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HYPOGLYCEMIC ANTIOXIDANT POTENTIAL OF TETRAHYDROXY FLAVONE (FISETIN) IN EXPERIMENTAL ANIMAL MODEL

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KEYWORDS:

Antioxidant, Fisetin, Streptozotocin, Diabetes, Oxidative Stress.

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ABSTRACT

Fisetin (3, 3 ′4 ′, 7- tetrahydroxyflavone) was evaluated for its protective effect against Streptozotocin induced diabetes in experimental rats. Fisetin is one of the naturally occurring flavonoids, it exhibits a wide variety of therapeutic benefits. Fisetin is a dietary flavonoid (widely distributed in strawberries, apples, persimmons, grapes, onions and cucumbers) which displays a variety of pharmacological properties including antioxidant. Our present study was carried out to evaluate the antidiabetic effect of Fisetin in Normal and Streptozotocin-induced diabetic rats. The oral feeding of Fisetin suspended in 0.5% DMSO and water administered orally to diabetic rats for 28 days at a dosage of 50mg/kg body weight exhibited a significant ($p<0.001$) reduction in FBG level and a remarkable increase in serum insulin level. There was a significant reduction ($p<0.001$) in serum parameters *viz.*, AST, ALT, lipids, TG, TC, urea, TBARS, and albumin in diabetic rats treated with Fisetin. Vitamin-C & E, ceruloplasmin, reduced glutathione and LPO levels were estimated in plasma of control and experimental groups of rats. The levels of lipid peroxides, reduced glutathione and activities of SOD, CAT and glutathione peroxidase were assayed in pancreatic tissue of control and experimental groups of rats. A significant increase in the levels of vit-E, ceruloplasmin, lipid peroxides and a concomitant decrease in levels of vit-C, reduced glutathione were observed in diabetic rats. The activities of pancreatic antioxidant enzymes were altered in diabetic rats. The body weight of diabetic rats was restored to normalcy state when treated with the Fisetin. Morphometric analysis of Fisetin treated rats islets of pancreas showed a significant ($p<0.001$) increase in the number and area of islets cells when compared with normal and diabetic control rats. Histopathology studies in liver and kidney of diabetic rats treated with Fisetin did not show any marked difference from normal which revealed the non-toxic effect of antioxidant. Based on the above results it is evident that Fisetin, has antidiabetic and antioxidant effect and must be considered as a potential candidate for future studies on diabetes mellitus and pharmacological studies.

INTRODUCTION:

Fisetin is a flavonol, a structurally distinct chemical substance that belongs to the flavonoid group of polyphenols. It can be found in many plants, where it serves as a colouring agent. Fisetin is one of the naturally occurring dietary flavonoids, it exhibits a wide variety of therapeutic benefits. Fisetin (3, 3′, 4′, 7- tetrahydroxyflavone) is a dietary flavonoid (widely distributed in strawberries, apples, persimmons, grapes, onions and cucumbers) which displays a variety of pharmacological properties including antioxidant [1], anti-allergic [2], anti-inflammatory [3], anti-cancer [4], neuroprotective [5,6] and neurotrophic [7] characteristics.

Streptozotocin (2-deoxy-2-[3-methyl-3-nitrosourea] 1-D-glucopyranose) is a permanent diabetogenic compound, produced by the gram positive soil bacterium *Streptomyces achromogenes* that exhibits broad spectrum of antibacterial properties [8]. STZ induces diabetes mellitus in laboratory animals by killing insulin-producing pancreatic β-cells. Streptozotocin is toxic glucose analogues that preferentially accumulate in pancreatic beta cells via the low affinity glucose transporter GLUT2. Streptozotocin is widely used to induce diabetes in rodent models by inhibition of β-cell O-GlcNAcase [9-13]. Streptozotocin features four important biological properties as evidenced by its antibiotic, β-cell (beta)-cytotoxic, oncolytic, as well as oncogenic effects [14-16]. This product is an antineoplastic antibiotic and is used mainly in the treatment of pancreatic (islet cell) tumors [13]. Used for treatment of malignant insulinoma [17].

Materials and Methods

Chemicals

Fisetin was purchased from Shanxi Jintai Biological (China). Streptozotocin (STZ) was purchased from Sigma (St Loius, MO, USA), Glucose Oxidase/Peroxidase (GOD/POD) reagent (glucose kits were obtained from Randox Laboratories Ltd, UK). Serum analysis was done using kit by Biocrest Systems. All other chemicals used were of good quality and analytical grade.

Oxidative Stress

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing [18-21]. These diseases fall into two groups: (i) the first group involves diseases characterized by

pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance the so-called “mitochondrial oxidative stress” conditions (cancer and diabetes mellitus), (ii) the second group involves disease characterized by “inflammatory oxidative conditions” and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase - induced formation of ROS (implicated in ischemia and reperfusion injury). The process of ageing is to large extent due to damaging consequence of free radical action [22], (lipid peroxidation, DNA damage, protein oxidation).

Animal Model

Male albino mice (150±200g), (*Rattus novergcus*) were procured from Tamil Nadu University for Veterinary and Animal Sciences, (TANUVAS) Chennai, India were used for the study. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water was provided *ad libitum*. The rats were housed under conditions of controlled temperature (22±2°C) and acclimatized to 12-h light, 12-h dark cycle. Animal experiments were conducted according to the guidelines of institutional animal ethical committee. All the drugs (standard and test as well as vehicle were administered per-orally using insulin syringe.

Induction of Diabetes

Animals were injected with Streptozotocin (STZ) dissolved in citrate buffer (pH 4.4) at a dose of 35 mg/kg body weight intraperitoneally. Diabetic state was confirmed by the 3rd day and rats whose fasting plasma glucose (FPG) levels > 200 mg/dl were considered to be diabetic.

Experimental Design

Seggregation of Groups

Experimental animals were divided into 3 groups of 14 rats as follows and the Fisetin were suspended in 0.5% DMSO and in water, were administered orally and Streptozotocin (STZ) dissolved in citrate buffer (pH4.4) at a dose of 35 mg/kg body weight intraperitoneally in the following way:

Group I **:** Served as Normal rats with a dose of Fisetin 50mg/kg body weight orally for 28 days.

Group II : (Diabetic Control) Rats were made Diabetic by a single i.p. injection of STZ at a dose of 35mg/kg body weight with normal water and diet for 28 days.

Group III : (Fisetin Treated) Rats were made Diabetic by a single i.p. of STZ and fed with Fisetin 50mg/kg body weight for 28 days.

Biochemical Assays

During the experimental period, on the 28th day of the animals were sacrificed under sodium pentathione anesthesia. The rats were sacrificed and blood was collected immediately for serum separation and organs *viz.*, pancreas, liver, and kidney were dissected out and stored in 10% formalin for histopathological studies and rest stored at -20˚C for biochemical analysis and assays. Plasma glucose level was estimated by GOD-POD method of Trinder [23]. Protein, urea, albumin, triglycerides, cholesterol, ALP, serum AST, ALT, was estimated using approximate kits by Biocrest Systems. Total lipids in serum, liver and kidney were estimated using the method of Frings [24]. Liver and kidney TBARS were estimated by the method of Okhawa [25]. Liver and kidney cholesterol, triglycerides estimated using kit by Biocrest systems.

Histological Study

After the last dose, animals were sacrificed and blood was collected in falcon tubes containing anticoagulant. For serum collection another vial was used without anticoagulant. The blood cells were allowed to settle and then serum was separated by centrifugation at 3000 g at 37ºC. Thus blood and serum were used for determination of various biochemical parameters. Organs *viz.*, Pancreas, Liver and Kidney were taken and fixed in 10% formalin. The specimens were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax. Sections of 5 µm in thickness were prepared and stained with haematoxylin and eosin then examined under microscope.

The pancreas were excised, rinsed in ice-cold saline and then homogenized in Tris-HCl buffer of pH7.4 (0.1M) using a Teflon homogenizer. The pancreatic 10% (w/v) tissue homogenate was then centrifuged in a cooling centrifuge at 500 g to remove the debris and the supernatant was used for the analysis of biochemical parameters. The tissue homogenate was placed at 20^{o}C until further use.

Morphometry Studies

Morphometric studies of islets of pancreas were done using image processing software (Image Analysis). Area and diameter of pancreatic islets was measured and results represented in micrometer and compared with control for statistical significance.

Statistical Analysis

Data were statistically evaluated using one way ANOVA and expressed as Mean±SD. Kruskall Wallis test and Mann Whitney U test using 11.0 version of SPSS Software were used when applicable. $p \leq 0.05$ was considered to be significant.

Results and Discussions

Changes in the Body Weight

Graph 1 shows the changes in body weight in the normal and experimental animals in each group. The mean body weight of the diabetic rats decreased with respect to Fisetin-treated rats and a significant decrease in body weight of the diabetic rats compared with normal and Fisetin-treated diabetic rats. After treatment with Fisetin for 28 days there was a significant increase in the body weight of diabetic rats ($p<0.001$).

Our study showed that oral administration of Fisetin decreased the blood glucose level in diabetic rats. We have reported that, the administration of Fisetin to STZ-induced diabetic rats optimized the activities of carbohydrate metabolizing enzymes and thus maintained blood glucose levels. Administration of medicinal plant extract to mildly STZ-diabetic rats resulted in activation of h-cells and granulation returns to normal giving insulinogenic effect [26]. Hence Fisetin may bring about its hypoglycemic action through stimulation of surviving h-cells of islets of langerhans to release more insulin. Since the percentage fall in plasma glucose levels was different in models with varying intensity of hyperglycemia it implies that the anti-hyperglycemic effect of that plant is dependent upon the dose of diabetogenic agent and therefore on the degree of h-cell destruction [27]. A number of other plants have also been observed to exert hypoglycaemic activity through insulin-release stimulatory effects [28,29]. Most of the tissue damage is consider to be mediated by these free radicals by attacking membranes through peroxidation of unsaturated fatty acids [30]. In the present study the

concentrations of lipid peroxides were increased in diabetic rats, which caused the development of diabetes, indicating an increase in the generation of free radicals. The increase in the levels of lipid peroxides in plasma generally is thought to be the consequence of increased production and liberation into the circulation of tissue lipid peroxides due to pathological changes [31]. An observed increase in the level of TBARS in pancreas may be due increased susceptibility of the tissue of diabetic rats to lipid peroxidation [32]. The present finding indicates significantly increased lipid peroxidation of rats exposed to STZ and its attenuation by Fisetin treatment. Our study suggests protective role of Fisetin, which could be due to the antioxidative effect hence act as strong superoxide radical and singlet oxygen quenchers. In our investigation low level of vitamin C and high level of vitamin E were observed in diabetic rats. Vitamin C is a key antioxidant, particularly protecting lipids from peroxidative damage by aqueous solution [33], has reported that vitamin C depletion leads to formation of hydroperoxides even when other antioxidants are still present. The low levels of vitamin C in diabetes may be due to increased utilization in trapping the oxyradicals.

Vitamin E is a lipophilic antioxidant and inhibits lipid peroxidation, scavenging lipid peroxyl radicals to yield lipid hydro-peroxides and the α-tocopheroxyl radical [34]. Vitamin E is used in combating free radicals and if vitamin C is present, vitamin E levels are preserved. The high levels may also be due to low levels of vitamin C or the storage of vitamin E by diabetic rats when compared with the controls [35].

The plasma protein, ceruloplasmin is a powerful free radical scavenger that oxidizes iron from the ferrous to ferric state. Ceruloplasmin levels increase under conditions leading to the generation of oxygen products such as the superoxide radical and hydrogen peroxides. The low levels of ceruloplasmin found in diabetic rats treated with Fisetin, in our study may be a protective response to a low level in circulating unbound Fe^{2+}, which may act as a inhibitor for further free radical induced lipid peroxidation.

GSH reacts with free radicals and is a crucial substrate for GPx and GST, which takes part in the cellular defense mechanisms against intermediate oxygenated products of metabolism. The observed decrease in GSH level in diabetic groups of rats represents high level of utilization due

to oxidative stress [36]. A relative decrease of NADPH due to aldose reductase activation and secondary to reduced production through the pentose cycle impairs GSH regeneration and leads to depletion of this free radical scavenger [37]. The effects of Fisetin on plasma glucose and pancreatic lipid peroxidation produced by STZ may be related to the significant rise in pancreatic and plasma glutathione induced and have proposed that the ratio of GSH/ GSSG plays a crucial role in the glucose homeostasis of diabetes. It has been suggested that thiol groups are important in the intracellular and membrane redox state of the secretory function of h-pancreatic cells. The Fisetin induced high level of glutathione content in plasma and pancreas, which may enhance the GSH/GSSG ratio and therefore improve plasma glucose regulation. The pancreas has been reported as the organ with the lowest levels antioxidant enzymes and pancreatic h-cells are considered to be exceptionally vulnerable to the cytotoxic actions of oxygen free radicals because of their relatively low levels of antioxidant enzymes [38]. In our findings, the high levels of CAT, SOD and GPx activities were observed in STZ-induced diabetic rats. The increase in CAT activity may be a compensatory response to an increase in endogenous H_2O_2 production in diabetic pancreas.

Insulin deficiency promotes the h-oxidation of fatty acids with resulting H_2O_2 formation [39]. Increase in SOD activity could be due to its induction by increased production of superoxide, which has been implicated in cell dysfunction. H_2O_2 has been reported to act as an inducer of tissue SOD [40], and hence high levels of pancreatic SOD is observed which correlates in our present study. Increased activity of GPx in pancreas of diabetic rats has been reported [41]. Hence, our study, has found that Fisetin maintains the pancreatic activity of SOD and CAT at control level. The altered SOD and CAT activity in diabetic rats treated with Fisetin indicates an adaptive mechanism in response to oxidative stress.

Biochemical Studies

Fisetin treated rats and there FPG level are shown in Graph 2, produced a significant reduction in the fasting blood plasma level in diabetic rats ($p<0.001$) and a decrease of 45% were compared with that of diabetic control.

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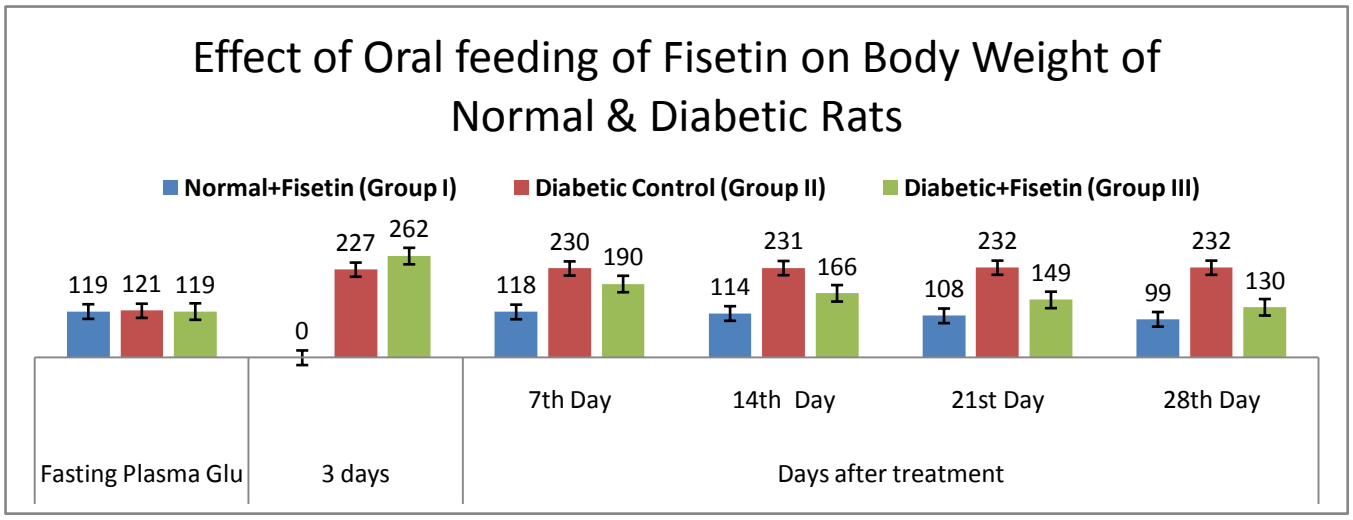
Significant reduction in serum parameters of protein, urea, albumin, lipids, total cholesterol, triglycerides, AST and ALT levels were seen in Fisetin treated group (Graph 3, 4, 5) when compared with diabetic control ($p<0.001$).

Morphometric Studies

Morphometric studies in rat islet pancreatic islets (n=6) showed an increase in area and diameter in Fisetin (3, 3′, 4′, 7- tetrahydroxyflavone) treated diabetic rats when compared with diabetic control which was then compared statistically using Mann Whitney U test & Kruskal Wallis test ($p<0.005$).



Graph 1. Effect of oral feeding of Fisetin on body weight of normal and diabetic rats.

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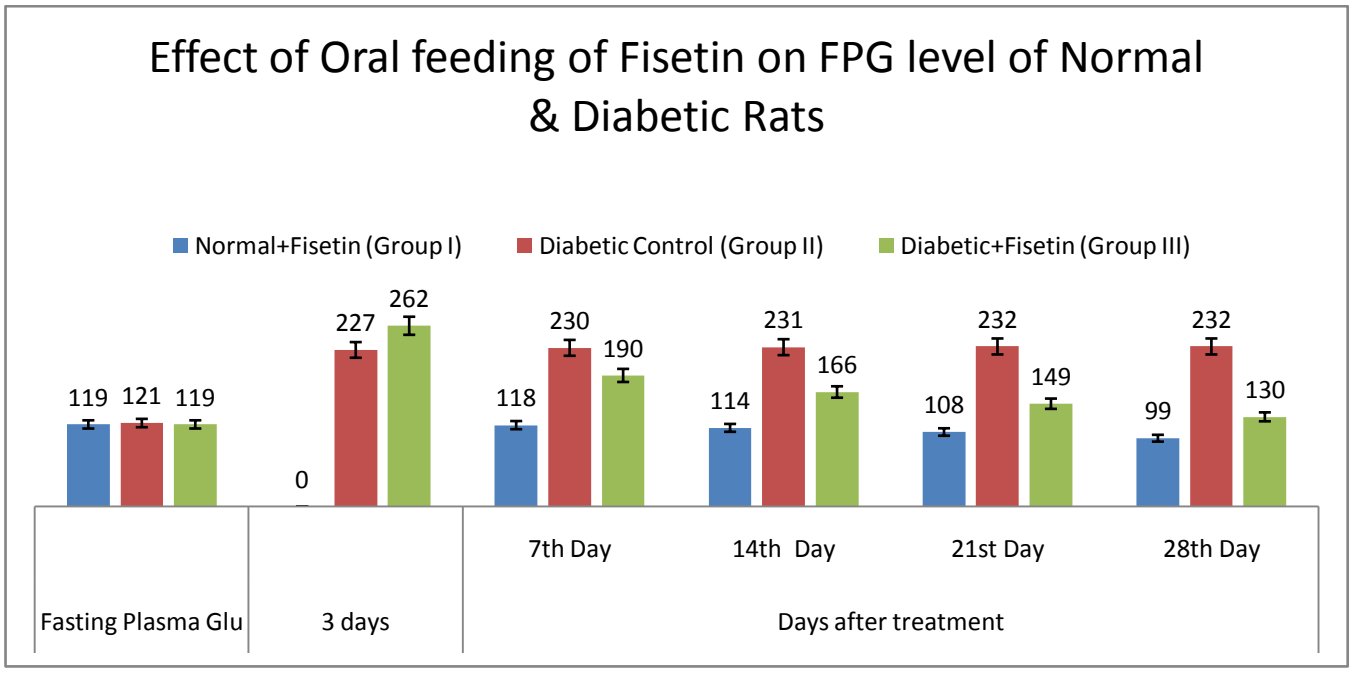
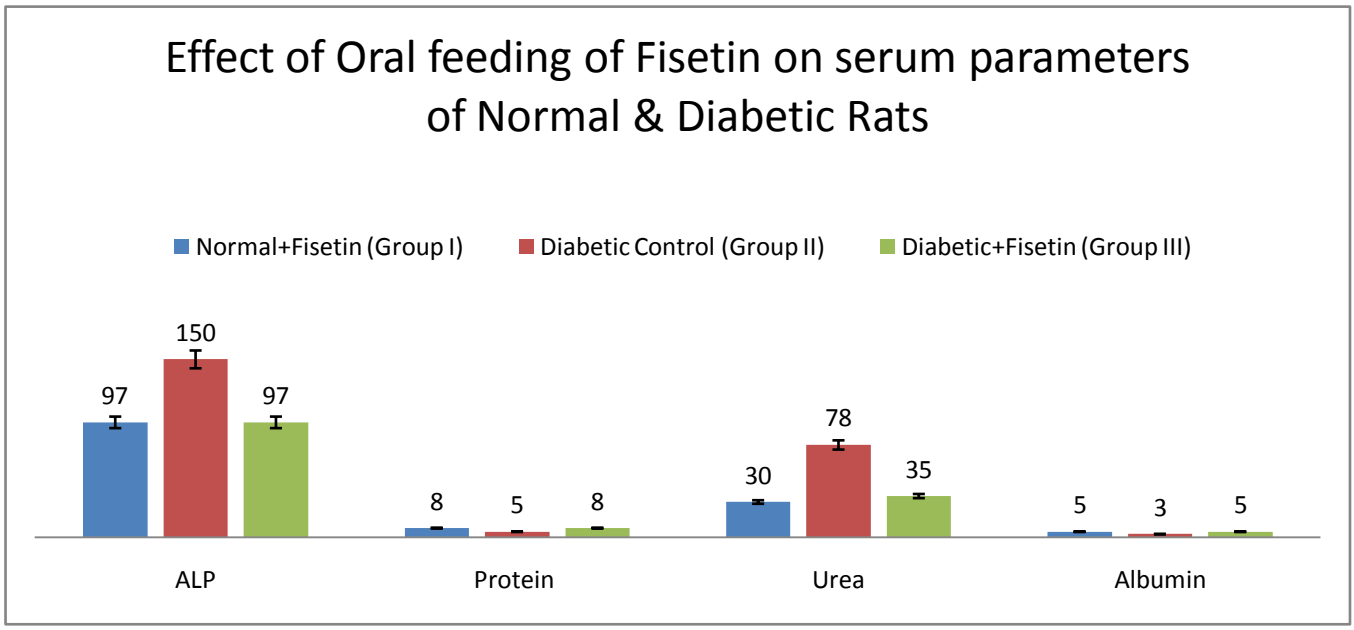


Graph 2. Effect of oral feeding of Fisetin on fasting plasma glucose level of normal and diabetic rats.



Graph 3. Effect of oral feeding of Fisetin on serum parameters of normal and diabetic rats.

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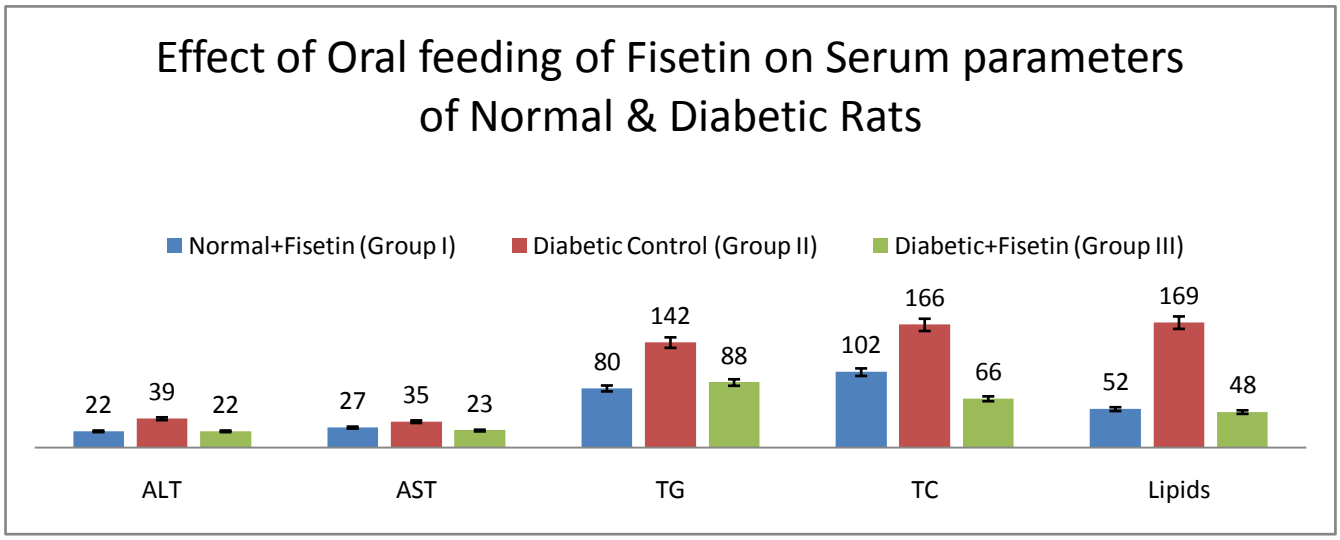
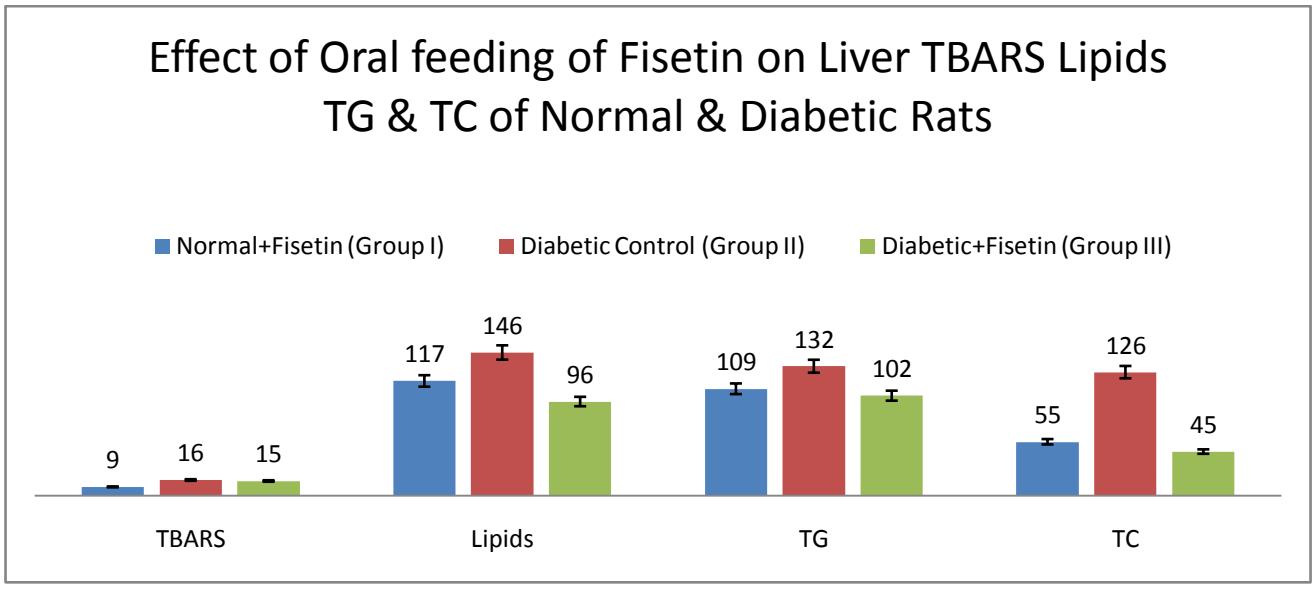


Graph 4. Effect of oral feeding of Fisetin on serum parameters of normal and diabetic rats.



Graph 5. Effect of oral feeding of Fisetin on liver TBARS, Lipids, TG and TC of normal and diabetic rats.

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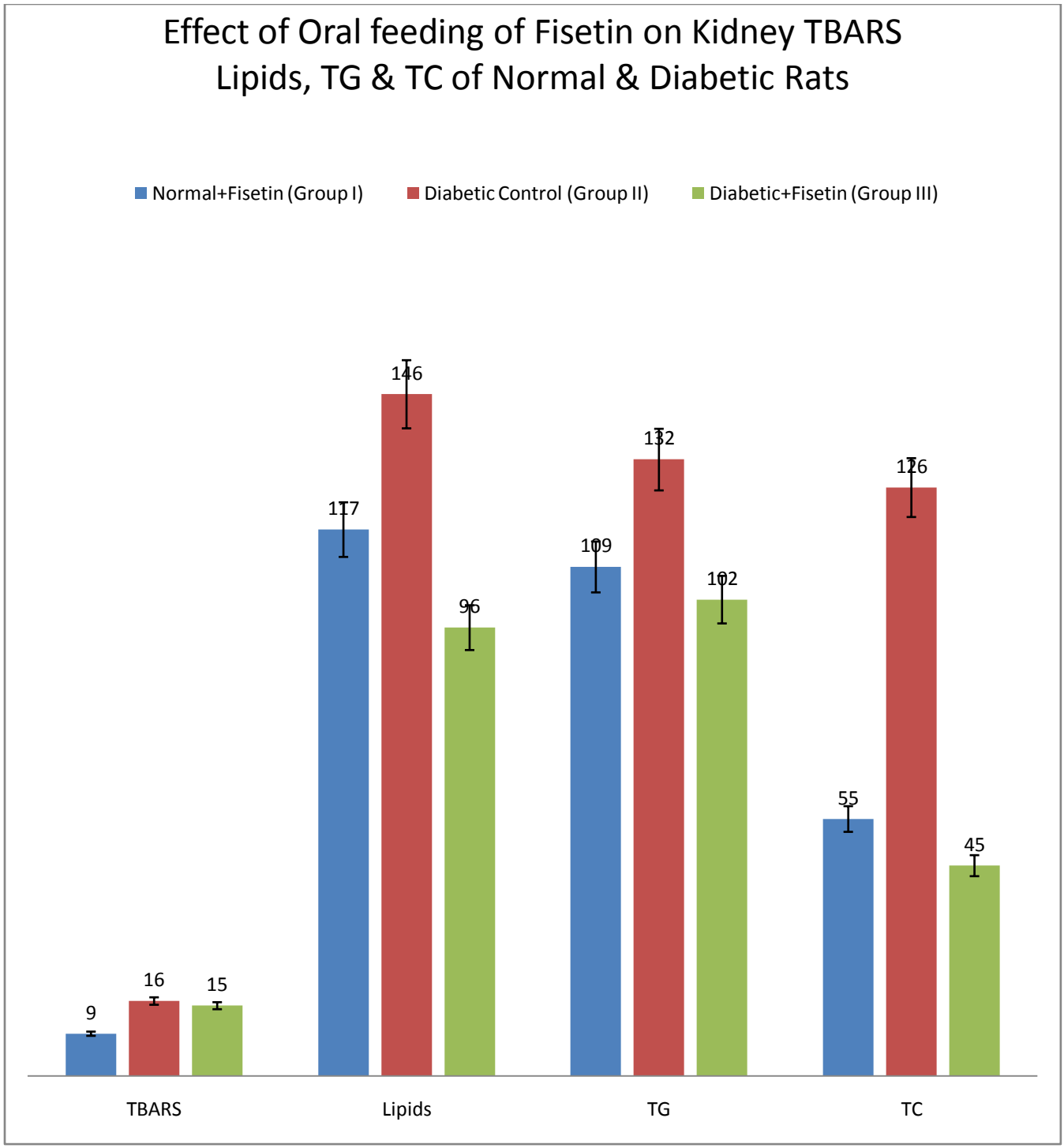

Graph 6. Effect of oral feeding of Fisetin on kidney TBARS, Lipids, TG and TC of normal

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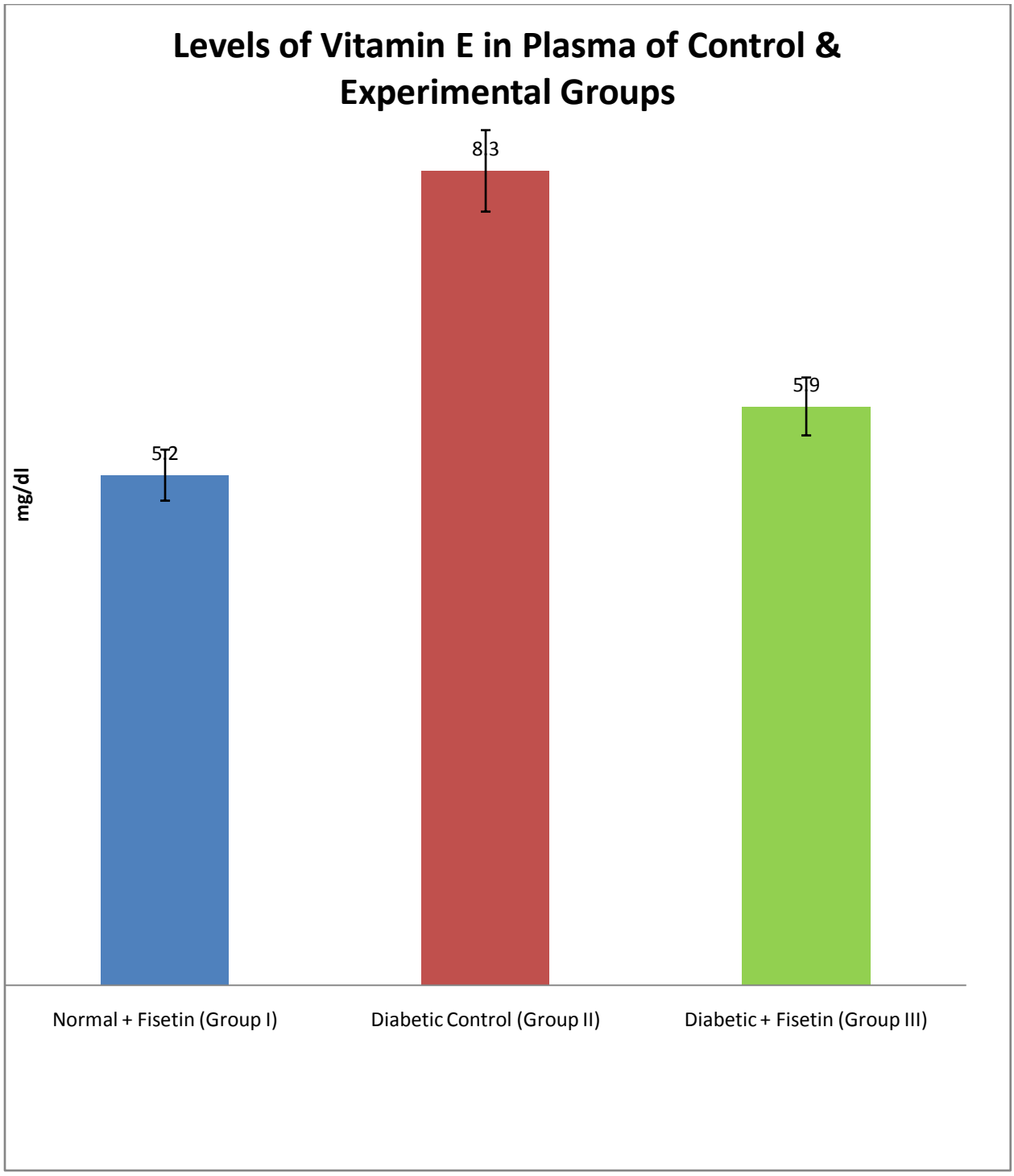

Graph 7. Levels of Vitamin E in plasma of control and experimental groups of rats.

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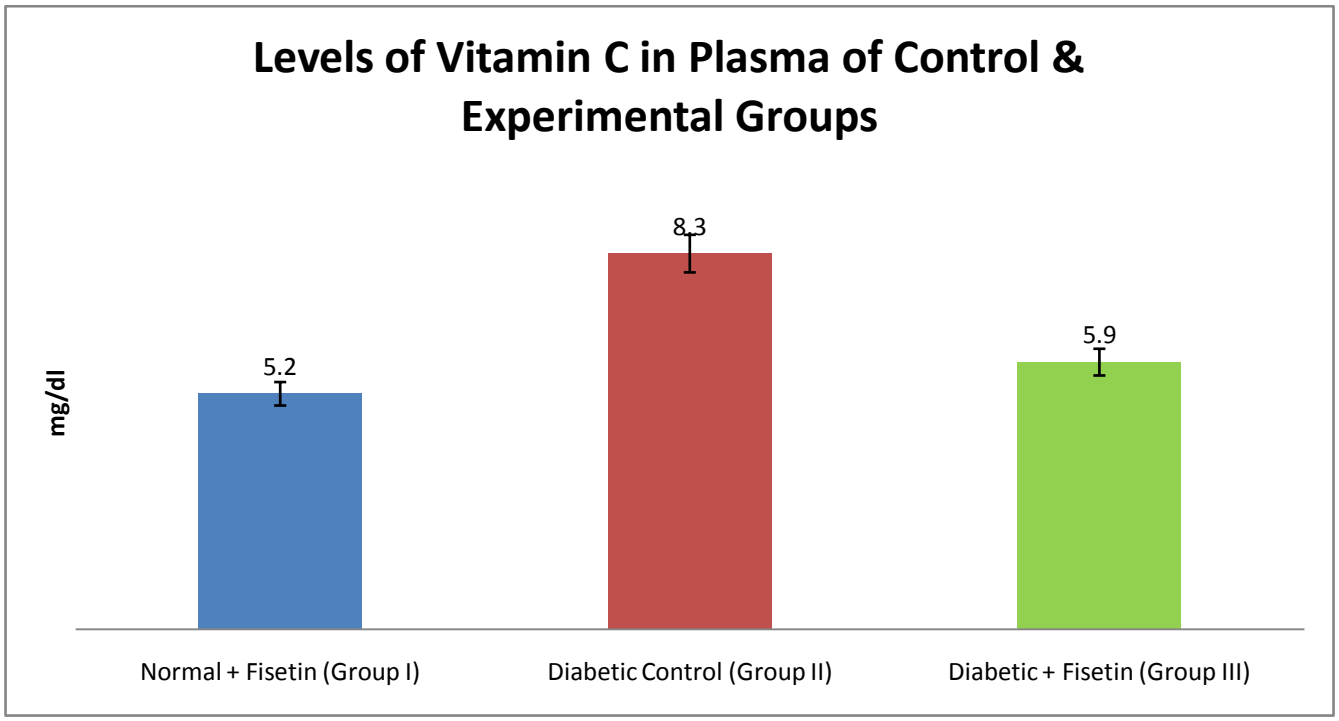
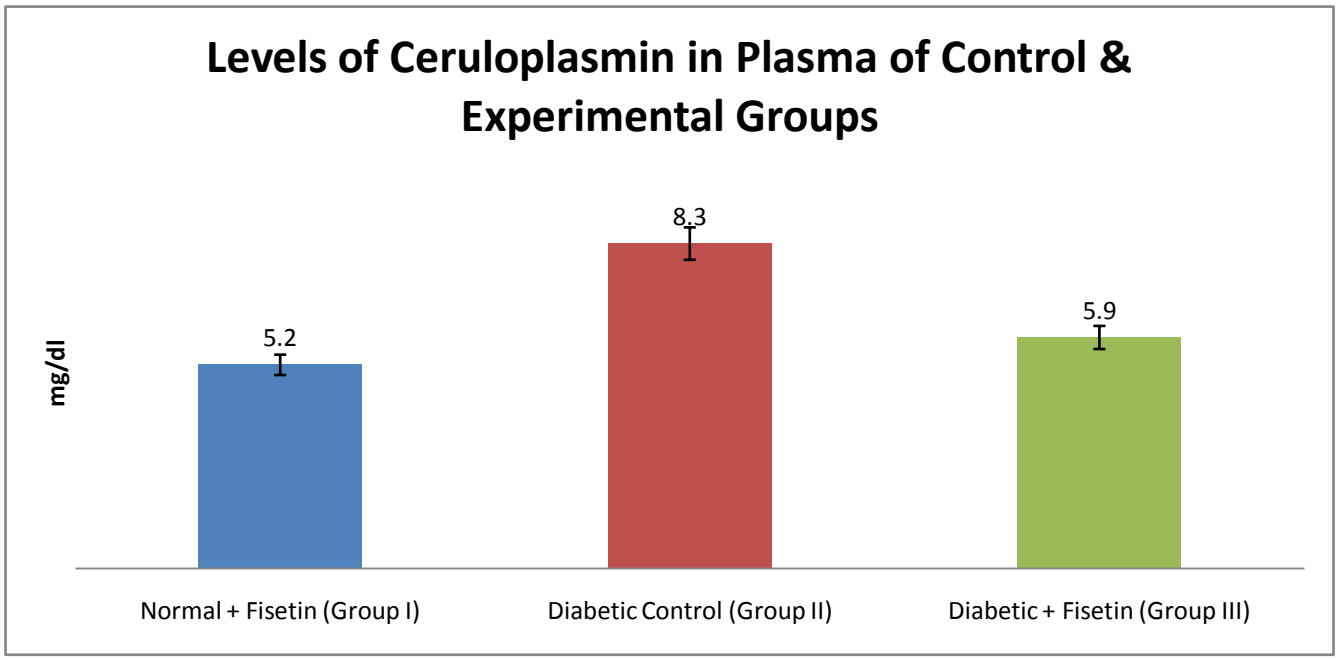


Graph 8. Levels of Vitamin C in plasma of control and experimental groups of rats.



Graph 9. Levels of Ceruloplasmin in plasma of control and experimental groups of rats

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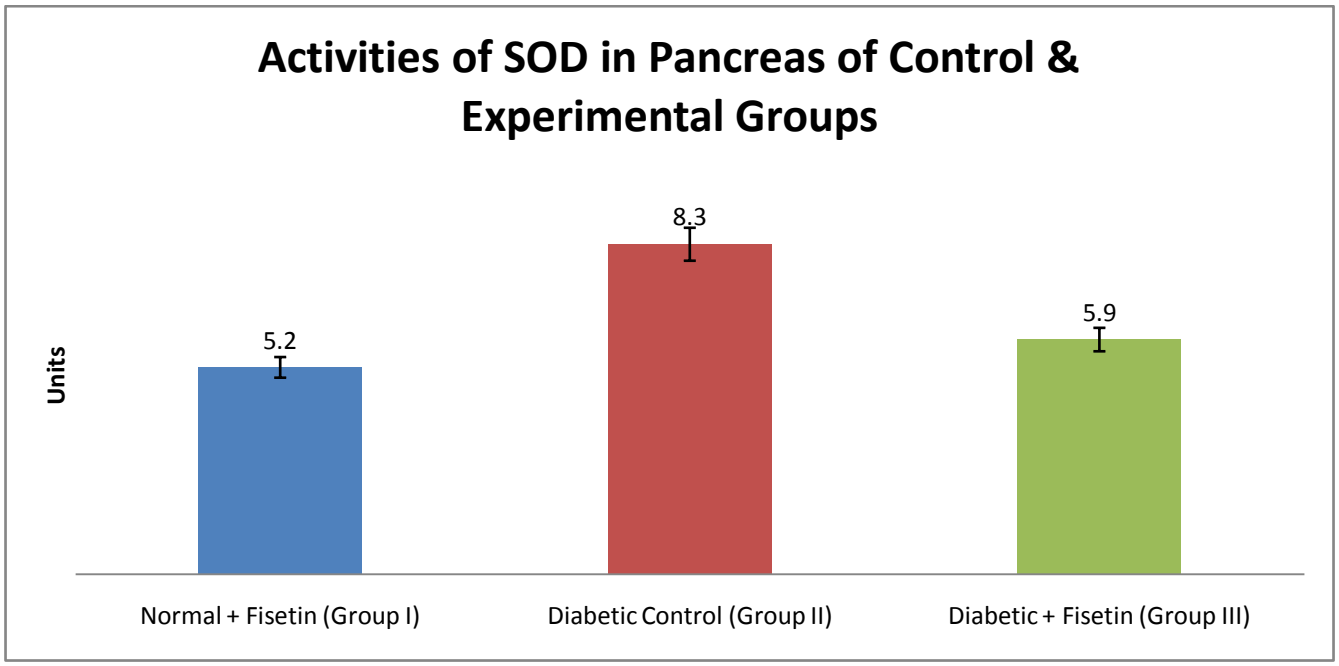
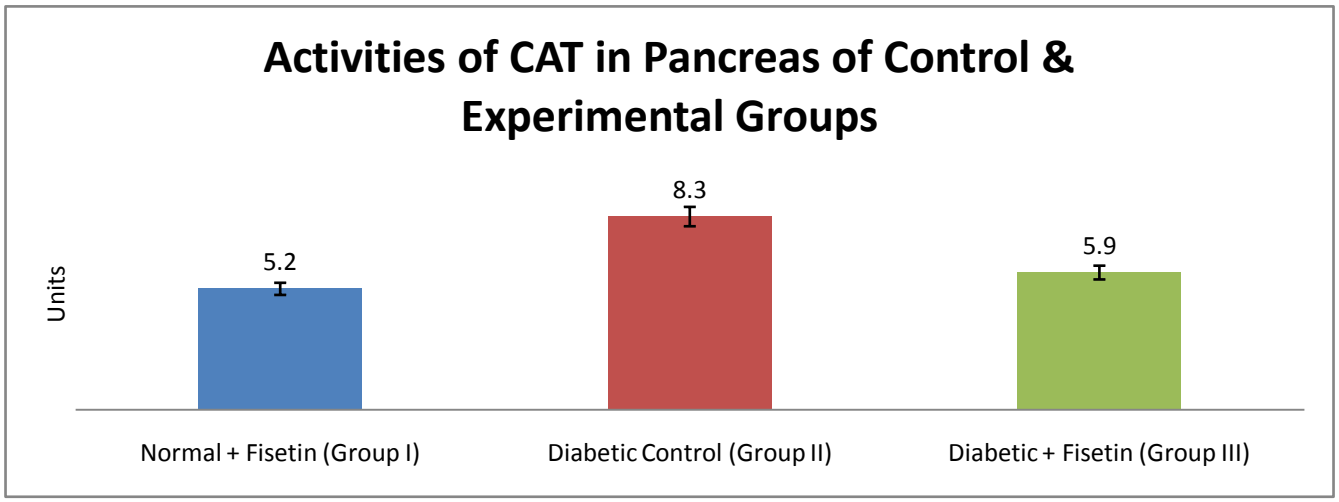
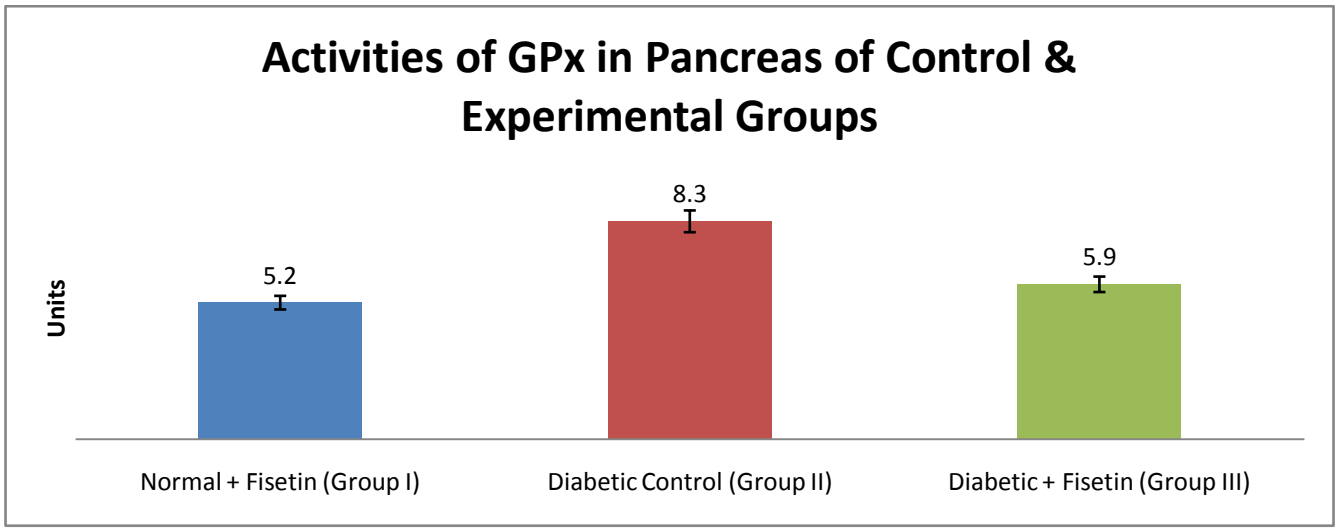


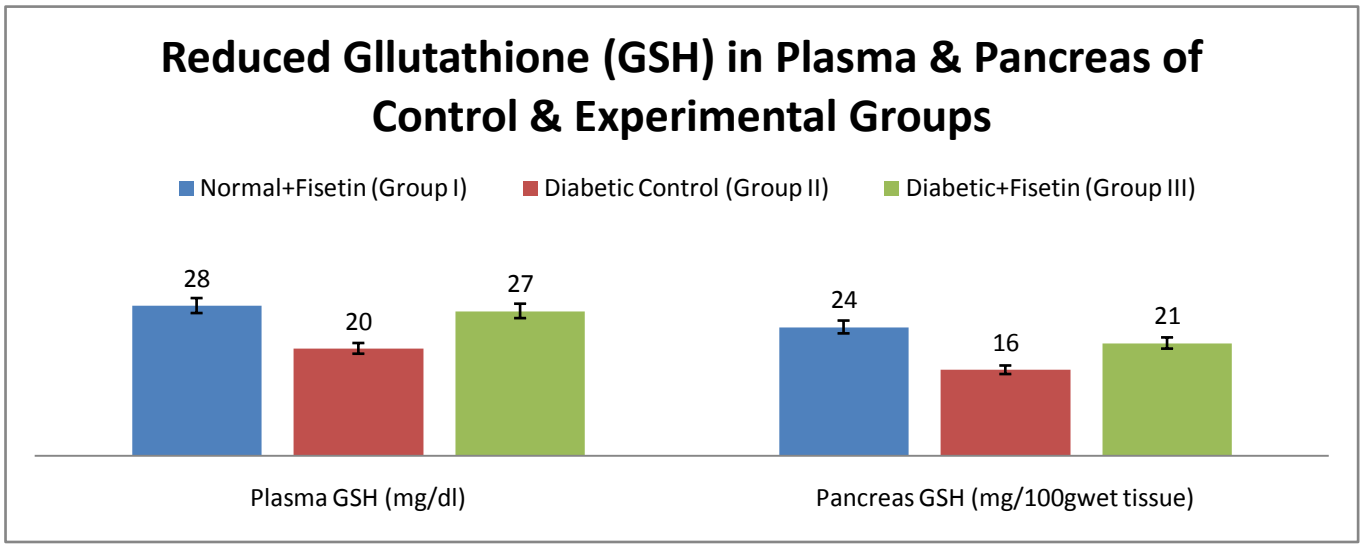
Graph 10. Levels of SOD in Pancreas of control and experimental groups of rats



Graph 11. Levels of CAT in Pancreas of control and experimental groups of rats



Graph 12. Levels of GPx in Pancreas of control and experimental groups of rats



Graph 13. Levels of GSH in plasma and Pancreas of control and experimental groups of rats

RESULTS AND DISCUSSIONS

Our study investigates the antidiabetic effect of Fisetin (3, 3′, 4′, 7- tetrahydroxyflavone) (antioxidant) on streptozotocin-induced diabetic rats. The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves over-production of glucose, from non carbohydrate sources and reduction in utilization of glucose by the tissues [42], 28 days administration of Fisetin resulted in significant reduction in the fasting blood glucose level compared to diabetic rats. The difference observed between the initial and final fasting plasma glucose levels of

different groups revealed a significant increase in blood glucose in diabetic control group compared with that of normal. Our study investigated that Fisetin is effective in controlling and maintaining the blood glucose levels in normal and STZ-nicotinamide-induced diabetic rats. There is a significant reduction in blood glucose levels.

There was no significant alteration was found in FPG level of control rats which justifies the antidiabetic activity of Fisetin. Antioxidant, Fisetin has not shown much reduction in the FPG level of normal rats. During the experimental period for 28 days the body weight is decreased in diabetic rats, hence there is a significant gain of body weight in treated rats. The failure of STZ-induced diabetic rat's gains weight has already been reported [43]. Administration of Fisetin restored these levels significantly ($P<0.001$) towards normalcy. The ability of the Fisetin to restore body weight seems to be a result of its ability to reduce hyperglycemia [44]. Diabetic rats treated with the Fisetin showed an increase in body weight compared to diabetic control. Oxidative stress has been shown to play a role in the pathogenesis of diabetes as such antioxidants may have a role in the alleviation of diabetes [45]. STZ produces oxygen radicals in the body, which cause pancreatic injury and could be responsible for increased blood sugar seen in animals [46]. We have investigated that Fisetin has lowered the TBARS levels in kidney and liver of treated rats by which tetrahydroxyflavone, have a major role in reducing oxidative stress associated with diabetes. The various mechanisms may involve improved glucose homeostasis *viz.*, increase of peripheral utilization of glucose, increase of synthesis of hepatic glycogen and/or decrease of glycogenolysis acting on enzymes, inhibition of intestinal glucose absorption, and reduction of glycaemic index of carbohydrates [47]. In diabetic rats there was a significant increase in lipids, total cholesterol, triglycerides ($p<0.001$). Fisetin-treated rats, there was a decrease in cholesterol, triglycerides, lipids, which shows the hypolipidemic effect also studied. This effect may be due to inhibition of fatty acid synthesis [48], were as in normal metabolism insulin activates the enzyme lipoprotein lipase and hydrolyses triglycerides and the deficiency in insulin results in inactivation of these enzymes thereby causing hypertriglyceridemia. The significant decrease in serum lipid levels in diabetic rats after Fisetin treatment may be directly attributed to improvements in insulin levels too. Fisetin lowered serum AST, an ALT level which

shows the protective effect and normal functioning of liver in reversing the organ damage due to diabetes which is clearly observed by high levels of AST and ALT in diabetic control [49]. Histopathology studies in liver and kidney of Fisetin-fed rats did not show any significant difference from normal rats, which suggest that this powerful (antioxidant) tetrahydroxyflavone not having any toxic effect. There was no significant difference between normal and Fisetin fed rats in protein level, hence, there was a significant reduction in the diabetic control and the protein level was restored in Fisetin fed rats ($p<0.001$)

Serum urea level in untreated diabetic rats was very high when compared with normal and treated rats, which shows renal dysfunction is associated with diabetes. Our study investigates and concludes that Fisetin has rich antioxidant and antidiabetic activity. From our well established results our study has shown that Fisetin can be considered as a potential source of required substance other than diet for patients with acute and as well as chronic diabetes.

Herewith we investigated and report that Fisetin a well-known antioxidant, which accounts for the scavenging of free radicals and ameliorative effect on antioxidant enzymes. High level of blood glucose is the prominent cause for the production of free radicals, which leads to development of diabetic complications. Along with the antioxidant potential, glucose homeostasis may be implicated as the major reason for the amelioration of oxidative stress in diabetes by Fisetin. So, further mechanistic research is needed to elucidate the exact mechanism of this antioxidant effect and to develop Fisetin as a potent oral antihyperglycemic drug in near future. In conclusion, that Fisetin exhibits potential characteristic feature on the glucose lowering activity observed in the diabetic animals due to the stimulation of the β- cells of the pancreatic islets, we strongly report that the body weights of diabetic treated with Fisetin group were significantly recovered when compared to the diabetic control and diabetic treated with Fisetin groups. At the same time decreased blood glucose levels were observed by the stimulation of the β- cells of the pancreatic islets naturally in diabetic group followed by increased oxidative levels were experimentally proved and no tissue damage were observed by the activity of Fisetin in STZ treated animals and that Fisetin could have a beneficial effect on liver and pancreas if used as a hypoglycemic agent in the treatment of diabetes.

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