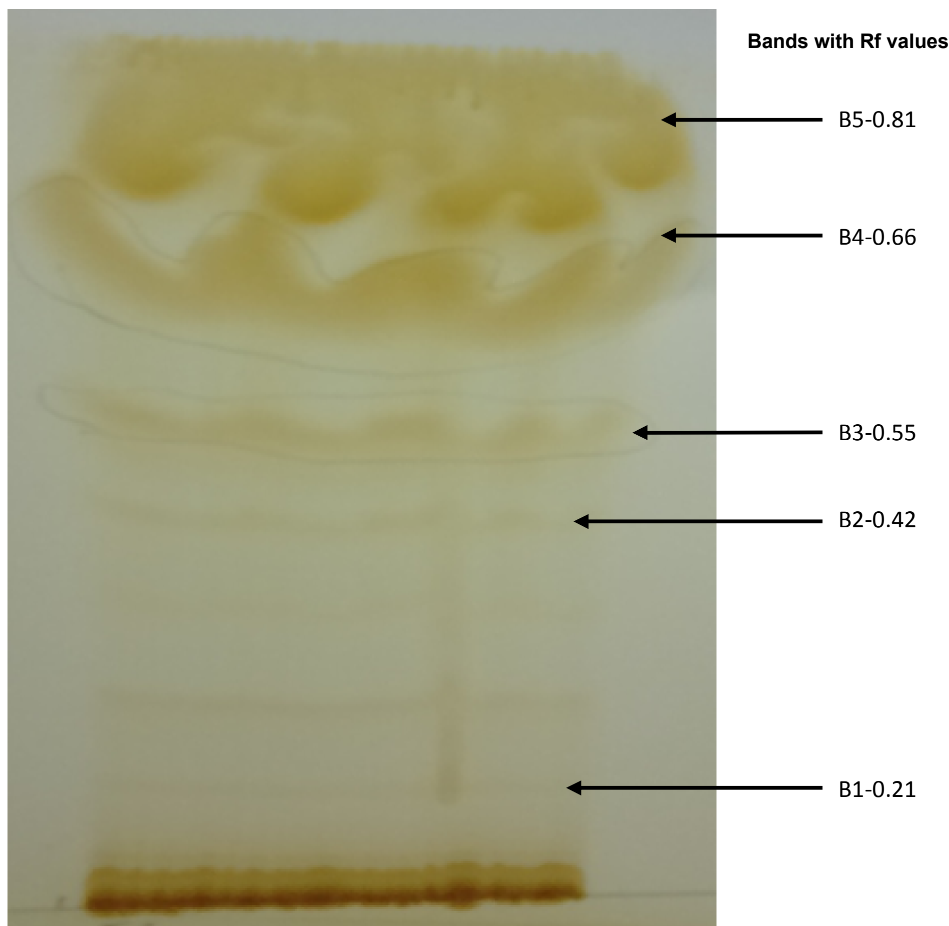


Plate 8

TLC profile of ethyl acetate extracts of *Trigonella foenum -graecum* seeds

TLC analysis provides an idea about the polarity of various chemical constituents in such a way that compounds showing high Rf value in less polar system have low polarity and vice versa. Analysis of potential bioactive compounds of leaves of *Gymnema Sylvestre* with methanol: Chloroform solvent revealed 6 fractions (Subashini *et al.,* 2015).

Thin layer chromatography provides an effective separation of the active compounds present in plant extracts (Kamalakar *et al*., 2014).The four fractions obtained from *Momordica charantia* and five fractions from *Trigonella foenum graecum* may contain bioactive compounds which may be responsible for antidiabetic action.

4.3.3.1 Alpha-amylase inhibitory activity of the TLC fractions of *Momordica charantia* and *Trigonella foenum graecum* seeds

Alpha-amylase inhibitory potential of the four TLC fractions of *Momordica charantia* seeds (MCS) and five fractions of *Trigonella foenum graecum* seeds (TGS) are presented in Table 22.

Table 22

Alpha- amylase inhibitory activity by TLC fractions of *Momordica charantia* and *Trigonella foenum -graecum* seeds

MCS Fraction	Rf value	Inhibition %	TGS Fraction	Rf value	Inhibition %
1	0.12	77	1	0.21	42
2	0.33	69	2	0.42	51
3	0.52	91	3	0.55	94
4	0.66	96	4	0.66	83
-	-	-	5	0.81	74

Values are mean of triplicates

MCS: *Momordica charantia* seeds TGS: *Trigonella foenum graecum* seeds

Alpha amylase inhibitory potentials of the fractions of MCS showed highest inhibition in Fraction 4 with 96 % followed by fractions 3, 1 and 2 with 91%, 77% and 69 % respectively. In TGS fractions, highest inhibition was found in fraction 3 with 94 % followed by fractions 4, 5, 2 and 1 with 83 %, 74 %, 51% and 42 % respectively. The inhibition percentage was found to be increased in TLC fraction 4 in MCS and TLC fraction 3 when compared to the crude ethyl acetate extracts of *Momordica charantia* and *Trigonella foenum graecum*. These fractions might contain the active compounds for alpha amylase inhibitiory property of the ethyl acetate extracts of seeds of *Momordica charantia* and *Trigonella foenum graecum*.

Twinkle *et al.* (2016) had reported *in vitro* alpha- amylase inhibitory activity and TLC profiling of five compounds in ethanol extracts of *Syzygium cumini* skeels. Poongunran *et al.* (2016) had reported bioassay-guided fractionation and identification of alpha-amylase inhibitors from ethyl acetate fraction of *Syzygium cumini* leaves from preparative TLC. The above studies support the fractionation of the seed extracts of *Momordica charantia* and *Trigonella foenum graecum* using preparative TLC for isolation of active compounds responsible for antidiabetic activity through alpha-amylase inhibition.

Based on the results obtained in preparative TLC and highest alpha -amylase inhibition in fraction 4 of *Momordica charantia* and fraction 3 of *Trigonella foenum graecum* ethyl acetate seed extracts, these fractions were selected for further studies.

4.3.4 Fourier Transform Infra-Red (FT- IR) Spectroscopic analysis of potent TLC fractions of *Momordica charantia* and *Trigonella foenum graecum* seeds

4.3.4.1 FTIR spectrum of potent TLC fraction 4 of *Momordica charantia* seed extract

FTIR spectrum of potent TLC fraction 4 of *Momordica charantia* seed extract was analyzed and the result of FTIR spectrum profile is depicted in Figure 28 and in Table 23.

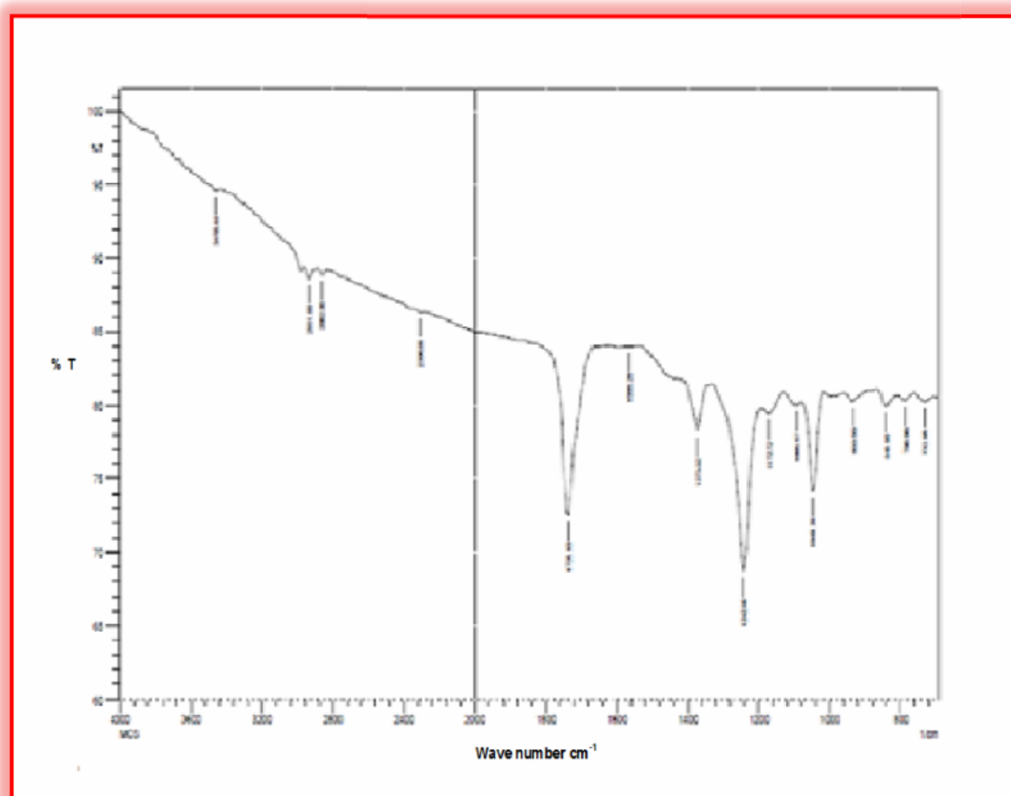


Figure 28

FT-IR spectrum of *Momordica charantia* seed extract

FTIR analysis was used to identify the functional group of active compounds based on the peak values in the region of infrared radiation. Ethyl acetate extracts of seeds of *Momordica charantia* showed characteristic absorption bands at 3456.44 cm^{-1} for N-H

stretching and O-H stretch, 2931.80 cm^{-1} and 2862.36 cm^{-1} for C-H stretch, 2306.86 cm^{-1} for N Ξ N stretch,1735.93 cm^{-1} for-CHO,1566.20 cm^{-1} for aromatic nitro compounds or carboxylate,1373.32 cm^{-1} for aliphatic nitro compounds C-O-C –Cyclic ethers, 1242.16 cm^{-1} and 1172.72 cm^{-1} for sulphate ions, C-O-C stretch in esters, aromatic amines-CN stretch, 2^0 amine NH stretch, 933.55 cm^{-1} for CH= CH_2 and CH_2 , 840.96 cm^{-1} for R-NH_2 primary amine aromatic CH in plane bend, 786.96 cm^{-1} for meta substituted aromatic compounds and 732.95 cm^{-1} for mono substituted aromatic hydro carbons.

Table 23

Interpretation of FT- IR absorptions of *Momordica charantia* seed extract

Group frequency wave number (cm^{-1})	Bond and functional groups
3456.44	> N-H Aromatic 2^0 amine NH stretch - OH Normal polymeric OH stretch-Alcohols/Phenols
2931.80 **2862.36**	> CH_2 Methylene asymmetric/symmetric stretch > CH- Methyne C-H stretch
2306.86	N Ξ N Diazonium salts N Ξ N Stretch
1735.93	-CHO Aldehyde or ester, six membered lactone
1566.20	Aromatic nitro compounds/carboxylate
1373.32	Nitrate ions,aliphatic nitro compounds C-O-C –Cyclic ethers
1242.16, **1172.72**	Sulphate ions, C-O-C stretch in esters Aromatic amines-CN stretch, 2^0 amine NH stretch
933.55	CH= CH_2 in vinyl groups CH_2 out of plane wag
840.96	R-NH_2 primary amine Aromatic CH in plane bend
786.96	Meta substituted aromatic compounds
732.95	-$(CH_2)_n$ Mono substituted aromatic hydro carbons

4.3.4.2 FTIR spectrum of potent TLC fraction 3 of *Trigonella foenum graecum* seed extract

FTIR spectrum of potent TLC fraction 4 of *Momordica charantia* seed extract was analyzed and the result of FTIR spectrum profile is depicted in Figure 29 and in Table 24.

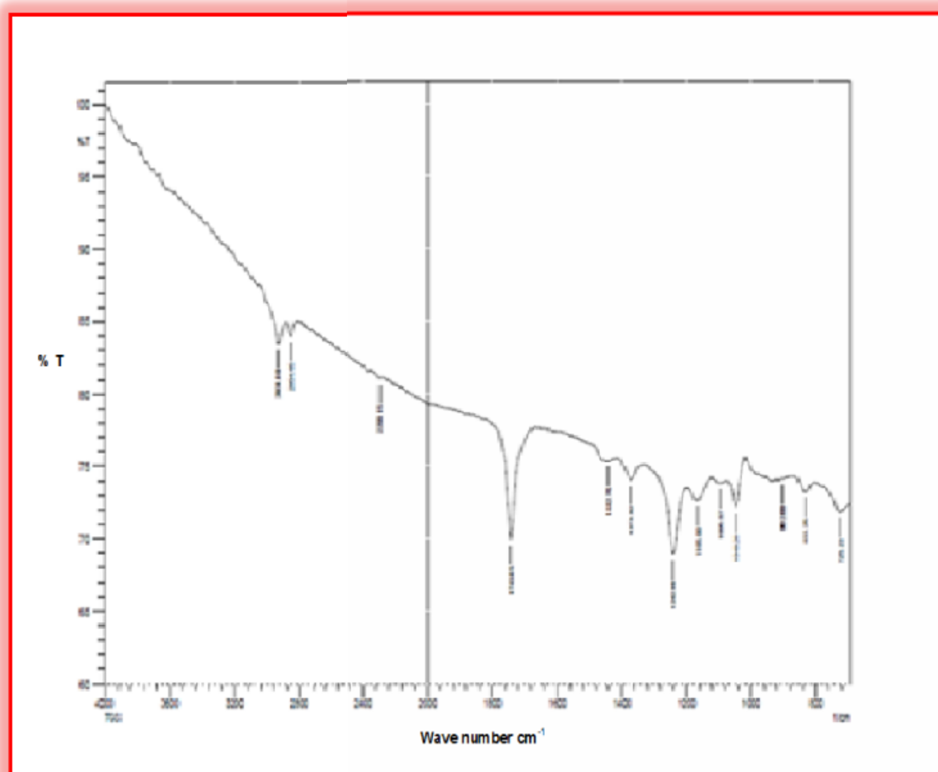


Figure 29

FT-IR spectrum of *Trigonella foenum -graecum* seed extract

FTIR analysis in ethyl acetate extracts of *Trigonella Foenum Graecum* seeds showed characteristic bands at 2931.80 cm^{-1} and 2854.65 cm^{-1} for CH_2 methylene and CH- methyne C-H stretch, 2299.15 cm^{-1} for N Ξ N Stretch, 1743.65 cm^{-1} for lactone C= O stretch, 1442.75 cm^{-1} for CH_3 aliphatic compounds, 1373.32 cm^{-1} for N=O and NO_2 in aliphatic nitro compounds, 1242.16 cm^{-1} for sulphate ions, C-O-C stretch in esters, aromatic amines-CN stretch and 2^0 amine NH stretch, 1095.57 cm^{-1} for C-OH in secondary, tertiary alcohols C-OH stretch and C-NH_2 in primary aliphatic amines C-N stretch, 1049.28 cm^{-1} for CH-OH in cyclic alcohols C-O stretch CH_2- OH in primary alcohols, 902.69 cm^{-1} for CH=CH_2 in vinyl compounds, CH_2 in out of plane wag, 833.25 cm^{-1} for 1,3,5 tri substituted benzene, CH out of plane deformation, 725.23 cm^{-1} for -$(CH_2)_n$ and aromatic OH in phenols, OH out of plane deformation.

Table 24

Interpretation of FT- IR absorptions of *Trigonella foenum- graecum* seed extract

Group frequency wave number (cm^{-1})	Bond and functional groups
2931.80 **2854.65**	> CH_2 Methylene asymmetric/symmetric stretch > CH- Methyne C-H stretch
2299.15	N Ξ N Diazonium salts N Ξ N Stretch
1743.65	Esters lower if unsaturated C=O in lactone C= O stretch
1442.75	CH_3 Aliphatic compounds
1373.32	N=O, NO_2 in aliphatic nitro compounds
1242.16	Sulphate ions, C-O-C stretch in esters Aromatic amines-CN stretch, 2^0 amine NH stretch
1165	C-O-C stretch in esters C-OH in alcohols C-O stretch
1095.57	C-OH in secondary and tertiary alcohols –C-OH stretch,C-NH_2 in primary aliphatic amines C-N stretch
1049.28	CH-OH in cyclic alcohols C-O stretch CH_2- OH in primary alcohols
902.69	CH=CH_2 in vinyl compounds, CH_2 in out of plane wag
833.25	1,3,5 trisubstituted benzene, CH out of plane deformation
725.23	-$(CH_2)_n$ in hydro carbons CH_2 rocking in methylene chain intensity depends on chain length Aromatic OH in phenols, OH out of plane deformation

Functional group analysis plays a vital role in understanding the overall physicochemical properties of the extract. Also, identification of the functional group helps to evaluate their structure–activity relationships. FT-IR spectral analysis of the root bark extracts of *Mammea suriga* showed the presence of phytochemicals carrying hydrogen bonded –OH functional group. It is well established that hydroxyl functionality is an integral part of most of the phenolic phytochemicals such as flavonoids and tannins (Mahesha *et al.,* 2015).

A study on aqueous extracts of *Gymnema sylvestre* by Sangeetha *et al*. (2014) revealed the presence of excess of aliphatic and aromatic amines and reported that the plant possessed highest antioxidant activity. Ragavendran *et al*. (2011) screened the functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, organic hydrocarbons and halogens that are responsible for various medicinal properties of *Aerva lanata.*

FTIR analysis of ethylacetate extracts of *Momordica charantia* seeds confirmed the presence of alcohols, phenols, alkanes, alkynes, aldehydes, aromatic compounds, aromatic amines and aromatic hydrocarbons. *Trigonella foenum graecum* seed extracts also showed the presence of esters, aliphatic compounds, primary alcohols, phenols, aromatic amines, cyclic and primary alcohols. The bioactive compounds with these functional groups might be responsible for antidiabetic and antioxidant activities.

4.3.5 Gas Chromatography Mass Spectroscopy (GCMS) profile of potent TLC fractions of *Momordica charantia* and *Trigonella foenum graecum* seeds

4.3.5.1 GCMS profile of potent TLC fractions of *Momordica charantia* seeds

Potent TLC fractions of ethyl acetate extracts of *Momordica charantia* seeds were subjected to gas chromatography and mass spectrum analysis. The identification of the compounds is based on the peak area, retention time and molecular weight. The bioactive compounds present in the ethyl acetate extract of *Momordica charantia* seeds were identified by GC-MS analysis and GC-MS running time was 47 minutes. The GC-MS chromatogram of MCS extract is presented in Figure 30.

The present study on GC-MS revealed the presence of about forty compounds. The identity of the components in the extract was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with the National Institute of Standards and Technology (NIST) 08 LIB and WILEY8 LIB library sources for matching the identified compounds from the plant material.

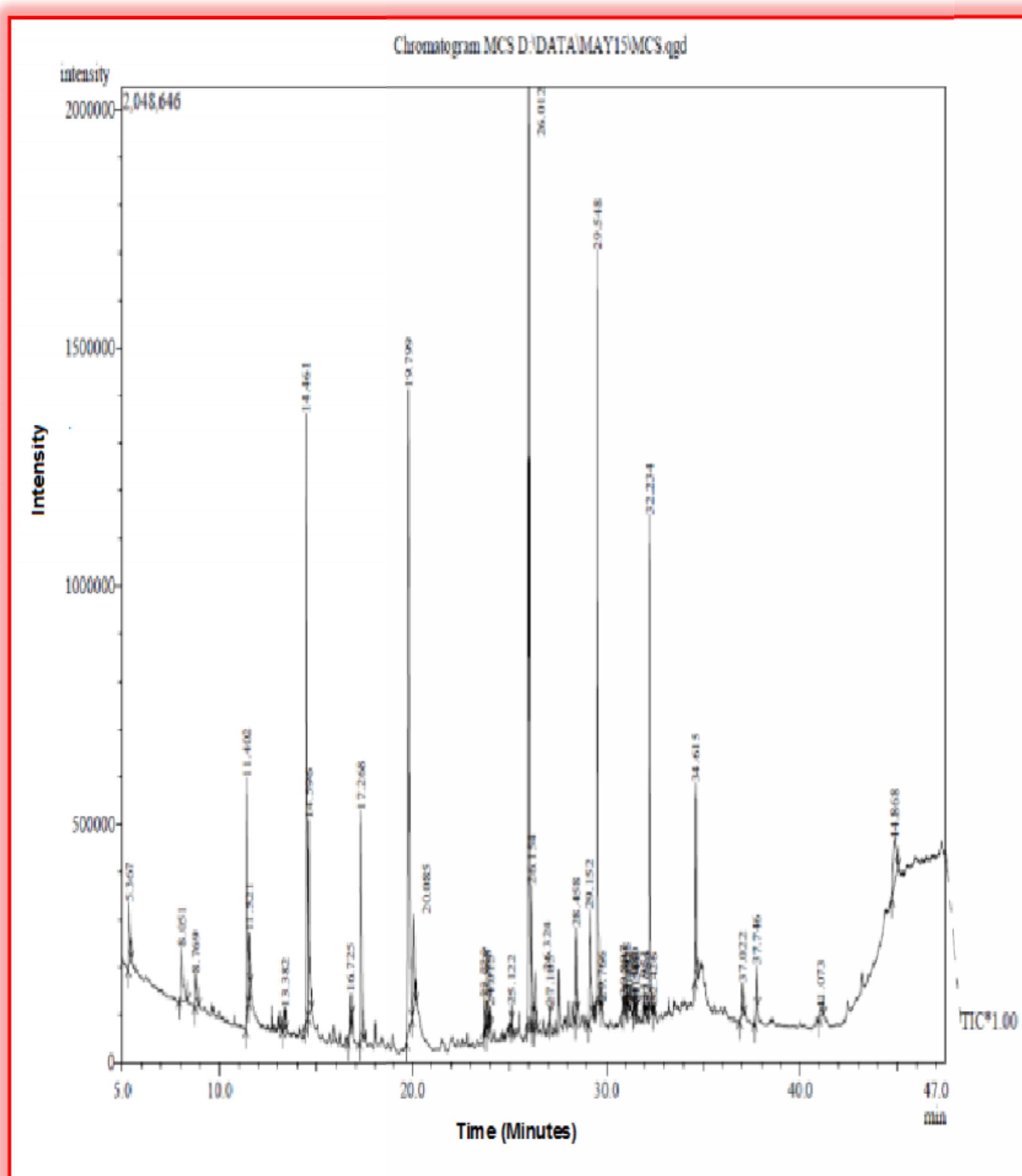


Figure 30

GCMS profile of potent TLC fractions of *Momordica charantia* seeds

Among the forty compounds identified eight of them were found to be biologically important as per literature studies because of their antidiabetic, antioxidant, antimicrobial and antifungal properties. The active compounds with their retention time, peak area, nature of the compound, molecular formula and molecular weight are depicted in Table 25.

Table 25

Compounds identified at various retention times from *Momordica charantia* seeds

Retention time	Peak Area	Name of the Compound	Nature of the compound and common name	Molecular formula	Molecular weight
8.769	337899	1-Methyl-4-(1-methylethenyl)cyclo hexene	Cyclic terpene Essential oil Limonene	$C_{10}H_{16}$	136
17.268	2113835	2,4-Di-tert-butylphenol	Alkylated phenol	$C_{14}H_{22}O$	206
25.122	59104	2,6,10-Trimethyl dodecane	Sesquiterpene Farnesane	$C_{15}H_{32}$	212
26.012	6593923	1-Nonadecene	Alkene	$C_{19}H_{38}$	266
31.420	7587	Nonahexacontanoic acid methyl ester	Fatty acid ester	$C_{70}H_{140}O_2$	1012
37.022	282371	1,2-Benzenedicarboxylic acid	Aromatic dicarboxylic acid	$C_8H_5NO_6$	211
41.073	98244	2-[(Decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)methyl 2,5-cyclohexadiene-1,4-dione	Benzoquinone	$C_{21}H_{28}O_2$	312
44.867	1014460	11-Methylspirostan-3,11-diol	Steroidal saponin	$C_{28}H_{46}O_4$	446

4.3.5.2 GCMS profile of potent TLC fractions of *Trigonella foenum graecum* seeds

Potent TLC fractions of ethyl acetate extracts of *Trigonella foenum graecum* seeds were subjected to gas chromatography and mass spectrum analysis, GC-MS running time was 38 minutes. The GC-MS chromatogram of MCS extract is presented in Figure 31.

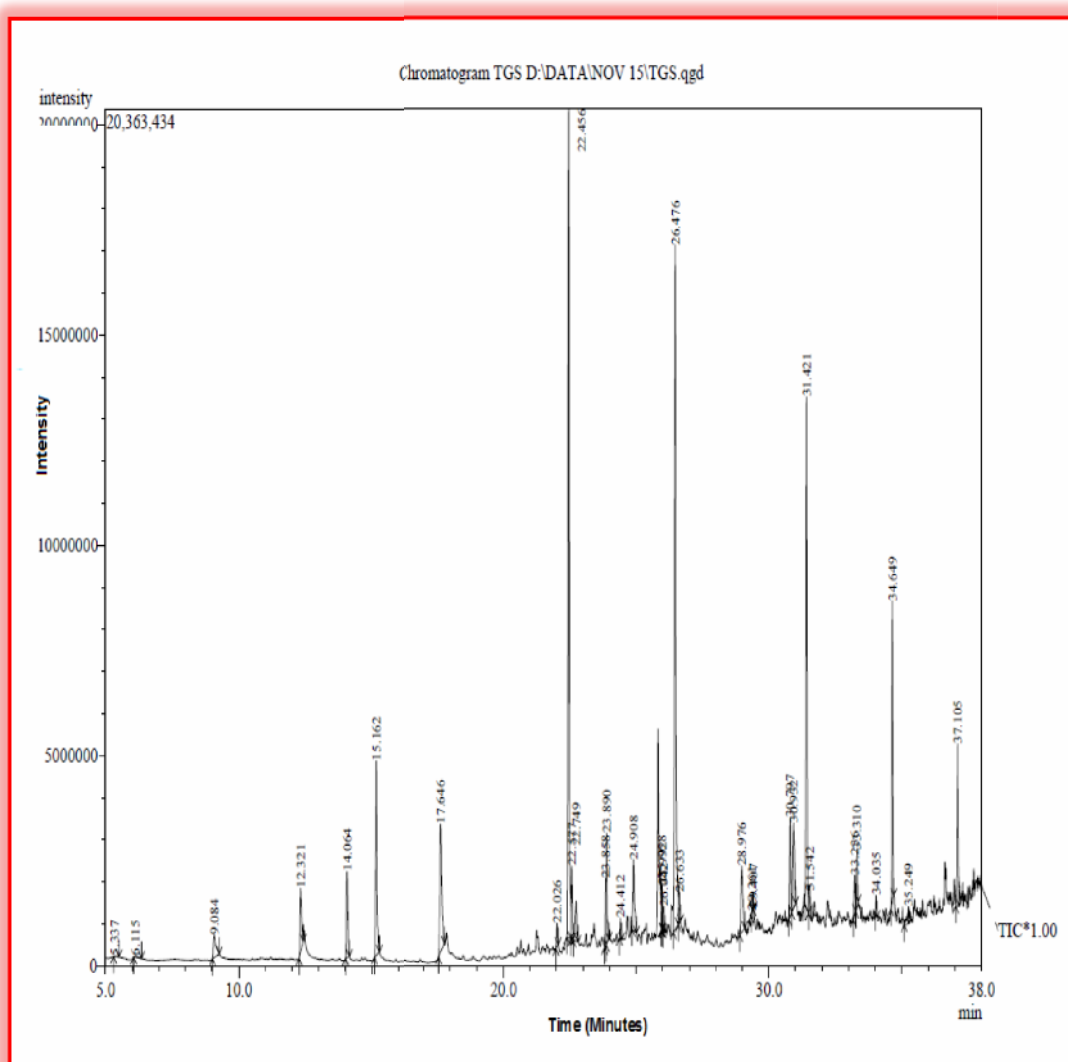


Figure 31

GCMS profile of potent TLC fractions of *Trigonella foenum- graecum* seeds

GCMS spectral analysis of *Trigonella foenum graecum* seed extract identified about thirty three compounds based on their retention indices and mass spectra fragmentation patterns with the National Institute of Standards and Technology (NIST) 08 LIB and WILEY8 LIB library sources for matching the identified components from the plant material. Seven out of thirty three compounds were found to be biologically important as per literature studies because of their antidiabetic, antioxidant, anti cancer and cholesterol lowering activity. The active compounds with their retention time, peak area, nature of the compound, molecular formula and molecular weight are shown in Table 26.

Table 26

Compounds identified at various retention times from *Trigonella foenum graecum* seeds

R/T	Peak Area	Name of the Compound	Nature of the compound and common name	Molecular formula	Molecular weight
14.064	7570047	2-[(Decahydro-5,5,8a-Trimethyl-2-Methylene-1Naphthalenyl)Methyl2, 5-Cyclohexadiene-1,4-dione	Benzoquinone	$C_{21}H_{28}O_2$	312
15.162	16811356	2,4-Di-tert-butylphenol	Alkylated phenol	$C_{14}H_{22}O$	206
26.476	67138510	1-Nonadecene	Alkene	$C_{19}H_{38}$	266
28.976	7970465	1-Heptacosanol	Aliphatic alcohol	$C_{27}H_{56}O$	396
30.797	7589735	i-Propyl5,9,17-hexacosatrienoate	Isopropyl esters of fatty acids	$C_{29}H_{52}O_2$	432
34.035	819973	1-Phenanthrene carboxylicacid,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester	Resin acid Dehydroabietic acid	$C_{21}H_{30}O_2$	314
35.249	1523064	2,5-Di-tert-butylhydroquinone	Phenol derivative	$C_{14}H_{22}O_2$	222

Murali *et al*. (2013) had recorded that D-limonene (1-methyl-4-(1-methylethenyl) cyclo hexane) reduces the oxidative stress in STZ-induced diabetic rats by decreasing lipid peroxidation and sparing the activities of antioxidant enzymes. Therefore, D-limonene can be considered as a safe food supplement with a potential as an antidiabetic agent in diabetic complications. Aadil *et a*l. (2012) had reported that 2, 4-di-tert-butylphenol an alkylated phenol identified in *Acacia arabica* and *Rouwolfia serpentina* extract had high phenolic content, potent antioxidant activity and were able to regulate glucose migration and alpha amylase activity.

Triterpenoids represent a promising and expanding source for biologically active natural compounds. Triterpenoids are widely distributed in plants but inhibitory α-amylase activity was related only for oleanane, ursane and lupane types (Sales *et al*., 2012). GC-MS analysis of *Petrea volubilis* root revealed the presence of 1, 2- benzenedicarboxylic acid, mono (2- ethyl hexyl) ester which might be responsible for antidiabetic activity through α-amylase inhibition (Parul and Rekha, 2015). Sumathy *et al*. (2013) had reported antioxidant and alpha-amylase inhibitory activities of *Croton bonplandianum* leaf extracts. GCMS analysis of ethanol extracts of *Croton bonplandianum* leaf extracts showed the presence of 1-nonadecene. Deepthy and Radhamany (2016) in their GCMS analysis of *Tamilnadia uliginosa* fruit extracts had identified 1-nonadecene and reported that the compound possess antioxidant, antimicrobial and anticancer properties.

Yadao *et al*. (2015) in their studies had identified hydroquinone in GC-MS analysis of methanol extract of *Cassia auriculata* flowers and also related that presence of these compounds might be responsible for antidiabetic and antioxidant property. Muhammad *et al.* (2013) identified monoterpenes in *Citrus sinensis* essential oils and also reported the hypoglycemic effect observed may be attributed to the presence of monoterpenes in the essential oil due to its insulin-mimetic properties or may be able to induce insulin production from the surviving beta cells enough to facilitate glucose uptake from the blood.

Mahendran *et al.* (2010) reported antihyperglycemic activity of embelin (benzoquinone) against alloxan induced diabetic rats at concentrations of 25 and 50 mg/kg body weight administered orally, reduces significantly fasting blood (serum) glucose levels and also added embelin treated rats improved significantly in body weights. Previous studies reported that sarsasapogenin a steroidal saponin had anti-diabetic effects, such as reducing blood glucose and reversing weight gain in diabetic animal models; however, diosgenin, with a Δ^5 double bond, had a much lower antidiabetic activity than did sarsasapogenin (Applezweig, 1987).

Abir *et al.* (2015) had reported three compounds, abietic acid, dehydroabietic acid and squalene isolated from the most active fraction in the bioassays (hexane) of balsam fir (*Abies balsamea* (L.) Mill.) which were able to decrease the activity of glucose-6-phosphatase and to stimulate glycogen synthase. Dehydroabietic acid was found to be most effective among the three compounds. Hence these compounds were suggested to have potential applications in the treatment of Type 2 Diabetes and insulin resistance. Genta *et al*. (2010) in their studies had reported about enhydrin, a major sesquiterpene

lactone from *Smallanthus sonchifolius* leaves which was found to be effective to reduce post-prandial glucose and found to be useful in the treatment of diabetic animals.

Unnikrishnan *et al.* (2014) had reported that GC-MS analysis of lead extracts of *Turbinaria ornate* showed the presence of major compounds, hentriacontane, z, z-6, 28-heptatriactontadien-2-one, 8-heptadecene, and 1-heptacosanol. Plant extracts were tested for their antidiabetic potential using enzyme inhibitory assays (α-amylase, α-glucosidase, and dipeptidyl peptidase-IV).Hence they suggested that these compounds can be used as a potential source for further *in vivo* studies in controlling hyperglycemia.

From the present study findings supported by previous reports from literature it may be suggested that the compounds identified through GCMS analysis from *Momordica Charantia and Trigonella foenum graecum* seed extracts might be responsible for antidiabetic and antioxidant activities.

Phase IV

4.4 *In Silico* Molecular Docking of Selected Compounds Identified from *Momordica charantia* and *Trigonella foenum graecum* Seeds against Human Pancreatic Alpha-amylase (HPA) and Porcine Pancreatic Alpha-amylase (PPA) Enzymes

Molecular docking is the computational method for structure-based drug designing which gives an idea about the proper and stable conformation of ligand and target protein and also tells about suitable protein ligand interactions. Hence, to get an insight about the mode of interaction between the binding site of alpha-amylase enzyme and the active compounds identified from seeds of *Momordica charantia* and *Trigonella foenum graecum,* docking studies were conducted.

Docking analysis of compounds, 11-methylspirostan-3,11-diol, 1-phenanthrene carboxylicacid,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methylester (dehydroabietic acid), 2,6,10-trimethyldodecane (farnesane), 2,4-di-tert-butylphenol, 1-nonadecene, 1-heptacosanol, 1-methyl-4-(1-methylethenyl) cyclo hexane (limonene), 1,2-benzenedicarboxylic acid and nona hexacontanoic acid methyl ester with selected enzymes human pancreatic alpha amylase (HPA) and porcine pancreatic alpha amylase (PPA) was performed using docking server.

The results of *in silico* molecular docking of selected compounds from *Momordica charantia* and *Trigonella foenum graecum* seeds with human pancreatic alpha amylase and porcine pancreatic alpha amylase enzymes and their binding energies are presented in Table 27.

Table 27

Binding energies of ligands with human pancreatic alpha- amylase (HPA) and porcine pancreatic alpha-amylase (PPA)

Ligand	11-Methyl spirostan-3,11-diol		Dehydro abietic acid		Farnesane		2,4-Di-tert-butylphenol		1-Nonadecene		1-Heptacosanol		Limonene		1,2 Benzene dicarboxylic acid		Nona hexacontanoic acid methyl ester	
Protein / Parameters	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA	HPA	PPA
Estimated free energy of Binding kcal/mol	-8.59	-6.02	-7.61	-5.52	-5.68	-4.05	-5.35	- 3.78	-5.20	-3.77	-4.80	-2.50	-4.79	-3.70	-4.61	-3.66	-4.43	-3.73
vdW + Hbond + desolv energy kcal/mol	-9.34	-6.62	-8.09	-6.07	-8.05	-6.63	-6.27	- 4.65	-9.55	-8.19	-11.38	-8.74	-5.09	-4.01	-5.12	-4.11	-8.02	-7.77
Electrostatic energy kcal/mol	+0.15	+0.00	-0.10	-0.02	-0.01	+0.01	+0.02	- 0.03	-0.00	-0.00	-0.13	-0.01	-0.00	+0.01	-0.18	-0.19	-0.06	-0.23
Total Intermolecular energy kcal/mol	-9.19	-6.62	-8.19	-6.09	-8.06	-6.63	-6.24	- 4.67	-9.55	-8.20	-11.51	-8.76	-5.09	-4.00	-5.30	-4.30	-8.08	-8.00

Chemical Information of docking calculations was carried out using molecular docking server. The docking energy values were calculated as the sum of the electrostatic, van der Waals energies and the flexibility of the ligand. The binding energies presented in negative values, indicate that the more negative values have more binding interactions.

Analysis of docking results showed the binding energies ranging from - 8.59 Kcal/mol to - 4.43 Kcal/ mol for ligands with human pancreatic alpha-amylase and - 6.02 Kcal/mol to -2.50 Kcal/ mol for ligands with porcine pancreatic alpha-amylase. The results of binding energy reveal that the ligand molecules were more potent in binding to human pancreatic alpha-amylase enzyme than to porcine pancreatic alpha amylase enzyme. Low docking energy indicates high binding ability. The variation in binding energies of the ligand molecules may be attributed to the intermolecular interaction energy between α-amylase and ligand molecule. The binding energy is nothing but the binding strength of the ligand which not only helps predicting the stable conformation of ligand-protein complex but also optimizes the newly formed bonds (Patil *et al*., 2010).

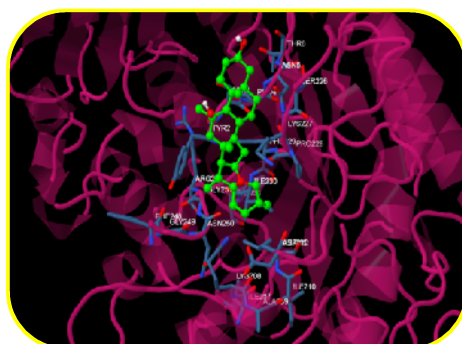
The docking results revealed that the compounds exhibited more favourable interactions with the targets. Plates 9, 10 and 11 represent promising binding pose and the effective binding of the selected ligands with proteins HPA and PPA.

Numerous interactions were observed between the proteins and the ligands docked. Aminoacid interactions with the ligands are shown in Table 28. After comparative analysis, it was understood that polar, hydrophobic, hydrogen bond and other interactions namely van der Waals and the electrostatic interactions are involved in binding of the ligands to the proteins.

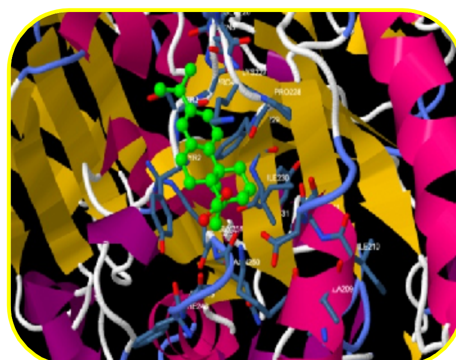
In view of findings it was observed that a polar interaction was through lysine and aspargine, hydrophobic interactions was through tyrosine, isoleucine, leucine and proline and amino acids lysine, serine and aspargine were involved in other type of interactions. So, these amino acids might be important in binding of human pancreatic alpha amylase with the ligands.

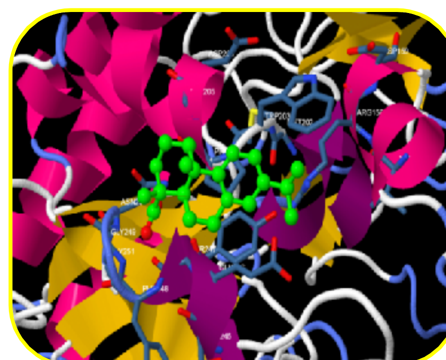
Amino acids arginine, aspargine, tyrosine, lysine and aspartic acid were involved in polar interactions, amino acids tryptophan, proline, leucine, tyrosine and alanine for hydrophobic interaction, hydrogen bonds through glycine and other interactions through amino acids aspartic acid, glutamic acid, arginine, lysine and aspargine in binding of the ligands to porcine pancreatic alpha amylase.

HPA | **PPA**

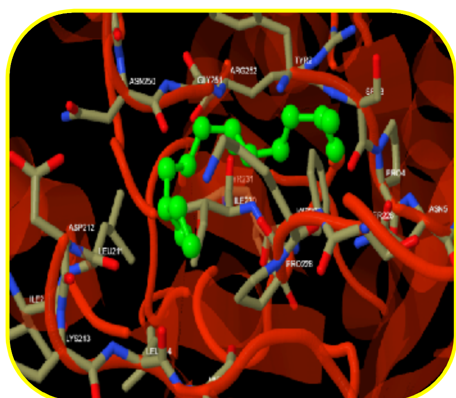


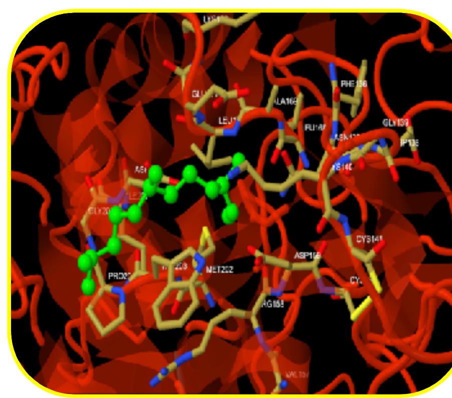
11-Methylspirostan-3, 11-diol





1-Phenanthrene carboxylicacid,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester



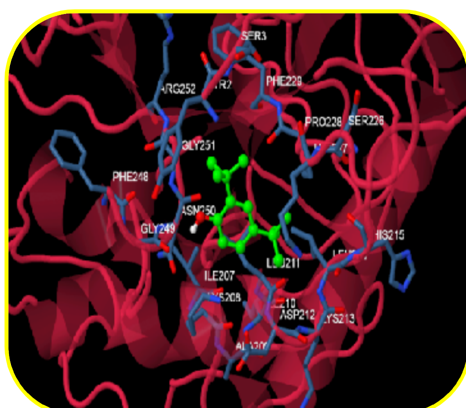


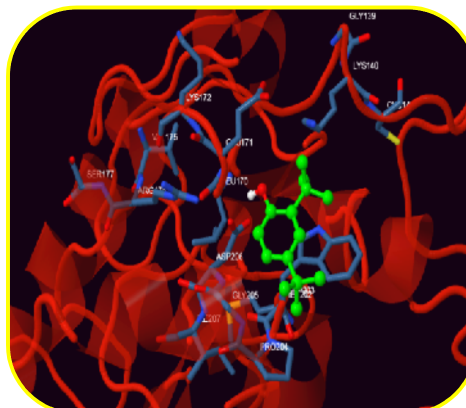
2, 6,10 -Trimethyldodecane

Plate 9

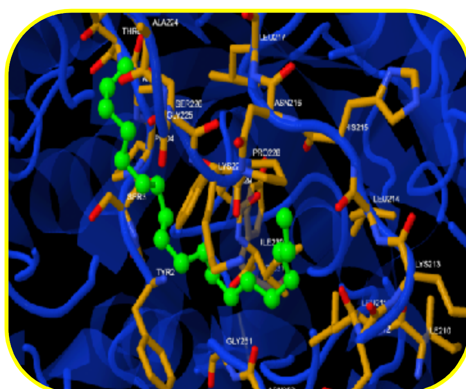
Molecular Docking of HPA and PPA with 11-Methylspirostan-3, 11-diol, 1-Phenanthrene carboxylicacid,1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester and 2,6,10-Trimethyldodecane

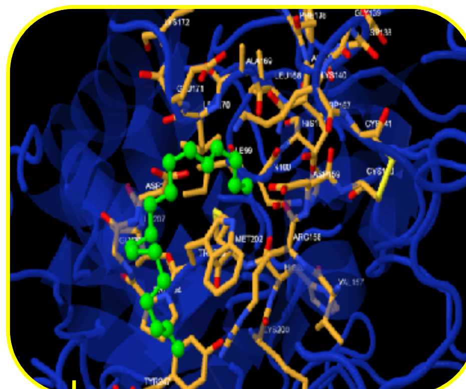
HPA | **PPA**



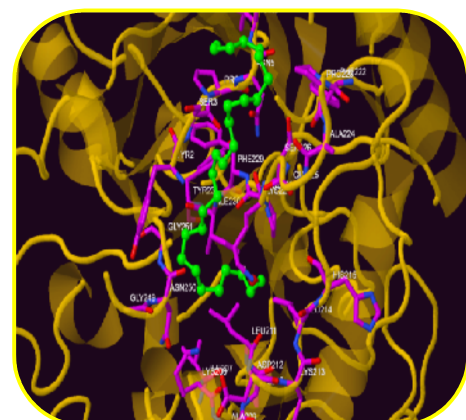


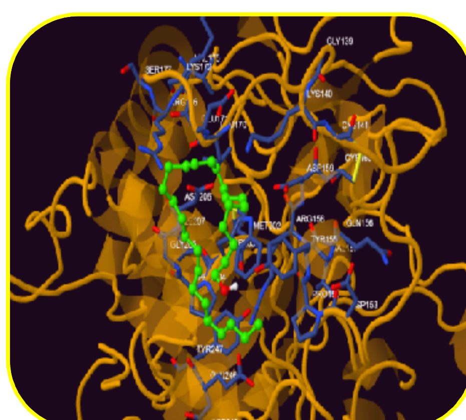
2, 4-Di-tert-butylphenol





1-Nonadecene

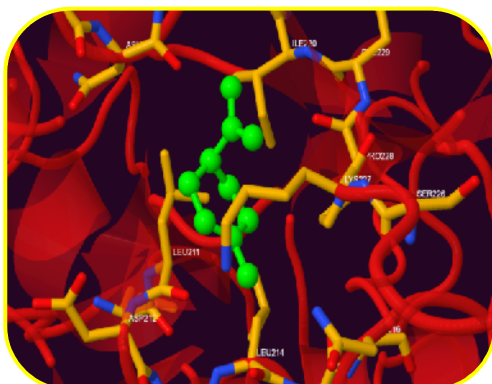
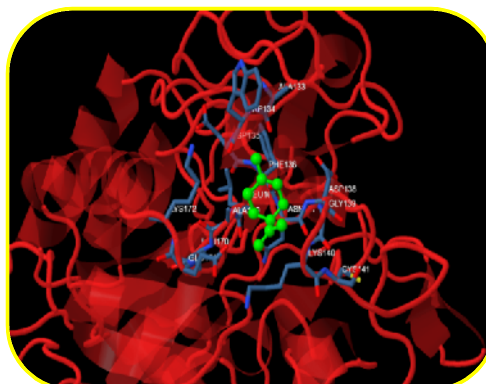




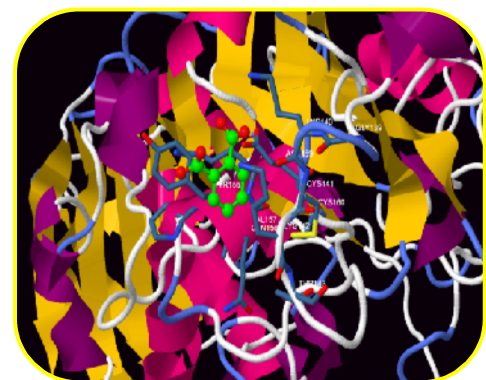
1-Heptacosanol

Plate 10

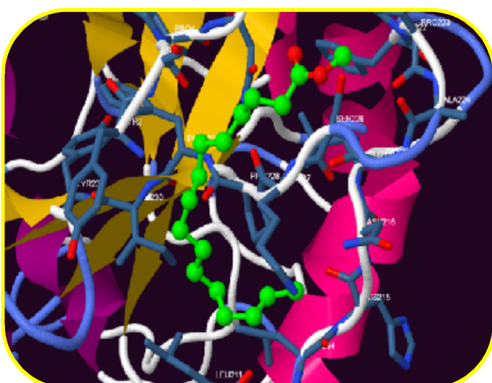
Molecular Docking of HPA and PPA with 2, 4-Di-tert-butylphenol, 1-Nonadecene and 1-Heptacosanol

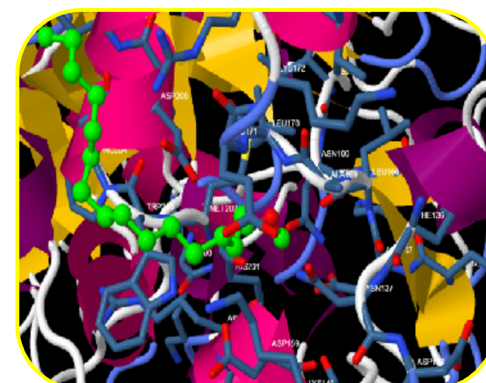
HPA	**PPA**
	

1-Methyl-4-(1-methylethenyl) cyclo hexane



1, 2 Benzene dicarboxylic acid





Nonahexacontanoic acid methyl ester

Plate 11

Molecular Docking of HPA and PPA with 1-Methyl-4-(1-methylethenyl) cyclo hexane, 1, 2 Benzene dicarboxylic acid and Nonahexacontanoic acid methyl ester

Table 28

Aminoacid interactions of human pancreatic alpha-amylase (HPA) and porcine pancreatic alpha- amylase (PPA) with the ligands

Ligand	Interacting amino acids of HPA			Interacting amino acids of PPA			
	Polar	Hydrophobic	Other Interactions	Polar	Hydrophobic	Hydrogen Bonds	Other Interactions
11-Methyl spirostan-3,11-diol	-	Tyr 2, Ile 230, Leu 211	Lys 227,Ser3, Lys 208, Asn 5	Arg 176	Trp 203,Pro 204	-	Asp 206,Glu 246, Arg 158,Glu 171
Dehydro abietic acid	Lys 208	Leu 211,Ile 230	Lys 227, Ser 3, Asn 5	Asn 250	Pro 204,Trp 203	Gly 249	Arg 158,Glu 246
Farnesane	-	Pro 228, Leu 211,Ile 230, Leu 214	Lys 227,Ser 3, Asn 5		Leu 170,Pro 204,Trp 203	-	Asn 137, Asp 159, Asp 206,Glu 171, Lys 140
2,4-Di-tert-butylphenol	-	Pro 228, Ile 230, Leu 214,Leu 211, Tyr 2	Lys 208, Lys 227	Asp 206, Arg 176	Trp 203,Pro 204	-	Glu 171,Lys 140
1-Nonadecene	-	Leu 211,Pro 228	Lys 227,Ser 3, Ser 226, Asn 5		Trp 203,Leu 170,Met 202, Pro 204	-	Lys 140, Glu 171, Arg 158, Asn 137, Asp 206,Asp 159, Asn 100,Glu 246
1-Heptacosanol	Lys 227	Leu 211,Pro 228, Pro 4, Ile 230, Pro 223, Leu 214	Lys 208,Ser 3, Ser 226, Asn 5	Tyr 155	Trp 203,Pro 204, Pro 154	-	Asp 206, Arg 158, Lys 140,Arg 176, Glu 171,Glu 246
Limonene	-	Leu 211,Leu 214,Ile 230, Pro 228	Lys 227	-	Tyr 155	-	Lys 140, Lys 142, Asp 159
1,2 Benzene dicarboxylic acid	Lys 208	Leu 211,Ile 230, Pro 228	Lys 227	Lys 142, Lys140, Asp159	Tyr 155	-	-
Nona hexacontanoic acid methyl ester	Asn 5	Leu 211,Leu 214, Pro 228	Lys 227,Lys 208, Ser 3	Asn 137, Asp167	T rp 203, Leu 170, Ala 209	-	Asp 206,Glu 171, Asp 159, Lys 140, Arg 176, Asn 100

Among the plant compounds used for docking 11-methyl spirostan-3, 11-diol (steroidal saponin) showed minimum energy and high binding affinity for both the enzymes. The present findings are supported by the findings of Sougata *et al*.(2014) who had reported that molecular docking of porcine pancreatic α-amylase and diosgenin, a phytosteroid sapogenin isolated and identified from *Dioscorea bulbifera* ethyl acetate extracts showed lowest energy (−7.4 kcal/mol) with highest binding affinity.

Docking results of cyclohexene, 1-methyl-4-(1-m / limonene with HPA and PPA are similar to the observations of Sudha *et al.* (2015) who had reported the involvement of Trp 58, Trp 59, Tyr 62 and Tyr 151 in interactions of free HPA and PPA-oligosaccharide with the neem limonoids, azadiradione and gedunin. These limonoids exhibited a mixed mode of inhibition as observed by kinetic experiments. The docking with free enzyme results suggested that both inhibitors likely bind near the active pocket. However, the active site residues were not involved in their binding. They also suggested that mixed inhibition would be of relevance for the regulated activity of HPA in order to slowly release glucose and thereby control post prandial hyper glycemia (PPHG).

Docking reveals the proper orientation of ligand–protein complex as well as various potential binding sites (Mobley and Dill, 2009). There has been a growing interest in the design of drugs forming a covalent bond with the target protein, with nearly 30% of the marketed drugs targeting enzymes known to act by covalent inhibition (Robertson, 2007 and Robertson, 2005). These types of inhibitors derive their activity from both non-covalent interactions and the formation of the covalent bond between the inhibitor and the target protein. The non-covalent interactions are conventional methods and are proved to be effective in prediction of different binding modes of protein ligand complexes (Kumalo *et al*., 2015). Non-covalent interactions include hydrophobic interactions, hydrogen bonding, van der Waals interaction and the electrostatics interaction (Gromiha *et al*., 2004). The binding affinity will be higher if the ability of ligands to form hydrophobic interactions with the binding site hydrophobic amino acids is higher (Mohapatra *et al.,* 2015).

Elucidating the mechanism behind the binding of small organic compounds to proteins is therefore highly relevant both for drug discovery and for understanding numerous biochemical processes that depend on the binding of a ligand to a protein. A fundamental understanding of the ligand-binding event requires insight into *the* non-covalent interactions that stabilize a protein-ligand complex as well as the dynamics and thermodynamics of the system. Non-covalent interactions differ from covalent bonds

in that no electrons are shared between the participating atoms. They are nonetheless specific, attractive and (importantly) reversible and can be formed and broken without being associated with a large energy cost. Non-covalent interactions play a key role in biological systems by impacting the structure, dynamics and function of biomolecules (Rezac and Hobza, 2016 and Panigrahi and Desiraju, 2007).

In a protein-ligand complex, non-covalent interactions may be formed both intra molecularly between the amino acids of the protein and inter molecularly between the protein and the ligand. The total interaction energy of a number of non-covalent interactions can be either greater or less than the sum of the interaction energies of the individual interactions. Non-covalent interactions can therefore interact in either a “positive cooperative” or “negative cooperative” manner (Mahadevi and Sastry, 2016 and Williams *et al*., 2004).

Specific non covalent interactions that are indicative of attractive, directional intermolecular forces have always been of key interest to medicinal chemists in their search for the "glue" that holds drugs and their targets together (Zhou *et al.,* 2012).

Acarbose, an established alpha amylase inhibitor and antidiabetic drug was docked with human pancreatic alpha amylase enzyme which showed a binding energy of - 7.1 Kcal/mol. Caffeic acid, chlorogenic acid and isorhamnetin characterized from *Corchorus olitorius* were docked with HPA and their binding energies were -6.5 Kcal/mol, -7.3 Kcal/mol and -8.5 Kcal /mol respectively as reported by Metibemu *et al.* (2016).

Hashim *et al.* (2013) had reported that docking simulations of α-amylase inhibitors from *Protomelas virgatus* methanol extract acrylic acid, 11 octadecenoic acid, 9–12 octadecodienoic acid, 6-oactadecynoic acid, hexadecanoic acid and phthalic acid involve interactions between ligand and Trp 59 / Try 62 suggesting these aminoacids to be important residues in binding of inhibitors to PPA.

In silico studies by Twinkle *et al.* (2016) revealed that ellagic acid (phenolic compound) isolated from *Syzygium cumini* (L.) has a potential to modulate the carbohydrate metabolizing enzyme activity showing higher affinity for the enzymes with much lesser binding energy, - 4.73 kcal/mol for alpha amylase, - 4.87 kcal/mol for beta-glucosidase, -4.79 kcal/mol for glycogen synthase kinase, - 4.18 kcal/mol for glucokinase and - 4.49 kcal/mol for alpha-glucosidase.

Ogunwa (2016) studied molecular interaction and inhibitory potential of plant - derived phenolic compounds scirpusin B, cassigarol E, epicatechin gallate and sarcoviolin on human α-amylase and compared to standard drugs used for the treatment of Type 2 Diabetes. Computational ligand docking revealed that these compounds possessed higher binding affinity (-9.2Kcal/mol, -9.0Kcal/mol, -8.9Kcal/mol and -8.3Kcal/mol respectively) and showed higher inhibitory potentials on human α-amylase as compared with reference compounds (acarbose and miglitol) having -7.5Kcal/mol and -5.1Kcal/mol binding energy respectively.

Bhuiyan *et al*. (2016) explored the α-amylase inhibitory activity by molecular docking analysis of beta-sitosterol, daucoterol and sitoindoside I isolated from *Pistia stratiotes*, to identify whether these compounds interact with α-amylase enzyme. Beta-sitosterol, daucoterol and sitoindoside I showed the docking score -3.783, -3.526 and -5.322 respectively.

Wulan *et a*l. (2014) in their *in silico* comparison between human and rat pancreatic amylase showed that molecular docking of betulin (Triterpene) had smaller $E_{binding}$ and Ki value (($E_{binding}$ -6.66 kcal/mol, Ki 13.12 μM to RPA and ($E_{binding}$ -8.42 kcal/mol, Ki 0.67μM to HPA) than betulinic acid ($E_{binding}$ -6.44 kcal/mol, Ki 18.97 μM to RPA and $E_{binding}$ -7.08 kcal/mol, Ki 6.48 μM to HPA).Since betulinic acid and betulin showed the same interaction with amino acid residue of alpha amylase, they also suggested that betulin could be a potential inhibitor with non-competitive pattern like betulinic acid.

Bharathi *et a*l. (2014) had reported in their studies that the docking calculations showed that van der Waals, electrostatic and desolvation energies play a key role in binding and these factors are considered for designing new inhibitors for α-amylase and α-glucosidase.

In silico molecular docking studies of the compounds identified from *Momordica charantia* and *Trigonella foenum graecum* seeds showed good docking efficiency through non covalent interactions and binding affinity with the enzymes human pancreatic alpha amylase and porcine pancreatic alpha amylase. The results of the present findings supported by literature and results of phase I, II and III reveal that the active compounds identified from the seeds of *Momordica charantia* and *Trigonella foenum graecum* seeds might act through inhibition of the pancreatic alpha- amylase enzymes in Diabetes Mellitus.