ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Gymnemic acid, a potent antidiabetic agent protects skeletal muscle from hyperglycemia mediated oxidative stress and apoptotic events in High fat and High fructose diet fed adult rats

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Article History:

Received on: 03.09.2019
Revised on: 15.12.2019
Accepted on: 29.12.2019

Keywords:

Type 2 diabetes,
High-fat diet,
Gymnemic acid,
Oxidative stress,
Apoptosis

ABSTRACT

Type 2 diabetes is delineated by impaired metabolic flexibility, and intramyocellular lipid accumulation, causing insulin resistance, particularly in skeletal muscle by reducing insulin-stimulated glucose uptake. High-fat diet and high fructose (HFD and HF) administration in rodents bestows a model for hyperlipidemia, insulin resistance, and Type 2 diabetes. The current study is focused on elucidating the role of Gymnemic acid in combating hyperglycemia mediated oxidative stress and apoptotic events in the skeletal muscle of HFD and HF induced Type 2 diabetes in Wistar albino rats by boosting antioxidant defense system. Gymnemic acid, a saponin of triterpene glycoside contained in leaves of *Gymnema Sylvestre*, has potent anti-diabetic properties. Treatment with Gymnemic acid restored the antioxidant status (Gpx, SOD, CAT, GR, Vit C & Vit E) with significant ($p<0.05$) decrease in free radical levels and reinvigorated the expression of apoptotic and antiapoptotic proteins in Type 2 diabetic rats. Histopathological data demonstrate that oral administration of Gymnemic acid protects skeletal muscle fibers from an oxidative niche in HFD and HF in Type 2 diabetic rats. In accordance with this, Gymnemic acid might be regarded as a promising therapeutic agent against Type 2 diabetes, thereby restoring skeletal muscle integrity and function.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i2.2029

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INTRODUCTION

Diabetes mellitus (DM), a metabolic disorder pronounced by a rise in the concentration of glucose in the blood because of absolute (type 1) or proportionate (type 2) lack of insulin leading to hyperglycemia. This is associated with the aberrant metabolism of protein, fat and carbohydrates (Ahmad, 2017). DM is one amongst the most important world health emergencies of the twenty-first century. According to WHO estimates universally, 422 million adults over eighteen years of age group had diabetes in 2014 (World Health Organizations, 2016) of which, 90% are type 2 diabetic. This alarming increase of diabetes occurring in developing countries, which is mostly due to rising living standards, sedentary habits, lifestyle changes like obesity and unhealthy diets (Jayaprasad *et al.*, 2018). Many factors are responsible for the cause of DM, including genetics, age, physical inactivity, obesity, autoimmunity, resistance to insulin, pancreatic β-cell defects, and low-grade inflammation (Leach, 2007).

Insulin resistance is broadly defined as a decline in the ability of the body to utilize glucose from the blood in response to insulin (Turcotte and Fisher, 2008). Resistance by target tissues (Liver, Skeletal muscle, Adipose tissue) to insulin (Sampath *et al.*, 2012) mediated by oxidative stress (Tiwari *et al.*, 2014) contributed by hyperglycemia and hyperlipidemia (Lim *et al.*, 2011) is the main physiological event causing type 2 DM. However, different types of orally ingested hypoglycemic agents (Biguanides, thiazolidinediones, sulphonylureas) are used along with insulin for DM treatment. Among them, Metformin, a biguanide antidiabetic drug potentiates insulin action, has been widely used for the treatment. The adverse effects of the above-mentioned agents instigate the researchers to identify naturally occurring products for their antidiabetic activity with little or no side effects (Dholi *et al.*, 2014). One such plant herb is *G. Sylvestre* (Asclepiadaceae), is a perennial, woody climber with distinct medicinal properties found predominantly in the middle and peninsular regions of India. Sweet taste suppressing factor, a property unique to its leaves and therefore called as 'Gurmar' (sugar destroying), has been utilized for more than 2000 years in DM treatment in the form of the crude extract (Kapoor, 1990; Dixit and Pandey, 1984; Gupta, 1961; Jain and Sharma, 1967) in India. (Reddy *et al.*, 1989). *G. Sylvestre* leaves are exploited as a diuretic, digestive, hypoglycemic, hypolipidemic, antiallergic, antiviral and antiobesity agent and also to treat dental caries (Leach, 2007).

The leaves contain the active compound Gymnemic acids, a triterpenoid Oleanane saponins which may possess the predicted antidiabetic property through inhibition of glucose absorption in the small intestine. The Gymnemic acids, a deacylgymnemic acid (DAGA) derivative, contain many acylated groups (methylbutyroyl, tiglolyl, etc.) (Thakur *et al.*, 2012), which has been described for its multifaceted antihyperglycemic agent with antiobesity effect (Zarrelli *et al.*, 2013).

Diseases that adversely impact on skeletal muscle health such as DM may negatively affect progenitor cell quantity and/or functionality. Functional impairments, as shown by a reduction in muscle strength (Sugihara *et al.*, 2000; Anderson *et al.*, 2009), are strongly related to intramuscular fat storage. The impact of Gymnemic acid on skeletal muscle has not been investigated so far during the diabetic condition. Hence the current study is aimed to elucidate the protective activity of Gymnemic acid in skeletal muscle of HFD+HF induced Type 2 diabetic rats.

MATERIALS AND METHODS

Source of chemicals

Gymnemic acid (90% pure, standardized to 75% Gymnemic acid IV) was purchased from Sant Clare herbals, Hungary. Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich, USA. Enhanced chemiluminescence (ECL) kit was procured from Millipore, USA. All other analytical grade chemicals were brought from Medox Biotech, India, Sisco Research Laboratories Pvt. Ltd (SRL), Genei, Bangalore and CDH (Central Drug House Pvt. Ltd., Mumbai, India).

Laboratory Animals

Male Wistar Albino rats were acquired from Central Animal House facility, University of Madras, Taramani campus and experiments were conducted in consonance with guidelines authorized by the Institutional Animal Ethical Committee (IAEC No: 01/10/2016). Two animals per large cavernous cage were housed under controlled temperature (25 $\pm$ 2 °C) conditions with 12/12 h light/dark cycle and were provided with water and food ad libitum. The animals were kept on commercial rat feed (GOLD MOHUR RAT FEED) manufactured by Hindustan Lever Ltd., Mumbai. The feed contained 21% protein, 5% fat, 55% nitrogen-free extract, 4% fibre (w/w) with sufficient vitamins and minerals.

Experimental Design

Young rats of 4 months old weighing around 130-150g were used for this study. Animals were sorted out into 5 groups with six rats in each group as follows,

Group 1

Rats fed with normal water and standard rat chow ad libitum served as normal healthy control.

Group 2

HFD and HF (25%) in drinking water-fed rats for 75 days and the disease was confirmed by the elevated glucose levels on day 45, served as diabetic control.

Group 3

HFD and HF(25%) in drinking water-fed rats for 75 days and Gymnemic acid (150mg/kg body weight/day) was supplemented for the last 30 days orally.

Group 4

HFD and HF(25%) in drinking water-fed rats for 75 days and Metformin (50mg/kg body weight/day) was supplemented for the last 30 days orally served as drug positive control.

Group 5

Normal water and standard rat chow adlibitum fed rats and supplemented with Gymnemic acid (150mg/kg body weight/day) for 30 days orally served as drug control.

HFD comprised of normal rat chow and 4% cholesterol, 1.5% cholic acid and 30% coconut oil supplementation. Gymnemic acid (150 mg/kg body weight /day) was dissolved in physiological saline (0.89%) and administered by oral gavage. As the experimental period ended, rats were made anesthetized with ketamine (22mg/kg bw/ip) and soleus muscle was immediately excised, drenched in physiological saline (ice-cold) and 10% homogenate of the muscle tissue was prepared using 0.01M Tris-HCl buffer with a pH of 7.4 and centrifugation is done for about 10 min at 12,000 rpm and analysis of various parameters were carried on the supernatant. The rest of the tissue was stored at -80 °C for protein expression studies.

Fasting Blood Glucose Measurements

Measurement of blood glucose levels at overnight fasting state on lateral tail vein blood samples were carried out weekly with the help of a One-touch select simple meter and system (Life scan, Scotland) all over the treatment period.

Determination of Glycosylated Hemoglobin (HbA1c)

Glycosylated hemoglobin (HbA1c) levels were analyzed by hemoglobin analyzer (D-10-Biorad) based on the ion exchange HPLC method and the absorbance changes measured at 415nm. From the collected blood samples, the glycosylated haemoglobin were separated and its content was calculated by the glycosylated haemoglobin peak area to the total haemoglobin peak area ratio. Results are expressed in gm%.

Lipoprotein Profile

Triglycerides (TG), Total cholesterol (TC) and high-density lipoproteins (HDL) in serum were analyzed using commercial kits purchased from spin react in semi-auto analyzer (Rx Monza, Randox, U.K). The amount of total cholesterol, triglycerides and HDL are indicated as mg/dl. Friedwald formula was used to calculate LDL cholesterol: LDL = Total cholesterol- (VLDL+ HDL cholesterol). VLDL (very low density lipoprotein) = TG/5. VLDL and LDL concentrations are shown as mg/dl.

Estimation of Reactive Oxygen Species (ROS)

Hydrogen peroxide, superoxide and hydroxyl radicals were estimated by (Jiang *et al.*, 1992; Park *et al.*, 2006; Nishikimi *et al.*, 1972; Puntarulo and Cederbaum, 1988) method respectively.

Biochemical Assessment of Protein Carbonyl and Lipid Peroxidation

Protein carbonyl in skeletal muscle tissue was determined using 2,4- dinitrophenyl hydrazine (DNPH) with slight modifications of Levine *et al.* (1990) method and expressed as nmols of protein carbonyl/mg protein/mL. The lipid peroxide levels was assayed by Devasagayam and Tarachand (Levine *et al.*, 1990) method and expressed as nmols of MDA released/g of tissue.

Assessment of Enzymic and nonenzymic Antioxidants

The activity of the enzyme SOD was assessed by Marklund and Marklund (1974); Devasagayam and Tarachand (1987) method. The activity of SOD is the amount of enzyme inhibiting 50% pyrogallol auto-oxidation rate and was conveyed as Units/mg protein/min. Sinha (1972) method was used to assess the catalase activity and expressed as moles of H_2O_2 consumed /min/ mg of protein. The modified methodology of Rotruck *et al.* (1973) was used to assess the GPx activity and expressed as μmoles of GSH utilized/ minute. Determination of Reduced glutathione (GSH) was carried out by Moron *et al.* (1979) method and was expressed as μg/mg protein. Vitamin C and Vitamin E were estimated by Roe and Kuether (1943); Jargar *et al.* (2012) method, respectively and expressed in μg/mg of protein.

Immuno Blot Analysis

Skeletal muscle tissue samples were homogenized in Tris-HCl buffer (0.01 M, pH 7.4) with 1mM Phenylmethylsulfonylfluoride as a protease inhibitor and protein concentrations were estimated using Lowry *et al.* (1951). Immunoblotting samples containing 50μg of proteins were separated by SDS−PAGE on 10% Polyacrylamide gels, followed by its transfer to polyvinyl difluoride membrane (PVDF). The membranes were incubated with primary antibodies (Bax, Bcl-2, and Cytochrome C) overnight at 4 °C and incubated 1 hour with horseradish peroxidase (HRP) conjugated secondary antibodies next day. Detection of Protein bands were done by standard, enhanced chemiluminescence (ECL) method in a Biorad Chemidoc XRS imaging system.

Statistical Analysis

The results are presented as the mean ± standard error of the mean (SEM) obtained from the average of three or six independent experiments. Analysis of Variance with one way ANOVA was done to analyze the differences between segregated groups using the Graph pad prism version 5.0 software package. Intergroup comparison testings were performed by Tukey's Post hoc testing. Values are significant sta-

Table 1: Effect of Gymnemic acid on Serum Lipid profile

Parameters (mg/dl)	Group I	Group II	Group III	Group IV	Group V
Cholesterol	107.1 ± 8.4	157.3 ± 12.5^a	116.1± 7.4^b	121.1±8.8^b	99.2 ± 6.5 ^{b}
Triglycerides	96.2 ± 7.6	227.3 ± 16.5^a	125.5 ± 8.6^b	135.5±10.8^b	105.1 ± 8.4 ^{b}
LDL	55.1 ± 3.6	87.6± 7.1^a	62.7 ±5.7^b	71.1±6.8^b	48.3 ± 2.9 ^{b}
VLDL	18.2 ± 2.2	48.1± 4.7^a	25.9 ± 2.3^b	30.4±2.9^b	20.1 ± 1.9^b
HDL	33.8 ± 3.3	22.8± 2.2^a	32.4± 3.2^b	29.5±2.9^b	33.1± 3.3 ^{b}

tistically at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Influence of Gymnemic acid on body weight changes, fasting blood glucose (FBG), and glycosylated hemoglobin (HbA1c) in Type 2 diabetic rats

Figure 1 A shows a significant increase (26%) of body weight in DM induced (group II) animals when compared to that of normal control rats (group I) ($p<0.05$). Treatment with Gymnemic acid or metformin (group III or group IV) profoundly show a significant decrease (18% and 14%) in body weight compared with that of group II diabetic rats ($P<0.05$). Figure 1 B shows fasting blood glucose levels estimated at various intervals during the experimental period. Levels of fasting blood glucose at the initial day were around 70 - 90 mg/dl in control and experimental animals. At the end of 45 days HFD and HF fed group II, III and IV animals showed a striking increase in the levels of the blood glucose 161 mg, 145 mg and 150 mg/dl when compared to that of the initial day 75 mg, 84 mg and 79 mg/dl respectively. Upon treatment with Gymnemic acid (group III) or metformin (group IV) from 45th day for 30 days lead to the decrement of blood glucose levels to 92 mg and 98 mg/dl significantly ($P<0.05$) when compared to that of diabetic control rats with 254 mg/dl (group II). No marked changes were noticed between normal control (group I) and drug control rats (group V). Figure 1 C depicts the glycosylated Hb levels in control and experimental animal models. About 50% increase in glycosylated Hb was recorded in HFD+HF fed animals at the end of the experimental period, while Gymnemic acid or metformin-treated groups explicitly showed a significant decrease in HbA1C levels, even though they did not revert to the normal range due to 30 days of short term treatment.

Figure 1 shows, (A) Bodyweight, (B) fasting plasma glucose levels and (C) glycosylated Hb levels are shown. Group I-control rats; Group II-diabetic rats; Group III-Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V-Gymnemic acid alone treated rats. Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p < 0.05$. Where a, compared with Group I; b, compared with Group II rats.

Effect of Gymnemic acid on Lipid Profile in Type 2 diabetic rats

Table 1 depicts the influence of Gymnemic acid or metformin on the serum lipid profile of control and experimental animal groups. Assessment of serum lipid profile reveals that a significant ($p < 0.05$) increase in the levels of serum triglycerides, LDL, VLDL, total cholesterol and a concomitant decrease in HDL was observed in DM induced group II compared to that of normal control rats. Gymnemic acid treatment to HFD and HF fed rats demonstrated a striking decrease ($p < 0.05$) in serum triglycerides, LDL, VLDL, total cholesterol with a concomitant increase ($p < 0.05$) in HDL, when compared to group II, moreover it was observed that Gymnemic acid was almost equally beneficial on reducing lipid levels in the serum compared to metformin.

In Table 1 shows, Group I-control rats; Group II-diabetic rats; Group III-Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V-Gymnemic acid alone treated rats. Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p < 0.05$. Where a, compared with Group I; b, compared with Group II rats.

Impact of Gymnemic acid on Markers of Oxidative Stress in Type 2 diabetic rats

Figure 2 A – Figure 2 C show the levels of hydrogen peroxide, superoxide and hydroxyl radicals in the skeletal muscle of control and experimental rats. HFD and HF fed rats (group II) demonstrated a 2 fold elevation in the free radicals level ($p<0.05$) when compared to control animals (group I). Administration of Gymnemic acid or metformin to HFD and HF fed rats decreased the levels of free radicals generated by 1.5 fold and 1.4 fold, respectively. Figure 3 A shows the impact of Gymnemic acid or metformin

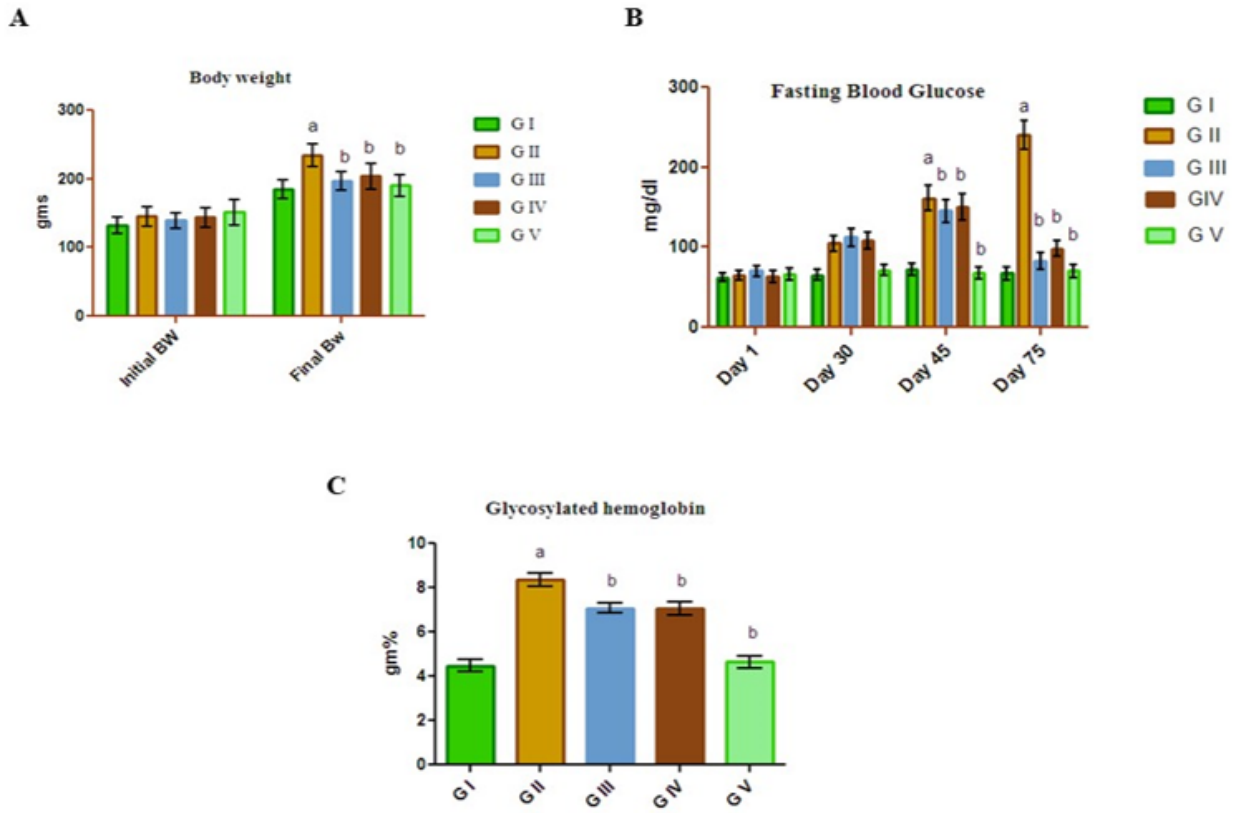

Figure 1: Influence of Gymnemic acid on body weight, fasting blood glucose levels and serum glycosylated Hb in Type 2 diabetic rats

on the levels of MDA in the muscle of control and experimental rats. Assessment of MDA levels were elevated in HFD and HF fed rats by about 3.5 fold when compared with that of control rats. On comparison with group II rats, the MDA levels were evidently brought down by 3.2 and 3.0 folds, respectively, upon Gymnemic acid or metformin treatment (group III or group IV). Figure 3 B shows the protein carbonyl levels in the skeletal muscle of control and experimental rats. HFD and HF (Group II) fed rats showed a significant increase (1.9 fold) in the protein carbonyls level when compared to that of control rats. Treatment with Gymnemic acid or metformin significantly brought down the protein carbonyl levels in HFD and HF fed rats by 1.4 and 1.2 fold correspondingly.

Figure 2 shows, (A) Hydrogen peroxide, (B) Superoxide and (C) Hydroxyl radical levels are shown. Group I-control rats; Group II-diabetic rats; Group III - Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V - Gymnemic acid alone treated rats. Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p < 0.05$. Where a, compared with Group I; b, compared with Group II rats

Figure 3 shows, (A) Lipid peroxidation (LPO) and (B) Protein carbonyl levels are shown. Group I-control rats; Group II-diabetic rats; Group III-Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V-Gymnemic acid alone treated rats. Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p < 0.05$. Where a, compared with Group I; b, compared with Group II rats

Impact of Gymnemic acid on levels of Enzymatic and Non Enzymatic Antioxidants in Type 2 diabetic rats

Figure 4 A – Figure 4 C shows the levels of enzymatic antioxidants in the skeletal muscle of HFD and HF fed rats, Gymnemic acid, or metformin-treated rats. On comparison with the control rats, there was a significant decrease ($p<0.05$) observed in the antioxidant enzymes, namely SOD, Catalase, GPx in HFD and HF fed (group II) rats by 50%, 61%, 40% respectively. Approximately 30-50% marked increase in these enzyme activities have been noticed in the HFD and HF fed rats when treated with Gymnemic acid or metformin. Figure 4 D and Figure 4 E shows the non-enzymatic antioxidants, namely Vit-C and Vit-E, GSH levels in the skeletal muscle of the various

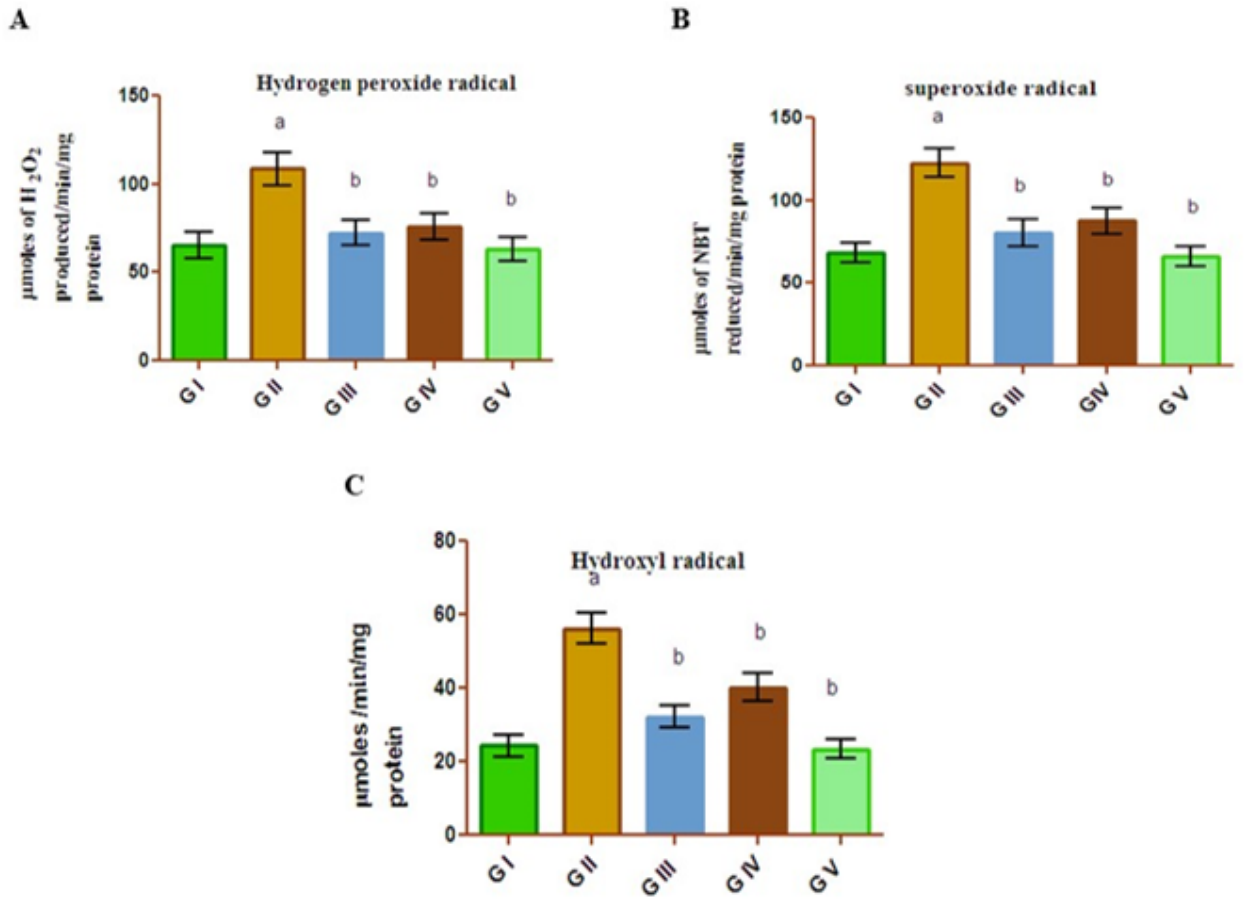

Figure 2: Influence of Gymnemic acid on free radicals in Type 2 diabetic rats

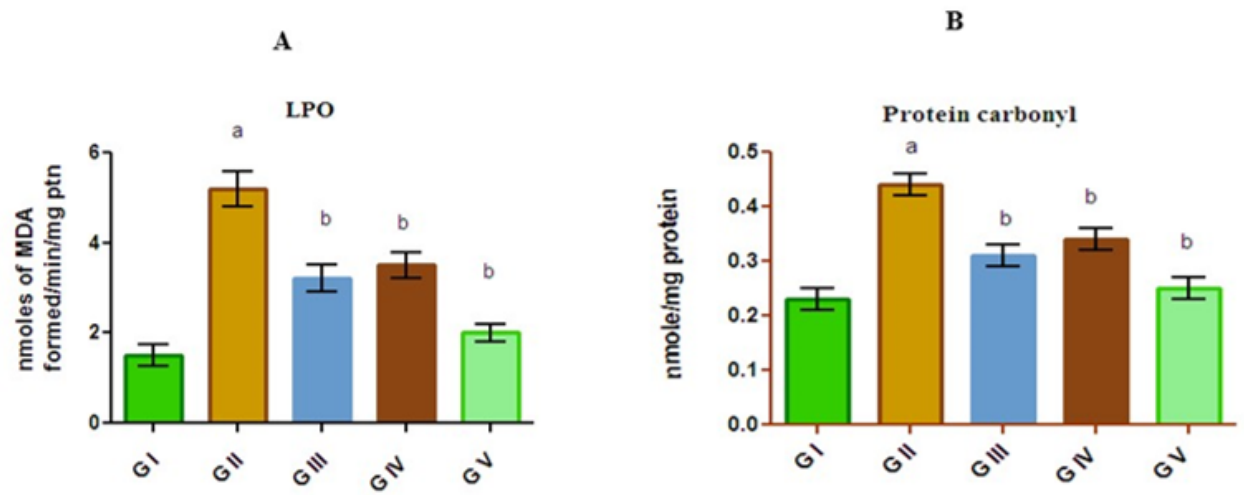

Figure 3: Influence of Gymnemic acid on oxidative stress markers in Type 2 diabetic rats

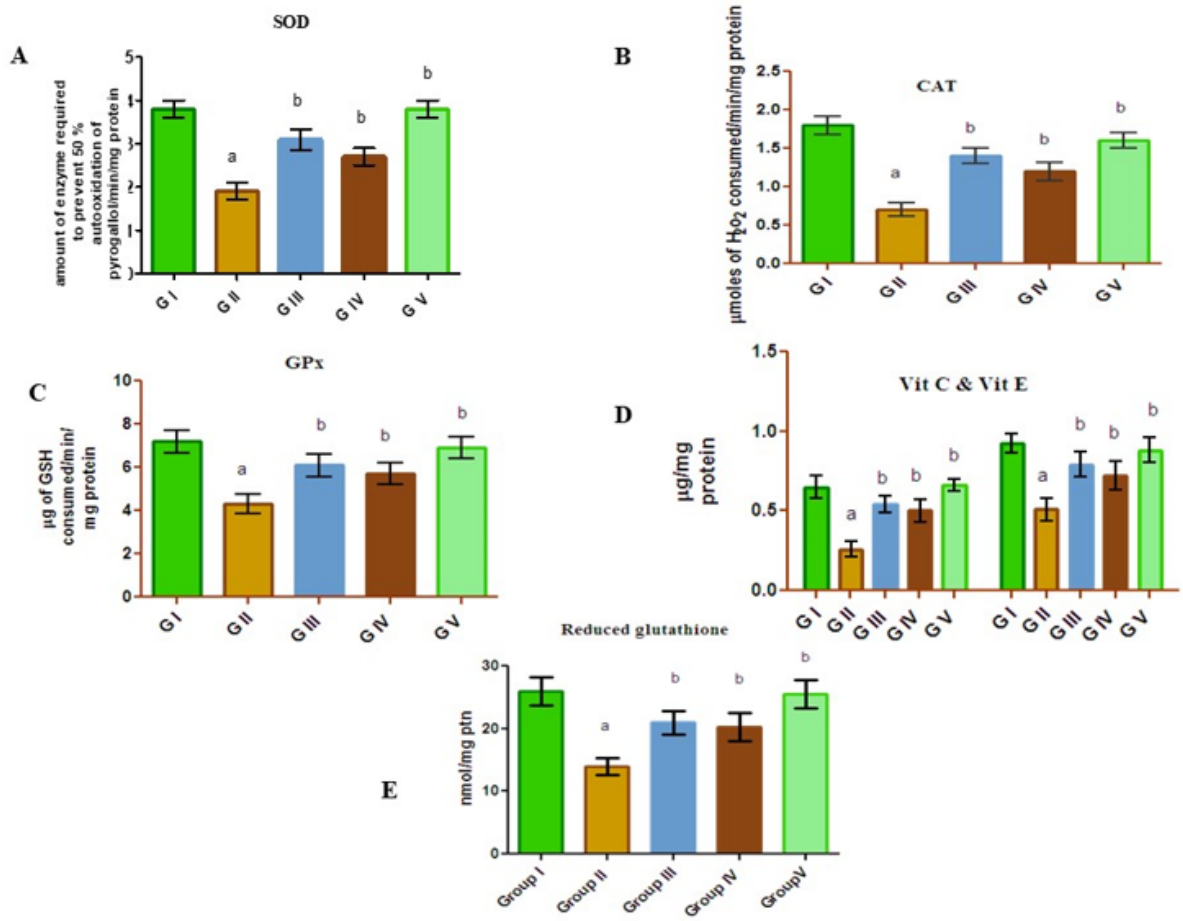

Figure 4: Influence of Gymnemic acid on enzymic and non-enzymic antioxidants in Type 2 diabetic rats

experimental groups showed a decreasing trend in group II rats by 60%, 44%, 46% respectively comparing with control rats (group I). Gymnemic acid or metformin-treated rats had shown a significant increase ($p<0.05$) in these antioxidant status.

Figure 4 shows, (A) SOD (B) CAT (C) GPx (D) Vit C and Vit E (E) reduced glutathione levels are shown. Group I-control rats; Group II-diabetic rats; Group III-Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V-Gymnemic acid alone treated rats. Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p < 0.05$. Where a, compared with Group I; b, compared with Group II rats.

Effect of Gymnemic acid on skeletal muscle Histoarchitecture of HFD and HF fed Type 2 diabetic rats (H&E 40×)

Figure 5 shows the histoarchitecture of control and experimental rats skeletal muscle. Control rats (Group I) show longitudinal muscle fibers exhibiting peripheral nuclei, Oval pale nuclei (p) and transverse sarcoplasmic striations. Diabetes induced rats (Group II) show wide skeletal myocytes separation and fragmentation of the skeletal muscles fibers. Gymnemic acid-treated rats (Group III) show narrower intercellular spaces between the skeletal muscle fibers, which appear relatively normal on comparison with the control group. Metformin treated rats (Group IV) display narrower intercellular spaces between the skeletal muscle fibers with a central nucleus. Gymnemic acid alone treated rats (Group V) show longitudinal muscle fibers exhibiting peripheral nuclei, which is similar to that of control rats.

Figure 5 shows, Group I (control rat) showing longitudinal muscle fibers exhibiting peripheral nuclei (arrows). Oval pale nuclei (p) and transverse striations in the (arrows) in the sarcoplasm. Group II (Diabetes induced) showing the wide separation of the skeletal myocytes and fragmentation of the skeletal muscle fibers (arrow). Group III (Gymnemic acid-treated) showing narrower intercellular spaces (arrow) between the skeletal muscle fibers, which appear relatively normal compared to the control group. Group IV (Metformin-treated) show-

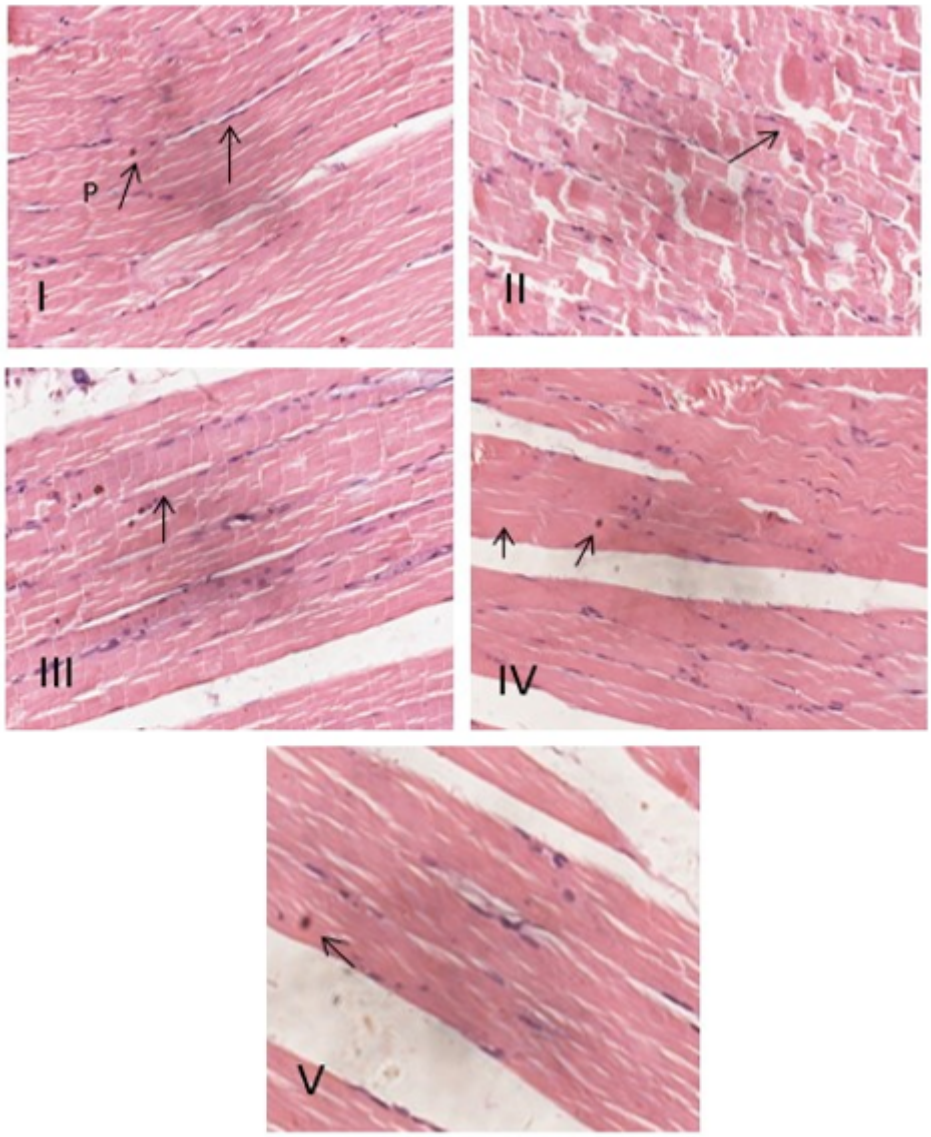

Figure 5: Effect of Gymnemic acid on skeletal muscle histoarchitecture of HFD+HF induced Type 2diabetes in rats (H&E 40×)

ing narrower intercellular spaces (arrow) between the skeletal muscle fibers with the central nucleus (arrow). Group V (Gymnemic acid control) showing longitudinal muscle fibers exhibiting peripheral nuclei (arrows) look like control.

Antiapoptotic potency of Gymnemic acid in HFD and HF fed Type 2 Diabetic Rats

ROS act as potential mediators of glucose-induced apoptosis, which involves the response of apoptotic proteins (Bax and Bcl-2) and initiated by caspase 3 cleavage activation. Immunoblot analysis depicts that Bax expression (2.2 fold) and released cytosolic Cyt C levels (2.8 fold) were significantly high in the HFD and HF fed (group II) diabetic skeletal muscle (Figure 6). However, treatment with Gymnemic acid or metformin significantly decreased the expression of Bax by 1.7 and 1.6 fold, respectively; Cyt C by 1.5 fold and 1.6 fold, respectively (Figure 6 A and Figure 6 C). The current study exhibited that HFD and HF induced diabetic conditions significantly decreased Bcl-2 expression to 1.6 fold (Figure 6 B), which upon Gymnemic acid or metformin treatment showed a 1.5 fold significant increase.

Figure 6 shows, (A) Bax (B) Bcl-2 and (C) Cytochrome C protein expressions are shown in the blot. β-actin was used as an internal control. * Represent the non-specific bands of Bcl-2. Densitometry analysis of each blots are shown. Group I-control rats; Group II-diabetic rats; Group III-Gymnemic acid-treated diabetic rats; Group IV-Metformin treated diabetic rats; Group V-Gymnemic acid alone treated rats. Values are expressed as mean $\pm$ SEM for three animals in each. Values are statistically significant at the level of $p < 0.05$.

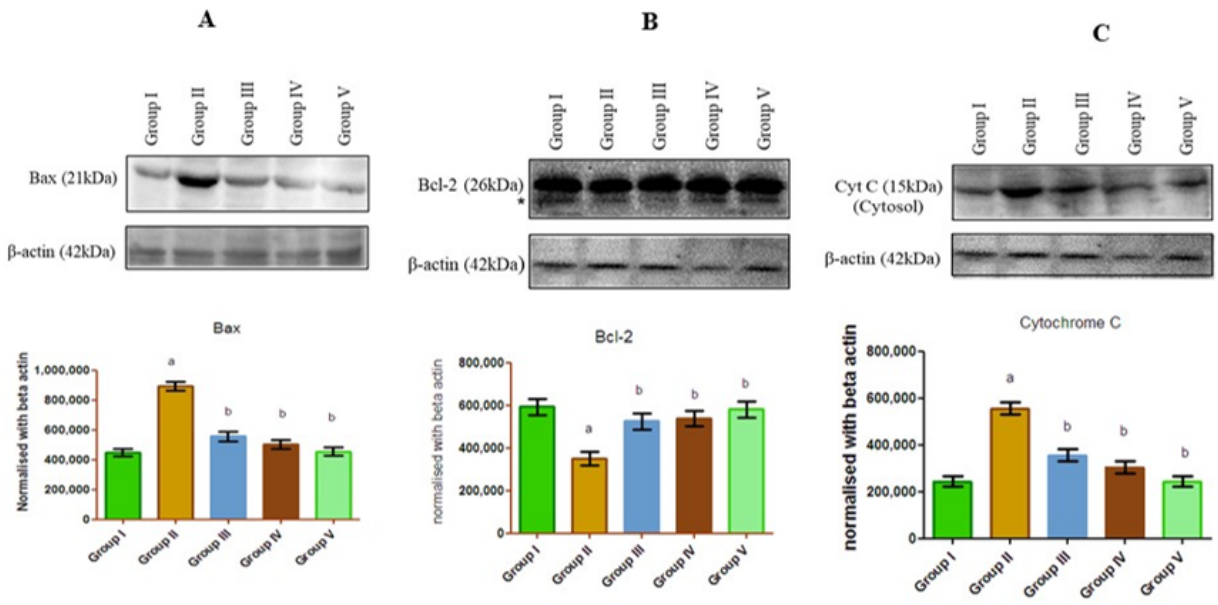

Figure 6: Impact of Gymnemic acid on the protein expression of Bax and Bcl-2 in skeletal muscleof Type 2 diabetic rats

Where a, compared with Group I; b, compared with Group II rats.

Skeletal muscle employs both glucose and free fatty acid (FFA) as sources of fuel for energy production (Lowry *et al.*, 1951). Thus, glucolipotoxicity causes obesity-related Type 2 diabetes in skeletal muscle. Our study showed raise in body weight, fasting blood glucose, glycosylated hemoglobin and elevation of lipids and lipoproteins except for HDL in HFD and HF fed rats when compared to that of control rats establishing glucolipotoxicity. These results also demonstrate that the rats are hyperglycemic and hyperlipidemic, a typical biochemical picture associated with Type 2 diabetes. Our results are supported by Singh *et al.* (2017); Abdul-Ghani and Defronzo (2010), who have demonstrated that HFD fed rats for four weeks provoked obesity and dyslipidaemia. Gymnemic acid or metformin treatment in group III or group IV rats in our study showed a significant decrease in body weight, fasting blood glucose and lipid profile. (Kim *et al.*, 2017; Singh *et al.*, 2017) have proved that mice fed with *G. Sylvestre* extract (500 mg/kg) demonstrate less food and energy efficiency ratios and less body weight gain when scrutinized with the HFD alone fed group.

Our results showed a remarkable increase in blood glucose levels upon HFD and HF feeding for 75 days in Group II rats, which is favored by the studies of (Nampurath *et al.*, 2008; Woods *et al.*, 2003; Kim *et al.*, 2017). Gymnemic acid lowered blood glucose levels, which might be mediated by its ability to inhibit the enzymes involved in the digestion of carbohydrates and delaying of absorption of glucose in the intestine (Nirmala *et al.*, 2016). In a study by (Baskaran *et al.*, 1990; Nakamura *et al.*, 1999) 22 DM patients (type 2) were administered orally with GS_4 extract (ethanolic extract of *G. Sylvestre* leaves) at 400 mg/day dose showed a substantial fall of glucose in the blood, glycosylated plasma proteins and glycosylated hemoglobin. The current study shows the significantly elevated levels of Glycosylated hemoglobin in HFD and HF (group II) rats and after Gymnemic acid treatment, these levels were reverted back to the normal.

We observed the significant restoration of amended lipid profile and circulating free fatty acids after administration of Gymnemic acid (group III) to type 2 diabetic rats is consistent with our previous studies by (Narasimhan and Kalaiselvi, 2018), which supported that Gymnemic acid administration to HFD and HF diet-induced Type 2 diabetic rats lowered the circulatory cholesterol, triglycerides, LDL and VLDL levels and also up-regulated HDL levels in serum. These results exhibit the antihyperlipidemic effect of Gymnemic acid. Wang *et al.* (1998) have also shown that Gymnemic acid inhibited potently oleic acid absorption in the small intestine.

Oxidative stress at the molecular level occurs when elevated ROS overcome the antioxidant defense capabilities systems. Chronic hyperglycemia increases the polyol pathway, formation of advanced glycation end products (AGE) and free radical generation rates. Hence we measured free radical levels such as hydroxyl radicals, hydro-

gen peroxide and superoxide radicals. Our study established a notable increase in free radicals level in skeletal muscle of the HFD and HF fed rats. Our results were in corroboration with findings of (Selvaraj *et al.*, 2016; Wang *et al.*, 1998) in which diabetic rats exhibited elevated levels of H_2O_2.OH, and LPO in the liver due to HFD and dihydroxy gymnemic triacetate treatment reduced the free radical levels. Hence our study represents that Gymnemic acid prevents hyperglycemia-induced advanced glycation end products (AGE) formation. Similarly, (Kang *et al.*, 2012; Shafey *et al.*, 2013) have reported a decline in the level of plasma lipid peroxidation and increase in superoxide dismutase level in rats treated with *G. Sylvestre* leaves extract after STZ-diabetic induction when compared to untreated diabetic rats by either directly decomposing the reactive oxygen species or by increasing the generation of antioxidant molecules. In order to authenticate the efficacy of Gymnemic acid on treating oxidative stress, we assessed the histopathology of skeletal muscle tissue. The histopathological observation showed a wide separation of skeletal myocytes and fragmentation of the skeletal muscle fibers in HFD and HF fed (group II) rats. This might be due to increased lipid peroxidation and protein carbonyls leading to myofibrillar fragmentation. Treatment with Gymnemic acid narrowed intercellular myofibrils and reduced significant pathological changes induced by hyperglycemia.

Our current findings shows that the antioxidant defense of control and experimental groups indicates that feeding HFD and HF reduce antioxidant enzyme activity and there was a marked increase in the activity of enzymes treated with Gymnemic acid suggesting the high antioxidant potential of this bioactive compound. Ohmori *et al.* (2005); Vasi and Austin (2009) have demonstrated that *G. Sylvestre* exhibited antioxidant potential over free radicals and oxidation of LDL in healthy volunteers. Supplementing diets incorporated with *G. Sylvestre* to experimental Wistar rats led to a significant increase in GSH levels, indicating that the leaves and bark have a free radical scavenging capacity, thereby reducing oxidative stress (Ohmori *et al.*, 2005).

The surplus production of ROS triggers hyperglycemia mediated apoptosis. Apoptosis is inhibited by Bcl-2, a major anti-apoptotic protein by forbidding the mitochondrial discharge of cyt C, finally resulting in caspase activity inhibition (Chauhan *et al.*, 2014). Lipid deposition in skeletal muscle can promote lipotoxicity and, as a result, lipoapoptosis. Our results demonstrate significantly increased expression of Bax and released cytosolic cyt C and decreased expression of Bcl-2 in the skeletal muscles of HFD and HF fed (group II) rats. (Li *et al.*, 2004; Kirkin *et al.*, 2004) have shown that increased Bax expression in the kidney cortex of diabetic experimental animal models. Sekiguchi *et al.* (2004) have elucidated apoptosis in endothelial cells *in vitro* by high glucose induction. Mizutani *et al.* (1998) have indicated low expressions of Bc1-2 in adult retinas of diabetic humans. Upon Gymnemic acid or metformin treatment in group III or group IV in the present study, the expressions of Bax, Bcl-2 and Cyt-C were reversed. These findings were coherent with the following observation. The antiapoptotic activity of Gymnemic acid by (Mizutani *et al.*, 1998; Pathan *et al.*, 2012) have explicated that Gymnemic acid phospholipid Complex's supplementation efficiently suppressed cardiomyopathy induced by Doxorubicin. Our study showed prominently the antiapoptotic potency of Gymnemic acid in skeletal muscle against HFD and HF induced type 2 diabetes.

CONCLUSIONS

The above findings indicate that Gymnemic acid attenuated Type 2 diabetes induced by HFD and HF through the amelioration of oxidative stress-mediated hyperglycemia-induced apoptosis. The Gymnemic acid maintains normoglycemia during diabetes, triggers not only liver and kidney to revert to their normal metabolic homeostasis but also skeletal muscle. Hence, Gymnemic acid may be considered as an adjuvant therapeutic agent for DM. However, studies are greatly needed further to establish its molecular mechanism of antidiabetic potency.

Conflicts of interest

The authors declare that they have no competing interests.

Contributions

All authors have contributed in this research work.

Abbreviations

HFD High Fat Diet

HF High fructose

SOD Superoxide dismutase

CAT Catalase

Gpx Glutathione Peroxidase

H&E Hematoxylin and Eosin

MD A Malondialdehyde

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