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Salutary Effects of Germinated *Glycine max* Seeds on Post Prandial Hyperglycemia and Dyslipidemia - Evidence from *In-vivo* and *In-vitro* Studies

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Abstract

Background: Germinated *Glycine max* seeds have been known to have anti-hyperglycemic effects; however, a comprehensive study pertaining to this has never been experimentally determined.

Methods: The aqueous extract of 12 h germinated *Glycine max* seeds (gGmax) were orally administered to Streptozotocin induced diabetic rats for 28 days and the effects were observed on glycemic index, lipid profile, antioxidant parameters alpha amylase and alpha glucosidase activity. Further the histomorphological changes were seen in the rat's pancreas. Finally, the metabolite profiling was done.

Results: There was a significant improvement in FBG and OGTT in diabetic rats treated with gGmax after a period of 28 days. The extract also led to a substantial increase in the levels of insulin and c-peptide as compared to diabetic control rats with marked improvement in insulin resistance. Lipid profile and atherogenic factors were significantly improved. Histomorphological examination of pancreatic tissue revealed increased number of islets and β-cells in treated rats as compared to diabetic controls. Subsequently, marked suppression in the activity of alpha-amylase and alpha- glucosidase was observed.

Conclusion: The antidiabetic property of the extract is attributed through the improvement in insulin secretion, suppression of post- prandial hyperglycemia and β-cell regeneration. Besides its antidiabetic properties, *Glycine max* seeds also demonstrated salutary effects on the management of dyslipidemia which may be mediated through scavenging of free radicals as well as suppression of atherogenic lipid variants and apolipoproteins. Overall this study represents *Glycine max* seeds as a promising therapeutic agent for diabetes and dyslipidemia.

Keywords: Diabetes; *Glycine max*; Post-prandial hyperglycemia; β-cell regeneration; Dyslipidemia

Abbreviation:

gGmax: 12 h Germinated *Glycine max* seeds; FBG: Fasting Blood Glucose; OGTT: Oral Glucose Tolerance Test; CVD: Cardio Vascular Disease; STZ: Streptozotocin; AYUSH: Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy; IAEC: Institutional Animal Ethical Committee; Hb1Ac: Glycosylated Hemoglobin; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; TG: Triglycerides; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; VLDL: Very Low Density Lipoprotein; Ox LDL: Oxidized Low Density Lipoprotein; IC_{50}: Half maximal Inhibitory Concentration; FRAP: Ferric Reducing Ability of Plasma; TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine; GC-MS: Gas Chromatography-Mass Spectrometry; ANOVA: Analysis of Variance

Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. It has caused significant morbidity and mortality due to microvascular and macrovascular complications [2]. It is one of the largest global health emergencies of the 21st century. Although the prevalence of diabetes is increasing at an alarming rate throughout the world, the occurrence and consequences associated with it is found to be high in countries like China (109.6 million), India (69.2 million), and United States (29.3 million). Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action [3].

Recently, considerable research has been focused on postprandial hyperglycemia because it doubles the risk of death from cardiovascular disease [4]. In diabetes, the postprandial phase is characterized by a rapid increase in blood glucose levels after meals [5]. The present day studies highlights on the possibility that postprandial "hyperglycemic spikes" may be relevant to the pathophysiology of late diabetes complications. In-fact Asian population displays marked increase in post-prandial hyperglycemia [6]. It has also been shown that, apart from oral glucose tolerance test (OGTT), activity of pancreatic α-amylase and α- glucosidasecorrelates to an increase in post-prandial glucose levels, the control of which is therefore an important aspect in treatment of type 2 diabetes [7].

Another important consequence of diabetes is exacerbated atherosclerosis and its recognized complications, which ultimately

leads to the development of cardio vascular diseases (CVD). Several mechanisms have been suggested to play a role in mediating the additive effects of hyperglycemiaon atherogenesis. These include increased oxidative stress and derangement in lipid and lipoprotein metabolism [8].

Currently there are several classes of synthetic drugs available to achieve better glycemic control but most of them are still not used for prevention of atherosclerosis, because of their potential toxicity and intolerance, thus the management of diabetes and its threatening complications are still a challenge. In this scenario, herbal drugs are offering great potential, as they exhibit multifarious approach. Moreover, they are considered to be safeand cost effective when compared to synthetic anti-hyperglycemic agents.

Compounds with both antidiabetic and anti-atherosclerotic properties are considered to be stupendous. Therefore, the present study demonstrates theantihyperglycemicand anti-atherosclerotic potential of aqueous extract of 12 h germinated *Glycine max* (gGmax) seeds in Streptozotocin (STZ)-induced diabetic rats models. The study elucidates its mechanism of action by observing its effect on glycemic index, insulin resistance, lipid profile, oxidized LDL, apolipoprotein and free radical suppression. Moreover, histomorphological studies of pancreas were also performed to assess the effect on islets of langerhans. Further, the role ofα amylase and α-glucosidase inhibition was also studied in order to assess its potential on post-prandial blood sugar control. Subsequent metabolite profiling was also performed.

Material and Methods

Plant material

The seeds of *Glycine max* were procured, and identified by department of Ayurveda, Yoga and Neuropathy, Unani, Siddha and Homoeopathy (AYUSH), Government of India. The voucher of the specimen was deposited in Botanic Garden of Indian Republic (BGIR), Noida and number was provided (Voucher specimen no-264).

Preparation of plant extracts

The seeds were germinated for 12 h at 37°C after which they were grinded to a fine paste. The seed paste was suspended in 500 ml of distilled water overnight, filtered and then lyophilized. The yield of aqueous extract was approximately 10% of the dried seeds. The stock solutions for inhibition assays were prepared by dissolving up to 1 mg of extract in 1 ml of DMSO and appropriately diluting it before use.

Preliminary screening of germinating *Glycine max* seed extracts: *In-vivo* studies

Induction of diabetes in rats

Diabetes was induced by injection of STZ [Sigma Aldrich, USA] (45 mg/kg body weight in 0.1 M citrate buffer, pH 4.5, intra peritoneally) to overnight fasting rats [9]. After 48 h of STZ administration, fasting blood glucose (FBG) levels were monitored and repeated twice at intervals of three days. The animals with blood glucose level above 200 mg/dL were considered to be diabetic. Institutional approval (IAEC) was taken for the study as per as the CPCSEA guidelines (Reg. No 508/2001/ CPCSEA, IAEC Approval no: UCMS/IAEC/27, dated 30th December 2009).

Experimental studies

The rats were divided into four groups, where group I was labeled as normal control. Group II was labeled as diabetic control and group III and IV comprised of diabetic rats. Group III rats were treated with glibenclamide (600 mg/kg b.wt.) and group IV rats were treated with gGmax seed extracts (200 mg/kg b.wt.).

The control rats received vehicle, i.e. distilled water, and the treated rats received gGmax seed extracts or glibenclamide in 1 ml of distilled water. The treatment was given daily for a period of 28 days using standard orogastric cannula. The blood was drawn from retro orbital plexus of overnight fasted rats by using micro capillary technique [10]. The blood collected in plain vial, was allowed to clot for separation of serum.

Glycemic control

The FBG level was measured by glucose oxidase peroxidase enzymatic method [11]. The estimation of Glycosylated hemoglobin (Hb1Ac) was done by method described by Goldstein et al. [12]. For OGTT, glucose (2 g/kg) was administered to non-diabetic control rats and was treated with glibenclamide (600 µg/kg) as well as gGmax (200 mg/kg) and improvement in glucose tolerance was estimated at an interval of 0, 60 and 120 minutes respectively. Serum insulin and C-peptide levels were measured by ELISA kits from Mercodia.

Calculation of insulin resistance indices [13,14]

- HOMA-IR=[fasting blood glucose (FBG; mg/dl)] × [fasting insulin; µU/ml)]/405.
- 20/(fasting C-peptide × fasting blood glucose)

Management of dyslipidemia

All the lipid profile parameters were performed in serum. Triglycerides (TG) were measured as per the method described [15]. Total cholesterol (TC) was assayed as per the method of Allain et al. [16]. High-density lipoprotein (HDL) was determined bythe method of Burstein [17]. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were calculated by using the formula from Friedwald et al. [18]. ApoB was estimated by immunoturbidity method. LDL/ApoB ratio was used to measure small-dense LDL. oxLDL was estimated by the baseline levels of diene conjugates in lipid fraction of LDL as described [19].

Histomorphological studies

Pancreas was dissected from rats and fixed in 10% neutral formaldehyde for histopathological evaluation. Tissue samples were processed, embedded in paraffin, sectioned (5 µ thick), and stained with haematoxylin and eosin.

In-vitro studies

Screening of gGmax for α-amylase inhibitory activity: Starch-Iodine colour assay

Screening of gGmax for α-amylase inhibitors was carried out in a microtitre plate according to Xiao et al. [20] based on the starch-iodine test. The total assay mixture composed of 40 µl 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.02 units of PPA solution and gGmax at concentration from 10-100 µg/ml (w/v) were incubated at 37°C for 10 min. Then soluble starch (1%, w/v)

was added to each reaction well and incubated at 37°C for 15 min. 1 M HCl (20 µl) was added to stop the enzymatic reaction, followed by the addition of 100 µl of iodine reagent (5 mM I2 and 5 mM KI). The color change was noted and the absorbance was read at 620 nm on a microplate reader. The control reaction representing 100% enzyme activity did not contain any plant extract.

To eliminate the absorbance produced by plant extract, appropriate extract controls without the enzyme were also included. The known PPA inhibitor, acarbose [Sigma Aldrich, USA; purity ≥ 95%], was used a positive control at a concentration range of 10-100 µg/ml. A dark-blue color indicates the presence of starch; a yellow color indicates the absence of starch while a brownish color indicates partially degraded starch in the reaction mixture. In the presence of inhibitors from the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark-blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

In-vitro α-amylase inhibitory assay

The assay was carried out following the standard protocol by Hansawasdi [21] with slight modifications. Starch azure (2 mg) was suspended in a tube containing 0.2 ml of 0.5 M Tris-HCl buffer (pH 6.9) containing 0.01 M calcium chloride (substrate). The tube was boiled for 5 min and then pre-incubated at 37°C for 5 min. gGmax (1mg) was dissolved in 1ml of 0.1% of dimethyl sulfoxide in order to obtain concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. Then 0.2 ml of extract of a particular concentration was put in the tube containing the substrate solution. 0.1 ml of porcine pancreatic amylase in Tris-HCl buffer (2 units/ml) was added to the tube containing the extract and substrate solution. The process was carried out at 37°C for 10 min.

The reaction was stopped by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4°C. The absorbance of resulting supernatant was measured at 595 nm using spectrophotometer. The concentration of the extract required to inhibit 50% of alpha amylase activity under the conditions was defined as the IC_{50} value. The experiments were repeated thrice with the same protocol. The α-amylase inhibitory activity was calculated as follows:

The α-amylase inhibitory activity = (Ac+) – (Ac-) – (As-Ab) / (Ac+) – (Ac-) × 100

Where, Ac+, Ac-, As, Ab are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively.

In-vitro α-glucosidase inhibitory assay

The assay was performed with the standard protocol by Brueggeman [22]. α-glucosidase (2U/ml) was premixed with 20 µl of gGmax at various concentrations (10, 20, 40, 60, 80 and 100 µg/ml) and incubated for 5 min at 37°C. 1 mM para-nitrophenylglucopyanoside (20 µl) in 50 mM of phosphate buffer (pH 6.8) was added to initiate the reaction. The mixture was further incubated at 37°C for 20 min.

The reaction was terminated by addition of 50 µl of 1 M sodium carbonate and the final volume was made up to 150 µl. α-glucosidase activity was determined spectrophotometrically at 405 nm by measuring the quantity of para-nitrophenol released from pNPG. The concentration of extract required to inhibit 50% of alpha glucosidase activity under the conditions was defined as the IC_{50} value. The assay was performed in triplicate.

Antioxidant activity

Ferric reducing ability of plasma (FRAP) was determined as described previously [23]. Briefly, the working FRAP reagent was prepared by adding 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l TPTZ in 40 mmol/l HCl and 20 mmol/l FeCl3.6H2O in the ratio of 10:1:1 respectively. Sample was added, thoroughly mixed and the absorbance was monitored at 593 nm for 4 min. The values are expressed as µmol/ml.

Preliminary Phytochemical Analysis

The gGmax extract was subjected to phytochemical analysis using Gas Chromatography-Mass Spectrometry.

GC-MS Analysis

Analysis of the gGmax extract was performed on a GC-MS-Triple quad (Agilent 7890 A) gas chromatograph equipped with DB 5 ms capillary column (30 m × 0.25 mm ID). Helium was the carrier gas with flow rate of 1 ml/min, the injector mode-split (1:11), the injection volume 2 µl, the temperature program used is as follows: 80°C (3 min), then increased to 280°C at 10°C/min, held at 280°C (10 min) and temperature scan, m/z 35-300 amu. Appropriate solvent controls were also run. The identification of the components was based on the comparison of their mass spectra with those of NIST-Wiley 2008 library.

Statistical analysis

Statistical analysis was done by PRISM-6 and MS-EXCEL. Values are expressed as the mean ± SEM for five animals in each group. The data was analysed by using repeated measure analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and one-way ANOVA followed by Tukey's multiple comparison test. The results were considered significant at $P<0.05$. The experiments for α-amylase and α- glucosidase were done in triplicate. IC_{50} values were calculated by plotting Lineweaverburk plot.

Results and Discussion

Oral administration of gGmax extract at dose of 200 mg/kg body weight for the period of 28 days exhibits remarkable glycemic control in the diabetic rats. The results summarized in the Table 1 shows statistically significant decrease in FBG ($p<0.0001$) and Hb1Ac ($p<0.001$) levels in diabetic rats treated with gGmax extract after a period of 28 days. Glucose tolerance was also found to be significantly improved after the treatment duration.

The effect of gGmax extract on serum insulin and C-peptide levels is recapitulated in Figure 1A. A significant decrease ($p<0.0001$) in the levels of insulin was observed in diabetic control rats as compared to normal rats. Administration of gGmax extract significantly increased ($p<0.001$) the levels of insulin. The drug glibenclamide also produced significant elevation in the insulin levels ($p<0.001$) in diabetic rats. However, in gGmax extract treated rats the increase in insulin level was found to be significantly higher when compared to ($p<0.001$) glibenclamide ($p<0.01$) treated rats. A significant depletion ($p<0.001$)

in the C-peptide levels was also observed in diabetic control (Figure 1B). The gGmax extract treated diabetic rats showed significant increase in ($p<0.01$) the C-peptide levels. The elevation in the level of insulin in gGmax extract treated rats suggests that it is able to potentiate the release of insulin from pancreatic β-cells. The antihyperglycemic activity of gGmax extract could be due to its insulinogenic action as increased levels of insulin and C-peptide were found in gGmax extract treated diabetic rats.

Insulin resistance indices are paraphrased in Figure1C and 1D. Both the indices indicate the improvement in insulin resistance after administration of the extract for 28 days. The improvement was significant when compared to the diabetic control ($p<0.001$).

The effect of gGmax extract on serum lipidprofile is summarized in (Table 2). A significant increase in the serum lipids, except HDL-c, was observed in diabetic rats when compared with the healthy rats ($p<0.001$). Treatment with gGmax extract for 28 days shows a marked reduction ($p<0.001$) in levels of TC, TG, LDL and VLDL. This lipid lowering effect produced by gGmax was found to be better than that of glibenclamide. The increased TG and TC levels and decreased HDL levels were known factors associated with coronary heart disease. Recent studies suggest that TG itself is independently related to CVD and most of the anti-hypercholesterolemic drugs do not decrease TG levels [24]. As the gGmax produced favourable effect on these factors, this suggests that the extract may help to prevent the progression of cardiovascular diseases.

Experimental groups	F86 (mg/dl)	GHb (%)	Body Weight (grams)		OGTT (mg/dl)
			Initial	Final	
Normal Control	107.6 ± 7.12	3.42	252 ± 2.00	301 ± 7.80	146 ± 332
Diabetic Control	265.8 ± 9.65$^{\#}$	5.94	175 ± 11.61	170 ± 6.89	390.6 ± 5.75$^{\#}$
Glib. treated	115.6 ± 9.34***	4.80	192 ± 6.63	243 ± 8.60	298.4 ± 13.96***
gGmax treated	114.4 ± 11.10***	4.12*	188 ± 4.63	246 ± 10.41	255 ± 8.83***
Values are given as means ± SEM. #p<0.0001 as compared with normal control group, *p<0.01, ***p<0.001 as compared with diabetic control group.					

Table 1: Effect of gGmax on glycemic response and body weight in STZ induced diabetic rats.

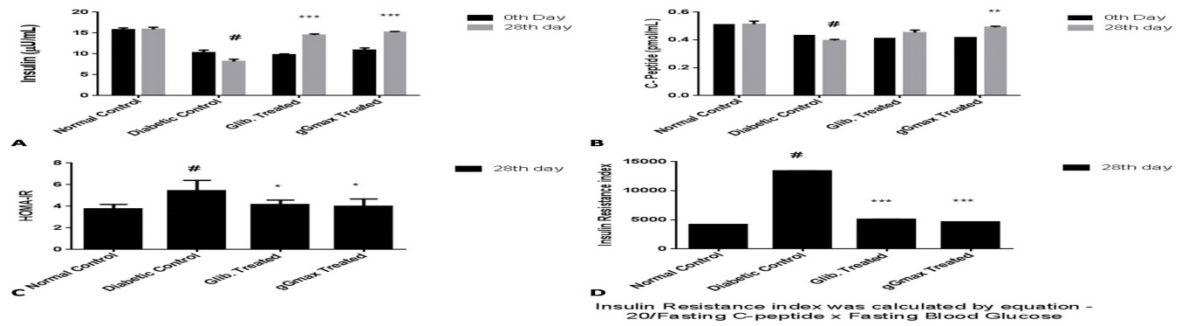

Figure 1: Effect of gGmax on (A) serum insulin, (B) serum c-peptide levels (C) HOMA- IR and (D) 20/Fasting C peptide X Fasting blood glucose in STZ induced diabetic rats. Values are given as means ± SEM. #p<0.0001 as compared with normal control group, *p<0.01, **p<0.001, ***p<0.0001 as compared with diabetic control group.

Groups	Serum Lipid profile (mg/dl)				
	TG	TC	HDL	LDL	VLDL
Normal control (veluele)	72.08 ± 6.91	77.8 ± 7.2	36.4 ± 3.3	26.84 ± 3.5	14.56 ± 1.23
Diabetic control (vehicle)	211.6 ± 9.3*	176.8 ± 8.7	21.4 ± 3.1*	113.08 ± 5.6*	42.32 ± 4.5*
Diabetic+Glib. (600 µg/kg)	100.8 ± 4.6****	97.6 ± 5.5	34 ± 3.6****	43.44 ± 6.03****	20.16 ± 1.9****
Diabetic+12 gGmax (200 mg/kg)	90 ± 2.7****	89.4 ± 3.7	32 ± 3.3****	39.4 ± 2.1****	18 ± 0.97****
Values are given as means ± SEM. # p<0.0001 as compared with normal control group, ****p<0.0001as compared with diabetic control group.					

Table 2: Effect of gGmax on serum lipid profile in STZ induced diabetic rats.

A significant reduction in Ox-LDL, sd-LDL and apoB levels was observed ingGmax extract treated rats (Figure 2). Moreover the antioxidant power of gGmax extract was found to be 136.35 µmol/ml (Figure 3).

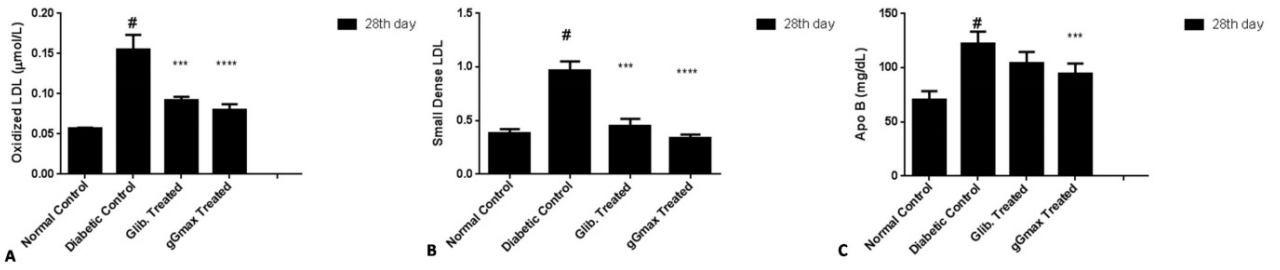

Figure 2: Effect of gGmax on (A) Ox LDL, (B) sd LDL, (C) Apo B in STZ induced diabetic rats. Values are given as means ± SEM. #p<0.0001 as compared with normal control group, ****p<0.0001, ***p<0.001 as compared with diabetic control group.

Histomorphological examination of pancreatic tissue from STZ-induced diabetic control rats (Figure 4A) showed reduction in the number of islets and β-cells, however pancreatic tissues of gGmax extract treated rats (Figure 4B) revealed an increase in the number of islets and β-cells as compared to the diabetic control rats. The reports suggest that STZ, a beta cytotoxin, acts through rapid depletion of pancreatic β-cells which leads to reduction in insulin secretion [25]. However, treatment with gGmax extract leads to the increased number of islets and β-cells. This interprets that gGmax extract produces insulinogenic effect through the regeneration of β-cells.

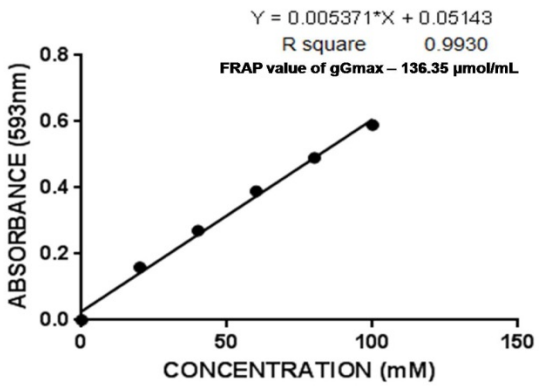

Figure 3: Linear nature of FRAP standards and antioxidant power of gGmax in STZ induced diabetic rats.

Screening for α-amylase inhibition was performed based on starch-iodine color complex formation. The rats treated with gGmax extract tested positive for inhibition of α-amylase and was further quantified with respect to PPA inhibition as per modified method given by Hansawasdi et al. [21]. It was noted that it exhibited significant α-amylase inhibitory activity. The percentage inhibition at varied concentrations (10, 20, 40, 60, 80, 100 µg/ml) of gGmax extract showed concentration dependant reduction in percentage inhibition (Figure 5A). The highest concentration of 100 µg/ml of gGmax extract showed maximum inhibition of 79.2%. Acarbose was taken as a positive control with a maximum inhibition of 81.1%. The IC_{50} values for the acarbose and gGmax extract were also calculated by plotting lineweaverburk plot. The IC_{50} of acarbose is found to be 67.77 µg/ml whereas gGmax extract exhibited an IC_{50} value of 28.01 µg/ml which is less than acarbose (Figure 5B and 5C).

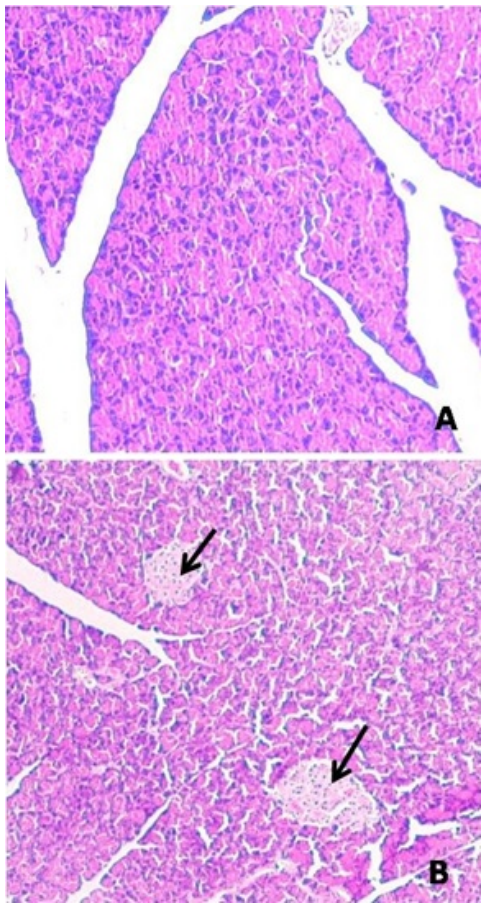

Figure 4: Histopathological findings of pancreas (haematoxylin and eosin stained figures). (A) Diabetic control rats showing reduced islets, (B) Diabetic rats treated with gGmax showing regeneration of islets.

Subsequently, the effect of gGmax was studied on inhibition of α-glucosidase activity. The *in-vitro* assay confirmed the powerful alpha

glucosidase inhibitory activity of gGmax when compared with that of acarbose. The IC_{50} values were found to be 14.01 µg/ml and 18.51 µg/ml of acarbose and gGmax respectively (Figure 6A-6C). The results confirmed that gGmax extracts hold a promising therapeutic value in controlling post-prandial hyperglycemia.

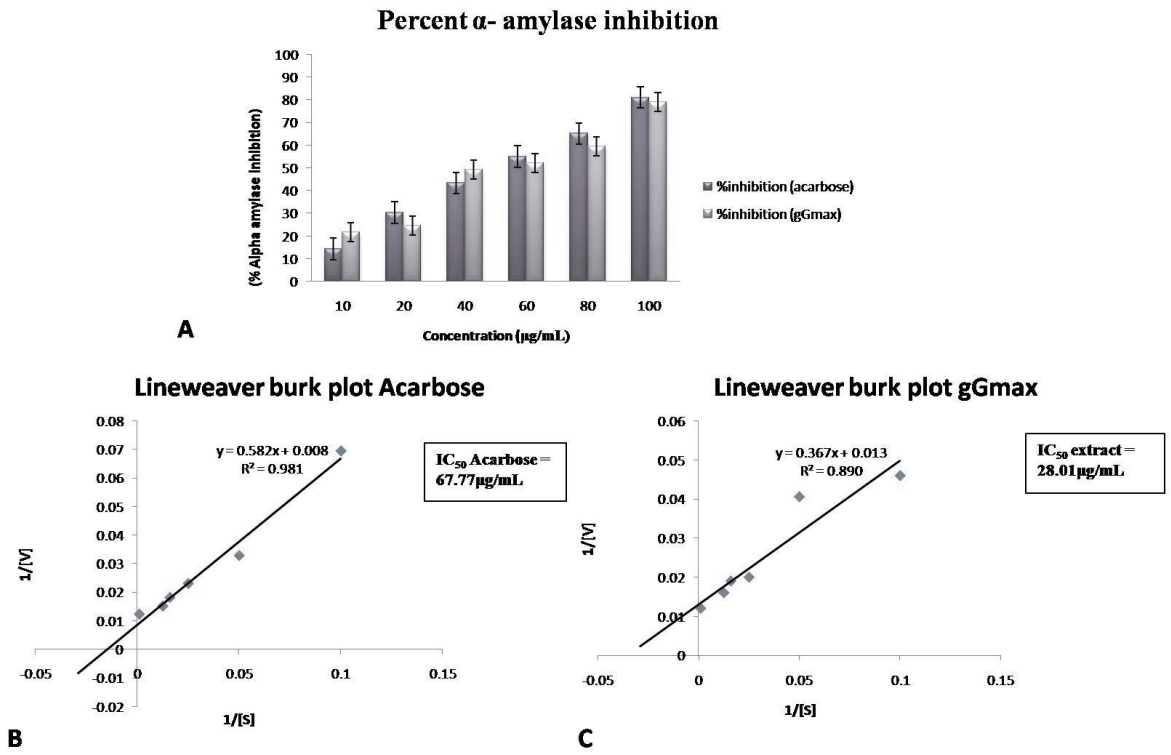

Figure 5: (A) The percentage inhibition of α-amylase at varied concentrations (10, 20, 40, 60, 80, 100 µg/ml) of plant gGmax and acarbose, (B) Lineweaverburk plot of Acarbose (standard drug) with calculation of IC_{50} which is 67.77 µg/ml for acarbose, (C) Lineweaverburk plot of gGmax with calculation of IC_{50} which is 28.01 µg/ml for gGmax.

Compounds	Molecular weight	Retention time	Area %
Undecanoic acid	186	20.35	96.02
Hexadecanoic acid or	256	27.82	5.33
Palmitic acid	270	28.32	7.13
Genistein Daidzein	254	28.51	5.12
Cinnamic acid ester	652	28.71	5.69
Isoquinoline alkaloid	403	29.1	48.74
4- ethoxy Benzoic acid, ethyl ester	194	29.23	16.28
Myristic acid	228	28.45	7.5
3-eicosene	280	29.67	6.15
Phthalic acid ester	278	31.46	50.01

Table 3: GC- MS analysis: Major identified phytochemicals present in gGmax.

The phytochemical analysis of gGmax extract was done toelucidate the major chemical constituents present in it. The extract prominently comprises of several fatty acids, alkaloids, saponins and flavonoids. The major identified constituents of gGmax extract are summarized in Table 3.

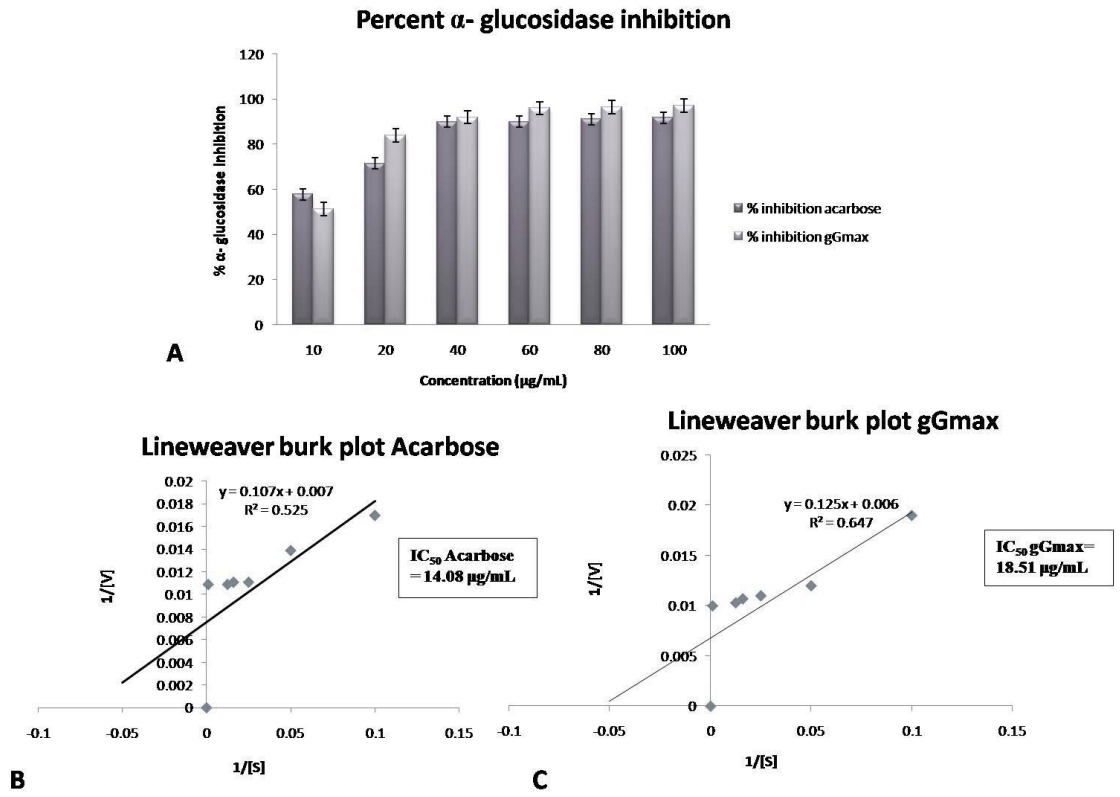

Figure 6: (A) The percentage inhibition of α-glucosidase at varied concentrations (10, 20, 40, 60, 80, 100 µg/ml) of plant gGmax and acarbose, (B) Lineweaverburk plot of Acarbose (standard drug) with calculation of IC_{50} which is 14.08 µg/ml for acarbose, (C) Lineweaverburk plot of gGmax with calculation of IC_{50} which is 18.51 µg/ml for gGmax.

Therefore, it is concluded that, gGmax extract exhibits a strong antihyperglycemic activity. It suppresses postprandial glycemic response by controlling OGTT as well as inhibiting α- amylase and α-glucosidase activity. It improves the glycemic response by regeneration of β- cells. It is evident from the results that gGmax possesses anti-hyperlipidemic and antioxidant potential also. These activities indicate the role of gGmax as a potent anti-atherosclerotic agent and thus may prevent the progression of cardiovascular diseases. Plant based therapeutics are prospective medications of future and gGmax presents a strong candidate in this pipeline.

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