

Role of chrysin on expression of insulin signaling molecules

Kottireddy Satyanarayana¹, Koora Sravanthi², Ivvala Anand Shaker¹, Rajagopal Ponnulakshmi³, Jayaraman Selvaraj⁴

¹Department of Medical Biochemistry, Bharath University, ³Department of Zoology, PG and Research, Ethiraj College for Women, Chennai,

⁴Department of Biotechnology, PG and Research, Holy Cross College, Trichy, Tamil Nadu, ²Department of Pharmacology, Sakshi Medical College and Research Centre, Guna, Madhya Pradesh, India

ABSTRACT

Background: Currently available drugs are unsuccessful for the treatment of type-2 diabetes due to their adverse side-effects. Hence, a search for novel drugs, especially of plant origin, continues. Chrysin (5,7-dihydroxyflavone) is a flavonoid, natural component of traditional medicinal herbs, present in honey, propolis and many plant extracts that has been used in traditional medicine around the world to treat numerous ailments. **Objective:** The present study was aimed to identify the protective role of chrysin on the expression of insulin-signaling molecules in the skeletal muscle of high fat and sucrose-induced type-2 diabetic adult male rats. **Materials and Methods:** The oral effective dose of chrysin (100 mg/kg body weight) was given once a day until the end of the study (30 days post-induction of diabetes) to high fat diet-induced diabetic rats. At the end of the experimental period, fasting blood glucose, oral glucose tolerance, serum lipid profile, lipid peroxidation (LPO) and free radical generation, as well as the levels of insulin signaling molecules and tissue glycogen in the gastrocnemius muscle were assessed. **Results:** Diabetic rats showed impaired glucose tolerance and impairment in insulin signaling molecules (IR, IRS-1, p-IRS-1^{Tyr632}, p-Akt^{Thr308}), glucose transporter subtype 4 [GLUT4] proteins and glycogen concentration. Serum insulin, lipid profile, LPO and free radical generation were found to be increased in diabetic control rats. The treatment with chrysin normalized the altered levels of blood glucose, serum insulin, lipid profile, LPO and insulin signaling molecules as well as GLUT4 proteins. **Conclusion:** Our present findings indicate that chrysin improves glycemic control through activation of insulin signal transduction in the gastrocnemius muscle of high fat and sucrose-induced type-2 diabetic male rats.

Key words: Chrysin, gastrocnemius muscle, high fat diet, insulin signaling, type-2 diabetes

INTRODUCTION

Sedentary lifestyle and energy-rich diet in the present industrialized world is aggressively contributing to a staggering increase in obesity resulting in insulin resistance. As a result, there is a steady increase in the pathogenesis

of type-2 diabetes.^[1,2] Diabetes mellitus (DM) is a growing problem worldwide, entailing enormous financial burden and medical care policy issues.^[3] According to the International Diabetes Federation (IDF), the number of individuals with diabetes in 2012 crossed 366 million, with an estimated 4.6 million deaths each year.^[4,5] Oral hypoglycemic agents are useful in the treatment of DM, but their use is restricted by the pharmacokinetic properties, secondary failure rates and accompanying side-effects, and there is a need to look for more efficacious agents with fewer side-effects.^[6] Flavonoids constitute the largest and most important group of polyphenolic compounds in plants.^[7,8] It is now widely

Address for correspondence:

Dr. Kottireddy Satyanarayana,
Department of Medical Biochemistry, Bharath University,
Selaiyur, Chennai - 600 073, Tamil Nadu, India.
E-mail: satya79700@gmail.com

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accepted that dietary polyphenolics may play an important role in protecting the body against chronic diseases, such as cancer, cardiovascular diseases^[9,10] and diabetes mellitus.^[11]

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid that is contained in many plant extracts, flowers such as the blue passion flower (*Passiflora caerulea*), honey and propolis,^[2] and is the major component of some traditional medicinal herbs.^[12] It possesses anti-inflammatory and antioxidant properties and is used as a dietary supplement.^[13] Most studies show the effects of chrysin in the regulation of the reproductive system and hormones; it was effective in antagonizing the enzyme aromatase thus preventing the conversion of testosterone to estradiol, a desirable effect for body builders to increase their muscle mass.^[14] Chrysin has also been shown to inhibit tumor angiogenesis *in vivo*;^[15] it presents anti-viral^[16] and anxiolytic,^[17] anti-allergic^[18] and antiestrogenic^[19] activities. It has remarkably beneficial pharmacological effects,^[20] with the most important and most reported being its anti-oxidant capacity.^[21] Pushpavalli *et al.*^[12] demonstrated that chrysin is able to decrease the levels of plasma lipids caused by D-galactosamine in rats.

Chrysin also has the potency for clinical and therapeutic application against the physiological and biochemical effects of aging.^[22] *In vivo* studies have indicated that chrysin offers protection against oxidative stress-mediated ethanol-induced liver injury in rats. It has been reported that oral administration of chrysin at a dose of 20 mg to streptozotocin-induced diabetic nephropathy in rats reduced the levels of blood glucose, urea, serum creatinine, urinary glucose, urea, creatinine and protein and elevated the level of glomerular filtration rate, suggesting that chrysin has a renoprotective effect against STZ-induced diabetic nephropathy in rats.^[23] Zarzecki *et al.*^[24] reported that oral administration of chrysin to Triton WR-1339-induced hyperlipidemia in female C57BL/6 mice decrease plasma lipids' concentration and that its antioxidant properties were involved in the hypolipidemic action of chrysin. However, there are no reports on the effect of chrysin against a high fat diet-induced skeletal muscle insulin resistance. In the present study, we have investigated the effects of chrysin on the expression of insulin signaling molecules in a high fat and sucrose-induced type-2 diabetic Wistar albino rat model. To evaluate the mechanism of action, we hypothesized that chrysin may improve the skeletal muscle insulin resistance in HFD-induced type-2 diabetic rat model by improve the insulin signaling molecules.

MATERIALS AND METHODS

Chemicals

Chrysin [Figure 1] was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and reagents used in this study were of molecular and analytical grade, and they were purchased from Sigma Chemical Company, St. Louis, MO, USA; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; and Sisco Research Laboratories, Mumbai, India. On-Call Plus Blood glucose test strips were obtained from ACON Laboratories Inc., San Diego, CA, USA. The ultra-sensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit was obtained from Crystal Chem Inc., A, Downers Grove, IL 60515, U.S.A. Biochemical kits used in the present study were purchased from Spinreact (Girona, Spain). β -actin monoclonal and polyclonal IR- β , IRS-1, phospho IRS-1 (Serine 632), Akt 1/2/3 and phosphoAkt (Threonine 308) and glucose transporter subtype 4 (GLUT4) antibodies were purchased from Santa Cruz Biotechnology, Inc., Dallas, Texas, U.S.A.

Animals

This study was approved by the Institutional Animal's Ethics Committee and by the regulatory body of the government (No. FN.41/2009). Adult male Wistar rats (180–200 g body weight) were obtained from the Laboratory of Animal Medicine, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences Studies, Madhavaram, Chennai, Tamil Nadu and kept at the Department of Medical Biochemistry, Bharath University, Selaiyur, Chennai - 600 073, Tamil Nadu under laboratory conditions of temperature (22 + 21°C), humidity (45 + 5%) and 12-h day: 12-h night cycle, and were allowed free access to food (standard pellet diet, Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*.

Induction of type-2 diabetes

Rats were subjected to 60 days of a high-fat diet containing cholesterol 3%, cholic acid 1%, coconut oil 30%, standard rat feed 66%^[25] and 30% sucrose through drinking water.^[26,27] On the 58th day of treatment, after overnight fasting, blood glucose was checked and the rats that had blood glucose above 120 mg/dL were chosen as type-2

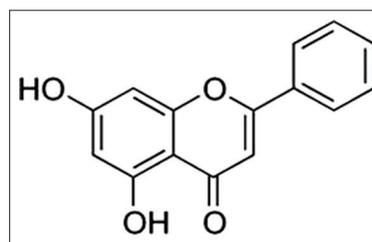


Figure 1: Structure of chrysin

diabetic rats. Sucrose feeding through drinking water with a high fat diet was continued until end of the study.

Dose fixation study

The optimal dose of chrysin was fixed on the basis of the following dose fixation study. Rats were randomly divided into the following groups of three rats each. Group I: Normal control rats (NC) received intragastric (i.g.) of vehicle (0.1% DMSO); Group II: Rats were made diabetic (type-2) after 60 days of a high fat diet feed and sucrose through drinking water (30%); Group III–VII: Diabetic (type-2) rats treated with chrysin (chrysin was dissolved in 0.1% DMSO at 25, 50, 100, 150 and 200 mg/kg body weight once a day, orally) for 30 days, respectively.

At the end of 30 days treatment, control and experimental animals were subjected to the oral glucose tolerance (OGT) test after overnight fasting. Blood was collected from the rat tail tip and blood glucose was estimated using On-Call Plus blood glucose test strips. The results are expressed in mg/dL. The dose of chrysin was fixed on the basis of reduction in the fasting blood glucose (FBG) level and glucose tolerance capacity. Chrysin at a dose of 100 mg/kg body weight effectively reduced the FBG and showed near-normal glucose tolerance when compared with the other doses [Figure 2a and Table 1]. Hence, the optimal dose (100 mg/kg body weight) was selected and used for further studies.

Experimental design

The following experimental design was framed and, accordingly, rats were subjected to chrysin treatment for 30 days. Rats were randomly divided into five groups of six rats each.

- Group I: Control (vehicle treated, rats treated with 0.1% dimethyl sulfoxide, DMSO).
- Group II: Diabetic (type-2) rats (vehicle treated,

rats were treated with 0.1% dimethyl sulfoxide DMSO).

- Group III: Diabetic (type-2) rats treated with chrysin 100 mg/kg body weight once a day (orally) for 30 days.
- Group IV: Diabetic (type-2) rats treated with metformin 50 mg/kg body weight once a day (orally) for 30 days.
- Group V: Control rats treated with chrysin 100 mg/kg body weight once a day (orally) for 30 days.

Two days prior to sacrifice, the control and experimental animals were subjected to an OGT test after overnight fasting. At the end of 30 days, blood was collected and animals were perfused with normal saline under ether anesthesia. The gastrocnemius muscle was dissected out for the assessment of various parameters.

Fasting blood glucose (FBG)

Blood glucose was estimated using the On-Call Plus Blood glucose test strips (ACON Laboratories Inc., USA) after overnight fasting. Blood was collected from the rat tail tip and the results are expressed in mg/dL.

OGT test

Blood glucose was estimated using the On-Call Plus blood glucose test strips at various time points (60, 120 and 180 min) after giving an oral glucose load (10 mL/kg bodyweight; 50% w/v). The blood glucose value before giving glucose is considered as the “0” minute value. Results are expressed in mg/dL.

Insulin assay

Serum insulin was assayed using an ultra-sensitive rat insulin ELISA kit obtained from Crystal Chem Inc., USA. The

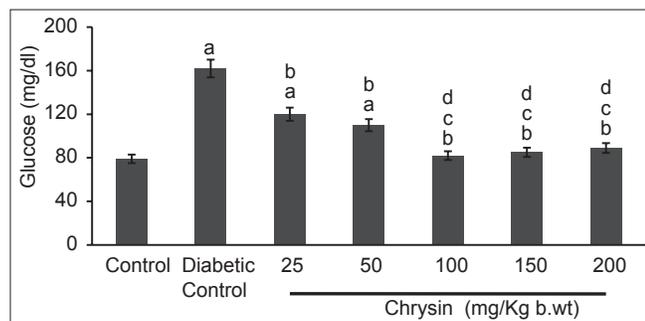


Figure 2: Dose-dependent effects of chrysin on fasting blood glucose of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (n=6) a-compared with control; b-compared with diabetic control; c-compared with diabetes + chrysin (25 mg/kg b.wt.); d-compared with diabetes + chrysin (50 mg/kg b.wt.)

Table 1: Dose dependent effects of chrysin on OGT of type-2 diabetic adult male rats

OGT	0 hr	1 hr	2 hr	3 hr
Control	79±2	140±5 ¹	120±6 ^{1,2}	69±2 ^{2,3}
Diabetic control	162±4 ^a	189±9 ^{a,1}	172±8 ^{a,1,2}	160±5 ^{a,1}
Diabetes+Chrysin (25 mg/Kg. b.wt)	120±4 ^{a,b}	172±11 ^{a,1}	169±4 ^{a,1,2}	130±4 ^{a,b,1,2}
Diabetes+Chrysin (50 mg/Kg. b.wt)	110±6 ^a	160±8 ^{b,c,1}	130±4 ^{b,c,1,2}	110±3 ^{a,b,c,2,3}
Diabetes+Chrysin (100 mg/Kg. b.wt)	82±2 ^{b,c,d}	169±6 ^{b,c,1}	120±9 ^{b,c,d}	77±5 ^{b,c,d,2,3}
Diabetes+Chrysin (150 mg/Kg. b.wt)	85±4 ^{b,c,d}	159±5 ^{b,c,1}	117±4 ^{b,c,1,2}	80±3 ^{b,c,d,2,3}
Diabetes+Chrysin (200 mg/Kg. b.wt)	89±5 ^{b,c,d}	170±6 ^{b,c,1}	120±6 ^{b,c,1,2}	74±2 ^{b,c,d,2,3}

OGT=Oral glucose tolerance, ^{a,b,c,d,1,2,3}=Each value is mean ± SEM of six animals (n=6). 1-compared with respective fasting blood glucose; 2-compared with respective 1-h blood glucose; 3-compared with respective 2-h blood glucose; a-compared with control; b-compared with diabetic control; c-compared with diabetes + chrysin (25 mg/kg b.wt.); d-compared with diabetes + chrysin (50 mg/kg b.wt.)

sensitivity of the assay was 0.005 ng/mL. The percentage reactivity of C-peptide was not detectable. Intra-assay CV was $\leq 10\%$ and inter-assay CV was $\leq 10\%$. Results are expressed as $\mu\text{IU/mL}$.

Serum lipid profile

Serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL), high-density lipoprotein cholesterol (HDL) and free fatty acids (FFA) were estimated using biochemical assay kits obtained from Spinreact, Spain according to the manufacturer's instructions.

Determination of lipid peroxidation (LPO) and reactive oxygen species (ROS) generation

LPO was measured by a previously published method.^[28] The malondialdehyde content of the sample is expressed as nanomoles of malondialdehyde formed/min/mg protein. Hydrogen peroxide generation was assessed by the spectrophotometric method.^[29] The hydrogen peroxide content of the sample is expressed as mmol/min/mg protein. Hydroxyl radical (OH^*) production was quantified^[30] and expressed as mmol/min/mg protein.

Estimation of glycogen content

Glycogen was estimated by the method of Hassid and Abraham.^[31] Five milligrams of tissue was digested with 1 mL of 30% KOH for 20 min in a boiling water bath. The contents were cooled on an ice bath and 1.25 mL of 95% ethanol was added, thoroughly mixed and gently brought to boil in a hot water bath, then cooled and centrifuged for 15 min at 3000 $\times g$. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few minutes. The precipitate was redissolved in 1 mL of distilled water, reprecipitated with 1 mL of 95% ethanol, centrifuged and drained as stated before. The precipitate was dissolved in 5 mL of distilled water and 10 mL of 0.2% anthrone reagent was added under ice cold conditions. Five milliliters of distilled water and series of standards with a final volume of 5 mL were treated with the anthrone reagent and subjected to the same procedure. The tubes were covered with glass marbles and heated for 10 min in a boiling water bath. The contents were cooled immediately and the color developed was read at 680 nm. The amount of glycogen is expressed as mg/g of wet tissue.

Western blot analysis

Isolation of plasma membrane and cytosolic fractions

Plasma membrane and cytosolic fractions from the skeletal muscle of control and experimental animals were prepared as described previously.^[32,33] Tissues were homogenized in buffer A containing 10 mM/L

NaHCO_3 (pH 7.0), 250 mM/L sucrose, 5 mM/L NaN_3 , protease inhibitor cocktail (Sigma Chemical Company, USA) and 100 $\mu\text{M/L}$ phenyl methyl sulfonyl fluoride (PMSF) using a Polytron-equipped homogenizer (Model PT 3000, Kinematica, Littau, Switzerland) at a precise low setting on ice. The resulting homogenate was clarified at 1300 $\times g$ for 10 min at 4°C. The resultant supernatant was centrifuged at 20,000 $\times g$ for 30 min at 4°C. The pellet was resuspended in buffer A, applied on discontinuous sucrose gradients (25, 32, and 35%, wt/wt) and centrifuged at 150,000 $\times g$ for 16 h at 4°C. Membranes at 25–32% (plasma membrane) and 32–35% (cytosolic fraction) interfaces were recovered, diluted with sucrose-free buffer A and centrifuged at 190,000 $\times g$ for 1 h at 4°C. Pellets were resuspended in buffer A and the protein concentration was estimated using bovine serum albumin (BSA) as a standard. The IR protein expression was evaluated in the plasma membrane fraction. IRS-1, p-IRS-1^{Tyr632}, Akt, p-Akt^{Thr308} and protein levels were seen in the cytosolic fraction. The GLUT4 level was assessed in the cytosolic fractions.

The lysate proteins (50 $\mu\text{g/}$ lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel) and transferred by electroblotting to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., 1000 Alfred Nobel Drive, Hercules, CA 94547). The membranes were blocked with 5% non-fat dry milk and probed with the primary antibodies (which were diluted to 1:1000). Following incubation, the blot was washed for three times (5 min each) with Tris-buffered saline containing Tween-20 (TBS-T). After washing with TBS-T, the membranes were incubated for 1 h with horseradish peroxidase-conjugated rabbit-antimouse or goat-anti-rabbit antibodies (which were diluted to 1:5000, GeNei, Bangalore, India). The specific signals were detected with an enhanced chemiluminescence detection system (Thermo Fisher Scientific Inc., 81 Wyman Street, Waltham, MA U.S.A 02451). The protein bands were captured using Chemidoc and quantified by Quantity One image analysis system (Bio-Rad Laboratories, CA). Later, the membranes were incubated in stripping buffer at 50°C for 30 min. After this, the membrane was reprobed using a β -actin antibody (1:5000). As the invariant control, the present study used rat β -actin.

Statistical analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) and Duncan's multiple comparison test to assess the significance of individual variations between the control and treatment groups using a computer-based software [SPSS 7.5 using Windows student version]. The significance was considered at the level of $P < 0.05$.

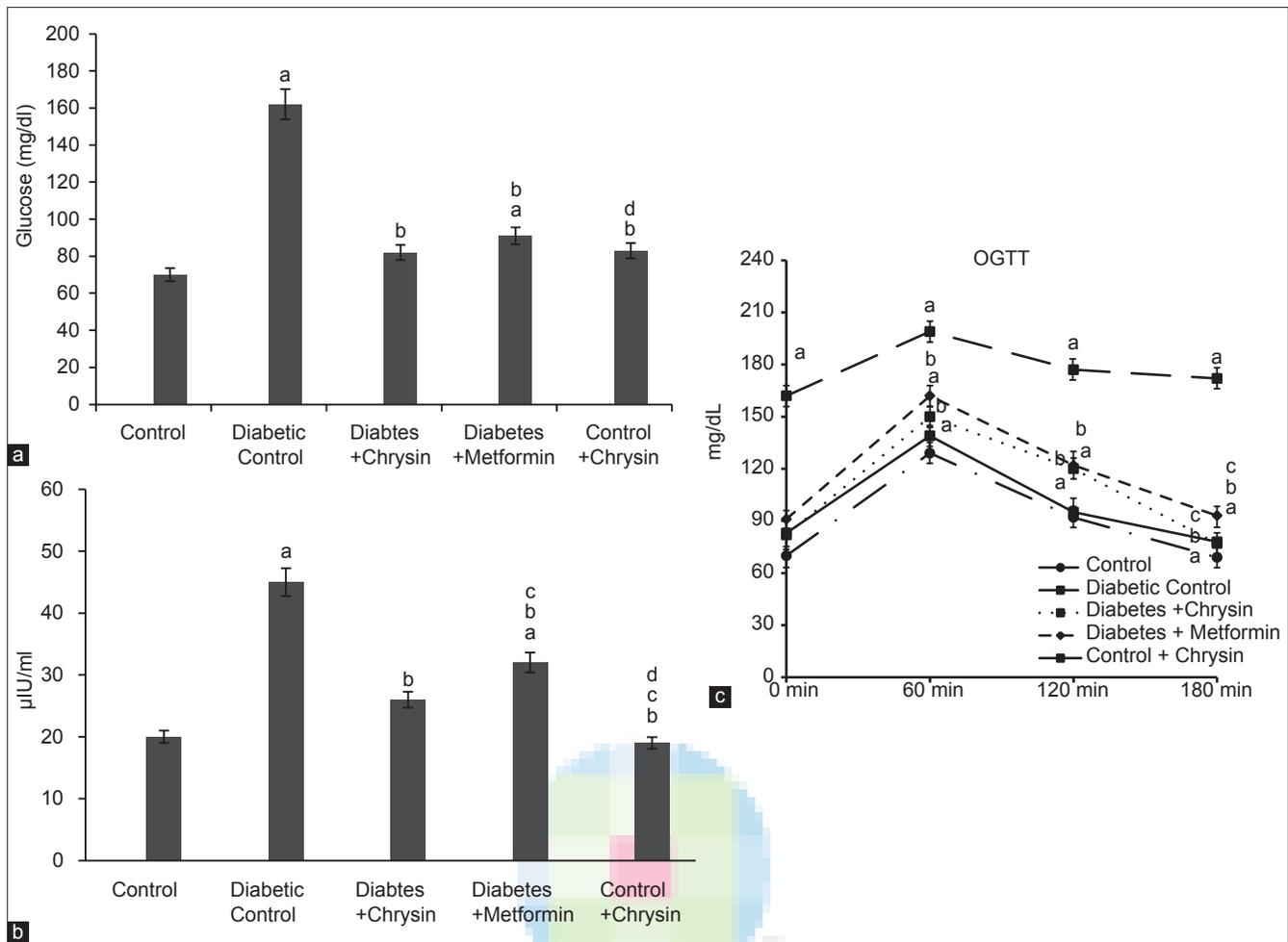


Figure 3: (a) Effect of chrysin on fasting blood glucose and (b) oral glucose tolerance of type-2 diabetic adult male rats. Each bar/point is mean \pm SEM of six animals ($n=6$). Significance at $P < 0.05$. a-compared with control; b-compared with diabetic control; c-compared with diabetes + chrysin; d-compared with diabetes + metformin (c) Effect of chrysin on serum insulin of type-2 diabetic adult male rats. Each bar is mean \pm SEM of six animals ($n=6$). Significance at $P < 0.05$. a-compared with control; b-compared with diabetic control; c-compared with diabetes + chrysin; d-compared with diabetes + metformin

RESULTS

FBG, glucose tolerance and serum insulin

Significant increase in blood glucose and insulin levels was noticed in the high fat and sucrose-induced type-2 diabetic rats than in the control rats [Figure 3a and c]. Chrysin treatment to diabetic rats reduced the blood glucose and insulin levels close to those seen in the control. Glucose tolerance test [Figure 3b] was performed for each group to investigate systemic insulin sensitivity. Exposure to high fat and sucrose feeding resulted in glucose intolerance in rats. Diabetic rats showed significantly higher glucose levels on glucose challenge than the control group. However, oral administration of chrysin to diabetic rats showed near-normal glucose tolerance. Chrysin treatment of the control rats did not show any change in the glucose and insulin levels compared with the control rats.

Effect of chrysin on lipid profile

Diabetic rats showed a significant increase in the levels of serum TC, TG, LDL cholesterol, VLDL cholesterol and FFA, but a significant decrease in HDL cholesterol level, whereas chrysin treatment reversed the same effectively [Table 2].

Effect of chrysin on LPO and ROS

ROS generation and LPO in the gastrocnemius muscle were found to be significantly elevated in diabetic rats when compared with control rats. Treatment with chrysin and metformin proved to be beneficial in reducing the free radical production and LPO in the tissues studied [Table 3].

Effect of chrysin on insulin receptor (IR) protein expression

Effect of chrysin on IR protein expression in the gastrocnemius muscle is depicted in Figure 4. In the

present study, the IR protein was significantly reduced in diabetic rats. Chrysin and metformin treatment significantly improved the IR protein in diabetic rats.

Effect of chrysin on insulin receptor substrate-1 (IRS-1) protein levels

In diabetic rats, IRS-1 and its phosphorylation (Tyr 632) protein and levels were found to be decreased compared with the control [Figure 5a and b]. Treatment with chrysin and metformin restored it to near-normal levels. There was no significant difference between the control and control rat treated with the chrysin groups.

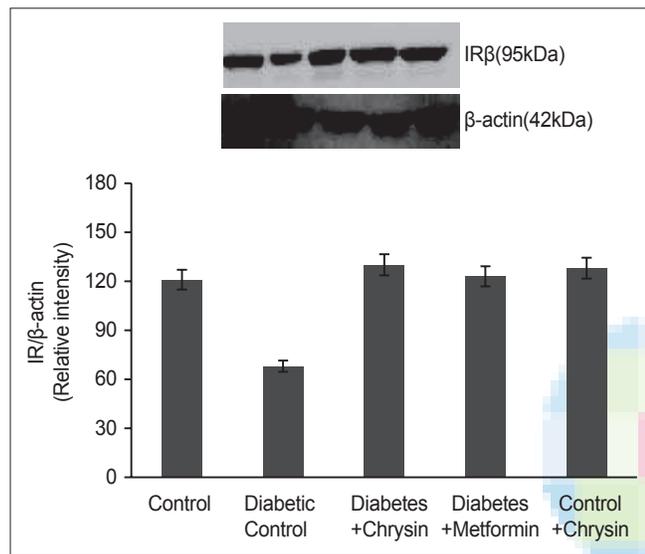


Figure 4: Effect of chrysin on insulin receptor protein expression in the gastrocnemius muscle of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (n=6). Significance at P<0.05, a-compared with control; b-compared with diabetic control

Effect of chrysin on Akt protein levels

Figure 6 depicts the effect of chrysin on Akt protein levels in the gastrocnemius muscle of diabetic rats. Akt protein expression was found to be decreased in diabetic rats [Figure 6a]. Similar to Akt, phosphorylation of Akt at Thr 308 was also decreased [Figure 6b]. Interestingly,

Table 2: Effect of chrysin on lipid profile of type-2 diabetic adult male rats

Parameters	Control	Diabetic control	Diabetes+ Chrysin	Diabetes+ Metformin	Control+ Chrysin
TC	52±2	136±7 ^a	62±3 ^b	70±3 ^{a,b}	56±6 ^{b,d}
TGs	92±6	193±7 ^a	113±6 ^{a,b}	119±8 ^{a,b}	90±5 ^{b,c,d}
HDL	31±2	14±1 ^a	37±2 ^b	41±3 ^b	39±1 ^{b,d}
VLDL	19±1	41±2 ^a	17±0.8 ^b	22±13 ^{a,b}	22±0.5 ^{b,d}
LDL	50±2	112±6 ^a	57±3 ^b	68±4 ^{a,b,c}	47±1 ^{b,d}
FFAs	93±4	167±9 ^a	88±4 ^b	96±6 ^{a,b}	88±6 ^{b,d}

TC=Total cholesterol, TGs=Triglycerides, HDL=High-density lipoprotein, VLDL=Very low-density lipoprotein, LDL=Low-density lipoprotein, FFAs=Free fatty acids, ^{a,b,c,d}=Each value represents mean ± SEM of six animals (n=6). Significance at P<0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes+chrysin; d-compared with diabetes + metformin

Table 3: Effect of chrysin on lipid profile of type-2 diabetic adult male rats

Group	Lipid Peroxidation (nmoles of Malondialdehyde Formed/min/mg protein)	Generation (mmol/min/mg protein)	
		Hydrogen Peroxide	Hydroxy Radical
Control	57±3.6	11±0.88	184±7.8
Diabetic control	145±5.7 ^a	26±1.2 ^a	245±10.2 ^a
Diabetes+Chrysin	68±3.2 ^{a,b}	12±1.3 ^b	179±7.2 ^b
Diabetes+Metformin	95±3.1 ^{a,b,c}	15±0.98 ^b	190±3.3 ^{b,c}
Control+Chrysin	63±4.2 ^{b,d}	12±0.89 ^b	177±4.4 ^{b,d}

^{a,b,c,d}=Each value represents mean ± SEM of six animals (n=6). Significance at P<0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes + chrysin; d-compared with diabetes + metformin

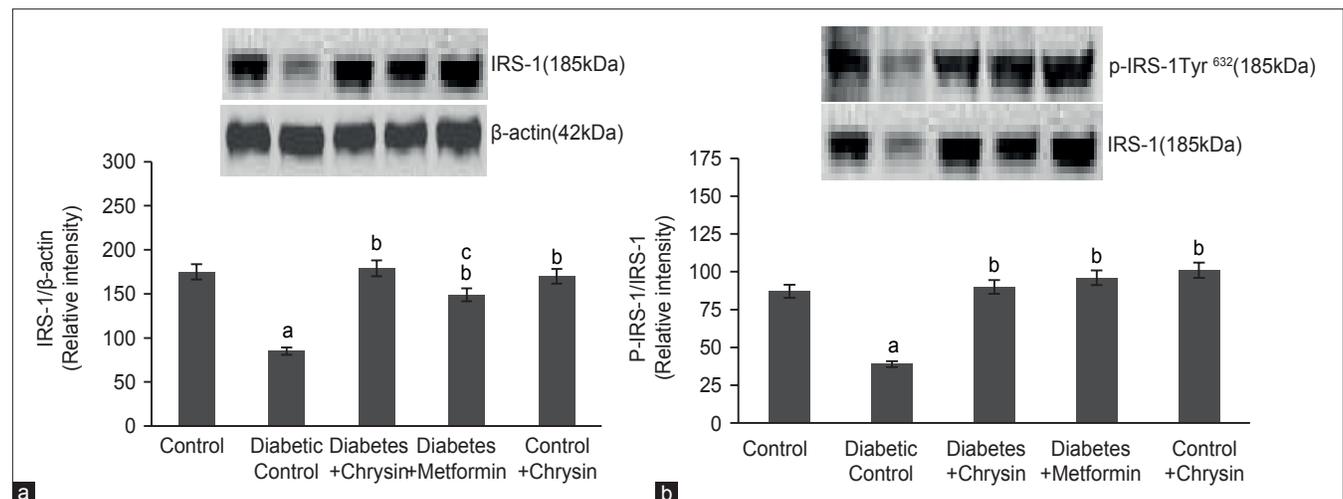


Figure 5: (a) Effect of chrysin on insulin receptor substrate-1 (IRS-1) and (b) p-IRS-1Tyr632 protein expression in the gastrocnemius muscle of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (n=6). Significance at P<0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes+chrysin

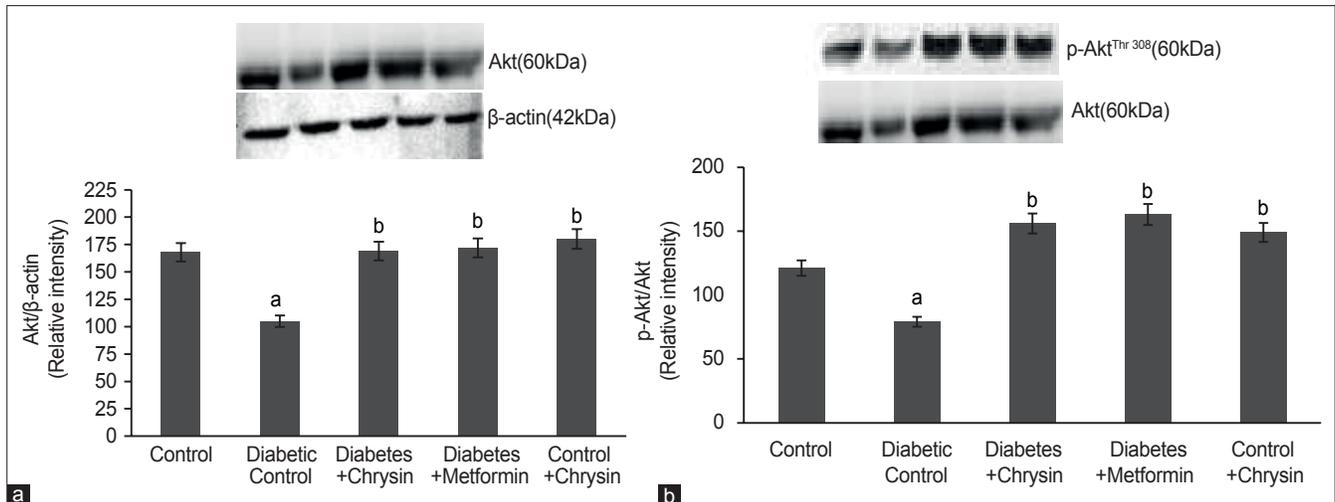


Figure 6: (a) Effect of chrysin on Akt and (b) p-Akt^{Thr308} protein expression in the gastrocnemius muscle of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (*n*=6). Significance at *P* < 0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes+control

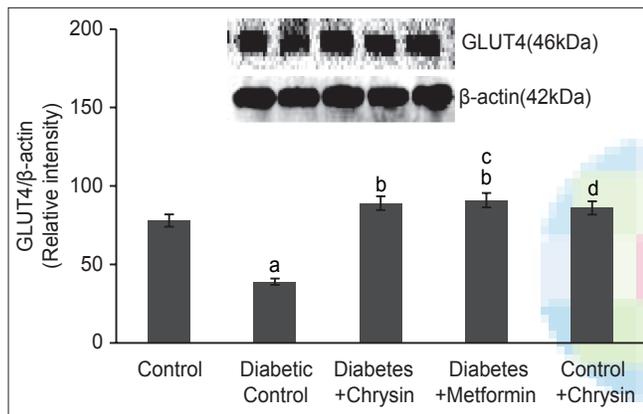


Figure 7: Effect of chrysin on glucose transporter 4 protein expression in the gastrocnemius muscle of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (*n*=6). Significance at *P* < 0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes+chrysin

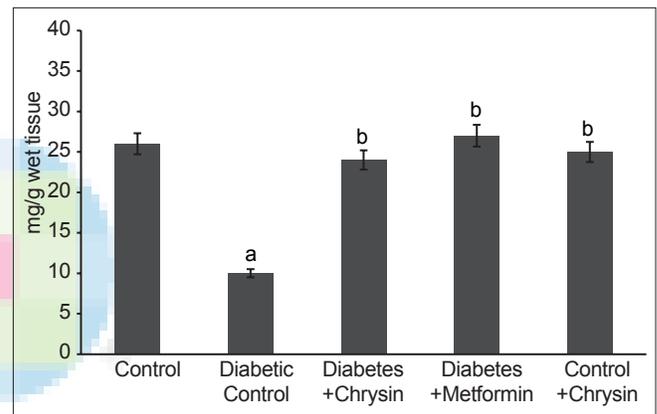


Figure 8: Effect of chrysin on tissue glycogen in the gastrocnemius muscle of type-2 diabetic adult male rats. Each value is mean ± SEM of six animals (*n*=6). Significance at *P* < 0.05, a-compared with control; b-compared with diabetic control; c-compared with diabetes+chrysin

chrysin treatment improved the phosphorylation of Akt better than metformin treatment [Figure 6]. Treatment with chrysin to control rats had no significant influence.

Effect of chrysin on GLUT4 protein expression

Figure 7 represents GLUT4 protein expression in the gastrocnemius muscle. In diabetic rats, GLUT4 protein expression was decreased whereas there was a significant rise in the chrysin- and metformin-treated rats. There was no significant difference in GLUT4 protein level between the control and control rats treated with chrysin groups.

Effect of chrysin tissue glycogen

Type-2 diabetes lowered the glycogen concentration in the gastrocnemius muscle, but treatment with chrysin was able to restore the same to the control levels [Figure 8].

No statistical difference was observed in the glycogen level between the control and chrysin control rats.

DISCUSSION

Naturally occurring compounds have been proposed to exert beneficial effects on health and have drawn attention because of their safety.^[34] Thus, evidences are accumulating to suggest the antidiabetic activity of flavonoids,^[34] although insufficient investigation at the molecular level has been performed to support these observations. In the present study, we have firstly shown that chrysin, a flavonoid, increased the IR, IRS-1, phosphorylation of IRS-1 (Tyr632) and Akt (Thr308) in the gastrocnemius muscle of type-2 diabetic rats. Further, chrysin restored the level of GLUT4 and glycogen content, which were decreased in high fat diet and sucrose-induced type-2 diabetic rats.

Skeletal muscle insulin resistance is considered to be the initiating or primary defect that is evident decades before β -cell failure and overt hyperglycemia develops.^[35] Binding of insulin to its receptor (IR) activates the receptor tyrosine kinase, which phosphorylates and recruits the IRS family of proteins. Tyrosine-phosphorylated IRS display binding sites for phosphatidylinositol-3 kinase (PI3K), which, in turn, activates Akt/protein kinase B, resulting in increased translocation of intracellular GLUT4 to the plasma membrane. The activation of the IRS-PI3K-Akt pathway facilitates glucose uptake by the skeletal muscle cells.^[36] In insulin-resistant states such as obesity, hypertension and type-2 diabetes, insulin-induced glucose transport is markedly decreased in the skeletal muscle due to an impaired expression and functionality of the insulin signaling pathway.^[37] In the present study, diabetic rats showed higher FBG and insulin levels than that of control rats. In this regard, studies have shown that high fat diet and sucrose feeding in rodents leads to the development of whole body insulin resistance and impaired ability of insulin to stimulate glucose uptake in the skeletal muscle.^[32,33] Therefore, high fat diet-induced increase in blood glucose and insulin levels recorded in the present study may be due to hyperglycemia-induced insulin resistance. However, chrysin administration decreased the levels of insulin and FBG to the normal range probably by improving insulin sensitivity and thus glucose tolerance, indicating that chrysin has an antidiabetic potential.

Elevated levels of TG and FFAs, TC in serum and altered lipoprotein profile (increased LDL cholesterol and VLDL cholesterol and decreased HDL cholesterol) were observed in high fat and sucrose-fed diabetic rats. Excess fat intake results in dyslipidemia, characterized by elevated levels of FFAs, TG and altered lipoprotein profile.^[38] Further, excess TG, FFA and their metabolites can interfere with insulin-stimulated phosphatidylinositol-3-kinase (PI3K)/Akt activation and thereby lower the downstream signaling events of insulin, leading to insulin resistance.^[38] Diabetic rats showed a significant increase in the levels of serum TC, TG, LDL cholesterol, VLDL cholesterol and FFA, but a significant decrease in HDL cholesterol level, whereas chrysin treatment reversed the same effectively. In accordance with the present study, Pushpavalli *et al.*^[12] demonstrated that treatment with chrysin at a dose of 25 mg/kg in rats was effective in decreasing TC, TG, FFA, LDL cholesterol and VLDL cholesterol and increasing HDL cholesterol levels in a model D-galactosamine-induced hepatotoxicity. In the study by Anandhi *et al.*^[39] chrysin at the dose of 200 mg/kg protected against hypercholesterolemia induced by Triton WR-1339 in rats. In the present study also chrysin treatment

reduced the lipid profile to the normal level, suggestive of an antihyperlipidemic activity.

ROS play an important role in the living system through their beneficial and detrimental effects.^[40] ROS can suppress the insulin response and contribute to the development of insulin resistance, a key pathological feature of type-2 diabetes mellitus. In the present study, diabetic rats showed an increase in the LPO *OH and H₂O₂ levels in the gastrocnemius muscle. However, diabetic rats treated with chrysin significantly reduced the levels of the same and brought it back to the normal levels, and this may be due to increased antioxidant activities. In this regard, Rice-Evans^[41] described the beneficial effects of chrysin by having the capability of free radicals scavenging. Sirovina *et al.*^[42] demonstrated that administration of quercetin and chrysin to diabetic mice resulted in a significant decrease in the LPO level in liver tissue.

The effect of insulin on glucose uptake is mediated by an efficient signal transduction process, which is initiated by its binding to the extracellular domain of IR.^[43] Insulin stimulated tyrosine kinase activity per IR was reported to be significantly lowered in highfat-fed animals, accompanied by diminished autophosphorylation of the β -subunit of the receptor and lower proportion of tyrosinephosphorylated receptors.^[44] In addition, it has been reported that FFA inhibits IR gene expression and contributes to a reduced amount of IR protein in the insulin target cells. In our study, diabetic rats showed a significant decrease in the IR protein level compared with the control.

The increased LPO and free radical generation recorded in the present study might have caused severe plasma membrane disruption thus leading to decreased IR in high fat and sucrose-induced diabetic rats. However, chrysin treatment reversed the same to the control level by reducing LPO and H₂O₂ generation. This is the first of a kind report of the role of chrysin on IR protein expression.

IRS-1 and IRS-2 are widely expressed and appear to specify diverse insulin signal cascades.^[45,46] IRS-1 plays an important role in insulin signaling in the skeletal muscle, whereas IRS-2 is important in the liver and ovary. In our study, IRS-1 protein and its phosphorylation at Tyr632 (essential for appropriate insulin signaling) were much decreased in diabetic rats. High fat diet was shown to increase the intracellular fatty acyl-CoA and DAG concentrations, which in turn result in activation of PKC- θ leading to increased IRS-1 Ser307 and Ser636 phosphorylation.^[47,48] Further, high fat diet has been shown to elevate the levels of c-Jun NH2-terminal kinase,^[32] inhibitor of nuclear factor kappa B kinase complex, extracellular signal-regulated kinases^[34] and cause serine phosphorylation (Ser636) of IRS-1, resulting in impaired

insulin-stimulated tyrosine phosphorylation (Tyr632) in IRS-1.^[49] Similarly, insulin-stimulated tyrosine phosphorylation of IRS proteins and activation of their downstream effectors are decreased in both genetic^[50] and induced^[51] rodent models of obesity and insulin resistance. Chrysin-treated type-2 diabetic rats showed an improved tyrosine phosphorylation (Tyr632) of IRS-1, and this may be attributed to an increase in IR protein expression recorded in the present investigation as a result of its anti-free radical scavenging and antihyperlipidemic activity.

The serine/threonine kinase Akt (also called protein kinase B) triggers insulin effects on the skeletal muscle, such as glycogen synthesis and activates GLUT4 translocation by phosphorylating Akt substrate 160kDa (AS160) and hence the Akt is considered to be one of the important downstream signaling molecules.^[52] In the present study, Akt protein was unaltered but there was a significant decrease in the phosphorylation of Akt protein at Ser473 in diabetic rats, and this may be due to defects in the IR and IRS-1 in the gastrocnemius muscle of high fat and fructose-induced type-2 diabetic rats, whereas chrysin treatment significantly improved the IR and IRS-1 phosphorylation thereby improving the activity of Akt.

GLUT4 is the major transporter that mediates glucose uptake in insulin-sensitive tissues, such as skeletal muscle and adipose tissue. Upon binding of insulin to its receptor, vesicles containing GLUT4 are translocated from the cytoplasm to the plasma membrane, inducing glucose uptake. Reduced GLUT4 translocation is one of the causes of insulin resistance in type-2 diabetes.^[53,54] GLUT4 expression was significantly reduced in high fat and sucrose induced-type-2 diabetic rats. Increased FFA and lipotoxicity have been shown to repress the activity of the GLUT4 promoter in cardiomyocytes and reduced the GLUT4 protein in human cardiac muscle biopsies and also attenuate insulin signaling and GLUT4 translocation through activation of the I κ B kinase (IKK) pathway.^[54] High levels of FFA are shown to reduce the GLUT4 gene expression and translocation of GLUT4 from cytosol to plasma membrane.^[55] Excess fatty acid in the diabetic rats was restored to normal after treatment with chrysin, and this might have been contributed to the restoration of GLUT4 protein expression. Thus, type-2 diabetic rats showed a decreased GLUT4 level in the gastrocnemius muscle. This amelioration in the GLUT4 protein following treatment with chrysin may also be due to enhancement in the activity of insulin signaling molecules and thus the improved insulin sensitivity. Glycogen concentration was also reduced in the gastrocnemius muscle of high fat and sucrose-fed rats, which may be the result of impairment in glycogenesis due to diminished levels of Akt phosphorylation at

Thr308 that is essential for the activation of glycogen synthase. Previous studies from our laboratory also showed a reduction in the muscle glycogen concentration of high fat fed rats.^[56]

CONCLUSION

The present study clearly reveals that chrysin improves glycemic control through activation of insulin signal transduction in the gastrocnemius muscle of high fat and sucrose-induced type-2 diabetic male rats. Treatment with this flavonoid in addition to dietary control may offer an effective alternative approach for the management of type-2 diabetes. The expression pattern of insulin signaling molecules observed in the present study ascertains the efficacy and therapeutic value of chrysin. Hence, it is concluded that supplementation of the chrysin, along with regulated dietary habits, can offer an effective approach for the management of type-2 diabetes. Clinical trials employing such flavonoids would be of great interest and beneficial to disease management and human welfare at large.

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Conflicts of interest

There are no conflicts of interest.

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