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Effects of flavonoid-rich extract from seeds of *Eugenia jambolana* (L.) on carbohydrate and lipid metabolism in diabetic mice

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Abstract

Eugenia jambolana (*EJ*) is long known for its antidiabetic activities in traditional medicines. The aim of this study was to determine the probable mechanism of action of a flavonoid-rich extract from seeds of *EJ* on streptozotocin-induced diabetic mice. Various biochemical parameters, e.g. glucose tolerance, lipid profile, glycogen biosynthesis, glucose uptake and insulin release *in vivo* and *in vitro*, were significantly improved in the extract-treated diabetic mice. Similarly, differential regulation and expression of glucose homeostatic enzymes, e.g. glucose-6-phosphatase and hexokinase, were also changed significantly in response to the flavonoid-rich extract, which clearly demonstrated the hypoglycemic and hypolipidemic effects in treated animals. Further, analysis of the extract using HPLC, demonstrated the presence of different flavonoids and their derivatives which are known for their antidiabetic and antioxidant potential. The data showed that this flavonoid-rich seed extract has a remarkable dual hypoglycemic and hypolipidemic effect.

Keywords: *Eugenia jambolana*; Glucose homeostasis; Serum lipids; Streptozotocin; Hypoglycemic; Hypolipidemic

1. Introduction

Diabetes is the world's largest endocrine disease, involving metabolic disorders of carbohydrate, fat and protein. According to WHO projections, the prevalence of diabetes is likely to increase by 35% (King, Aubert, & Herman, 1998). Currently there are over 150 million diabetics worldwide and this is likely to increase to 300 million or more by the year 2025 (Yadav, Vats, Dhunnoo, & Grover, 2002). Therefore, it is necessary to look for new drugs and interventions that can be used to manage this metabolic disorder. Although many drugs are available to manage diabetes, in most instances these are expensive for a developing country like India and they may also have adverse effects, e.g. hypoglycemia, obesity. On the other hand, India is a country with a vast reserve of natural resources and a rich history of traditional medicine (Grover, Yadav, & Vats, 2002). More than 400 plants with glucose-lowering effects are known. Among these plants, some have been reported to possess hypoglycemic effects (Babu, Prabuseenivasan, & Ignacimuthu, 2007; Wang & Ng, 1999) and some hypolipidemic effects (Sharma, Nasir, Prabhu, Dev, & Murthy, 2003). However, there is little information about plants with both hypoglycemic and hypolipidemic effects (Babu et al., 2007).

Eugenia jambolana (*EJ*) which belongs to the family Myrtaceae, is a large evergreen tree growing up to 30 m height, found widely in India and the Asian subcontinent. The seeds of this plant have been reported to possess many medicinal properties in the Ayurveda system of medicine. The fresh seeds are most effective in diabetes as they quickly reduce sugar in urine (Ashok & Daradka, 2001; Sharma, Nasir, Prabhu, & Murthy, 2006). Achrekar, Kakij, Pote, and Kelkar (1991) reported the hypoglycemic response of seed and pulp extract on diabetic mice. Although *EJ* is established for its antidiabetic potential in ayurveda, as well as in the modern scientific community, active constituents from the seed core have not as yet been isolated or identified.

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Flavonoids, low molecular weight substances, found in all vascular plants, are phenyl-benzopyrones (phenylchromones) with an assortment of structures based on a common three-ring nucleus. They are primarily recognized as the pigments responsible for the autumnal burst of hues and many shades of yellow, orange and red in flowers and food. The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities (Carroll, Guthrie, So, & Chambers, 1998; Welton, Hurley, & Will, 1988). However, little is known about their effect in regulating glycemic control in the body.

The aim of this study was to understand the molecular mechanism behind the hypoglycemic and hypolipidemic nature of the flavonoid-rich fraction of *EJ* seeds. Out of various fractions (alkaloids, flavonoids, steroids, saponins, terpenoids) isolated from seeds of this plant, the study was narrowed down to the flavonoid-rich fraction as it was found to be the most effective for hypoglycemic and antidiabetic effects. Glucose tolerance test (GTT), lipid profile, *in vivo* and *in vitro* insulin secretion, glycogen content and enzyme activity were analyzed to evaluate its effect on biochemical parameters. Further, its actions at cellular and molecular levels were analyzed by determining its effects on expression of various target genes, e.g. glucokinase (GK), glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), glut 4 and aldose reductase (AR) in different target tissues, such as liver, muscle, adipose and kidney. Finally, we attempted to identify the exact molecules responsible for this dual hypoglycemic and hypolipidemic effect by HPLC analysis of the flavonoid-rich extract. To the best of our knowledge, this is the first ever report on the role of flavonoids demonstrating both hypoglycemic and hypolipidemic effects at cellular levels.

2. Materials and methods

2.1. Plant material

The seeds were collected from in and around the campus of the Indian Institute of Technology, Roorkee during the months of July–September. The collected plant materials were identified from the Ayurveda literature and by local in charge of herbal gardens and also confirmed by Dr. H.S. Dhaliwal, Professor of Plant Biotechnology, Department of Biotechnology, Indian Institute of Technology Roorkee, India.

2.2. Preparation of flavonoid-rich extract of the seed

EJ seeds were thoroughly washed with water, and dried in the shade. One hundred grammes of air-dried seeds were ground to fine powder and soaked in 70% ethanol for 24 h with continuous stirring. After soaking, the mixture was filtered with Whatmann No. 1 filter paper. The filtrate obtained was centrifuged at 10,000 rpm at room temperature (25 °C) and the pellet was discarded. The supernatant was concentrated *in vacuo* by means of rotavapor .The concentrated extract was then dissolved in as little water as possible and washed three times with chloroform. The residual layer was extracted three times with ethyl acetate. All the extracts were finally pooled and concentrated using the rotavapor.

2.3. Selection of animals

The study was conducted on mature albino mice (Jamia Hamdard University, New Delhi, India), five to six weeks of age, weighing 35 ± 5 g, which were housed in colony cages (five mice per cage) at an ambient temperature of 25 ± 2 °C with 12 h light and 12 h dark cycle. The mice were fed normal diets purchased commercially from vendors. The animals were allowed to acclimatize to the laboratory environment for 1 week and then randomly divided into three groups ($n = 5$ mice per group) as given below: Group I – untreated (control); Group II – streptozotocin-treated (diabetic); Group III – streptozotocin-induced diabetic mice treated with *EJ* flavonoid-rich seed extract (diabetic treated). After randomization into various groups, the mice were acclimatized for a period of a further 7 days in the new environment before initiation of the experiment. Animals described as fasting had been deprived of food for at least 16 h but were allowed free access to drinking water. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee and had prior approval.

2.4. Induction of diabetes mellitus, followed by flavonoid-rich EJ seed extracts treatment

Overnight-starved experimental mice from groups II and III (as described above) were injected with streptozotocin (Sigma, USA) at a dose of 60 mg/kg bw. The chemical was injected intraperitoneally (i.p.) within 10 min after dissolving in 0.025 M sodium citrate at pH 4.0. The mice in group I were injected with sodium citrate buffer as vehicle control. Fasting blood glucose (FBG) was estimated at the time of induction of diabetes and postprandial glucose (PPG) was checked regularly until stable hyperglycemia was achieved. The mice exhibiting blood glucose levels of ~250 mg/dl were included in the study as stable hyperglycemic animals. Once the stable hyperglycemia was achieved, the mice belonging to group III were treated with an oral dose of *EJ* flavonoid-rich extract once every day for 15 consecutive days while groups I and II mice received only 0.01% ethanol (as vehicle control).

2.5. Effect of fasting blood glucose level

Fasting blood glucose was measured after 15 days of treatment with flavonoid-rich extract of *EJ*, during which the animals were fed with normal diets. For the determination of FBG, on completion of the 15 days of treatment,

the mice were fasted overnight, the blood was collected from the tip of the tail vein and the blood glucose was measured using a GOD–POD glucose estimation kit (Excel Diagnostics Pvt. Ltd., India). The results were expressed as milligrammes per decilitre of blood.

2.6. Glucose tolerance test (GTT)

In order to determine the effect of *EJ* flavonoid-rich extract on insulin activity, the GTT was carried out on all three groups of mice, namely control, diabetic and diabetic treated. GTT was performed by oral administration of glucose load of 1 g/kg bw in 0.1 ml water to overnight-fasted animals. Blood samples were collected from the tail vein at 30, 60, 90 and 120 min after the oral glucose load and treated as before for plasma glucose analysis.

2.7. Estimation of lipid profile in blood samples

On completion of the treatment, blood samples were collected and lipid profiles for all the three groups of animals were obtained by using commercially available kits. Total cholesterol, high density lipoprotein (HDL) cholesterol and triglyceride (TG) levels in serum were determined according to the instructions of the manufacturer (Transasia Bio Medical Limited, Mumbai, India). For the determination of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, Friedwald's formula was used which states: VLDL cholesterol = Triglyceride/5 and LDL cholesterol = Total cholesterol – (VLDL + HDL cholesterol).

2.8. Biochemical estimation of enzyme activities and tissue glycogen content

2.8.1. Glucose-6-phosphatase in liver

Glucose-6-phosphatase catalyzes the conversion of glucose-6-phosphate to glucose (Schaftingen & Gerin, 2002). The mice from all three groups were sacrificed and the collected liver was homogenized in ice-cold sucrose solution (250 mM). To 0.1 ml of sucrose/EDTA buffer, 0.1 ml of 100 mM glucose-6-phosphate, 0.1 ml of imidazole buffer (100 mM, pH 6.5) and 0.1 ml of liver homogenate were added with thorough mixing. The tubes were then incubated at 37 °C for 15 min. The reaction was terminated by the addition of 2 ml of TCA:ascorbate (10%:3%, w/v), and the solution was centrifuged at 3000 rpm for 10 min. To 1 ml of clear supernatant, 0.5 ml of ammonium molybdate (1%, w/v) and 1 ml sodium citrate (2%, w/v) were added and the absorbance was measured at 700 nm. The enzyme activity was expressed as units per gramme of wet tissue.

2.8.2. Hexokinase activity in liver

The hexokinase activity was determined based on the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase, according to the method described earlier (Brandstrup, Kirk, & Bruni, 1957). Briefly, the excised liver tissue homogenate was prepared in normal saline. To 0.1 ml of homogenate, 2.28 ml of Tris–magnesium chloride buffer (200 mM Tris and 20 M $MgCl_2$, pH 8.0) was added, along with 0.5 ml of 0.67 M glucose, 0.1 ml of 16 mM ATP, 0.1 ml of 6.8 mM NAD and 0.01 ml of 300 U/ml glucose-6-phospate dehydrogenase solution. The solution was mixed thoroughly, and the absorbance was measured at 340 nm after 5 min of incubation. The activity was expressed as units per gramme of wet tissue.

2.8.3. Tissue levels of glycogen

Glycogen contents of liver and skeletal muscles were measured according to earlier established methods (Sadasivam & Manikam, 1996). Briefly, the samples were homogenized separately in warm 80% ethanol at a concentration of 100 mg/ml and then centrifuged at 10,000 rpm for 20 min. The residue was collected and allowed to dry in a boiling water bath. To each residue, 5 ml of distilled water and 6 ml of perchloric acid were added. The extraction was further carried out at low temperature for 20 min. The collected extract was centrifuged at 10,000 rpm for 15 min and 0.2 ml of the supernatant was transferred to a test tube and the volume was made up to 1 ml by addition of distilled water. To each tube, 4 ml of anthrone reagent was added and the whole incubated at 95 °C in a boiling water bath for 10 min. The absorbance of the samples was measured at 630 nm after cooling the tubes to room temperature. The amount of glycogen in tissue samples was expressed as microgrammes of glucose per milligramme of tissue.

2.9. Effect of flavonoid-rich extract on the release of insulin

2.9.1. In vivo studies

In order to discover whether the flavonoid-rich seed extract could stimulate the release of insulin, serum insulin levels were measured in diabetic mice treated with the extract for 15 days. Serum insulin levels were estimated in each sample of blood using enzyme linked immunosorbant assay kits (Boehringer Manheim Diagnostic, Manheim, Germany).

2.9.2. In vitro studies

Isolation of pancreatic cells was done according to methods reported earlier (Gupta et al., 2005; Xia & Laychok, 1993) with slight modification. After removing the pancreas from the mice, they were perfused with Hank's balanced salt solution (HBSS) (pH 7.4) for about 15 min to remove blood and endogenous insulin. This was followed by finely mincing the tissue and then they were incubated for 30 min at 37 °C with rapid magnetic stirring in a solution of crude collagenase (4 mg/ml) in HBSS containing 0.3% glucose and 1% bovine serum albumin (BSA) pH 7.4. In the next step, the separation of the islet cells from acinar tissues was done with ficoll (Type-400)

(Himedia, Mumbai, India) with varying gradients, followed by centrifugation. The islets were picked up from the interphase of 20–11% gradient by Pasteur pipette. The purity of the islet cells were checked by Gomori's chromium hematoxylin phloxin stain (Gomori, 1941). After dividing into 10 islets/batch they were pre-incubated with glucose – Krebs Ringer bicarbonate buffer (KRB), along with $NaHCO_3$ (0.2%), HEPES (0.38%), insulin-free BSA (0.1%) and 10 mM glucose for 5 min at 37 °C in a CO_2 incubator. The incubation was continued for a further 1 h after adding 50 µl of plant extract (final concentration of 30 mg/l) or buffer for controls. Aliquots of 50 µl were removed from the incubation mixture at the end of incubation (i.e. 1 h) and were stored at −20 °C prior to insulin assay.

2.10. Semiquantitative RT–PCR

Total RNA was extracted from the liver, muscle, kidney and adipose tissue of the mice treated with 300 mg/kg bw of flavonoid-rich extract and diabetic control mice according to the method described earlier (Chomczynski & Sacchi, 1987). The isolated RNA samples were quantified and equal amount of it were reverse transcribed with the help of the RT–PCR kit purchased from Bangalore Genei (Bangalore, India) according to the manufacturer's instructions. PCR was performed by denaturing at 94 °C for 60 s, annealing at various temperatures (depending on primer pairs) and by extension at 72 °C for 60 s and 35 additional cycles were used for amplification. The primer pairs used for analysis were: GK-F 5′-TTCACCTTCTCCTTCCCT-GTAAGGC-3′; GK-R5′-TACCAGCTTGAGCAGCAC-AAGTCG-3′; G6Pase–F5′-AAGACTCCCAGGACTGG-TTCATCC-3′; G6Pase–R5′-TAGCAGGTAGAATCCA-AGCGCG-3′; PEPCK-F5′-TGCTGATCCTGGGCATA-ACTAACC-3; PEPCK-R5′-TGGGTACTCCTTCTGGA-GATTCCC-3′; AR-F5′GGTGCAAGTTCCATGACAA-GAGC3′; AR-R5′CGTCCAAGTGTCCACACAATCG3′; Glut-4-F5′-TGGCCATCTTCTCTGTGGGTGC-3′; Glut-4-R5′-GGCATTGGCTAGGCCATGAGG-3′. PCR products of β-actin, using β-Actin-F 5'-TCACCCACACTGT-GCCCCATCTACGA-3' and β-Actin-R 5'-CAGCGGAA-CCGCTCATTGCCAATGG-3' primers gene, were used as internal standards. The PCR products were then separated on 2% agarose gel and visualized in a gel documentation system (Bio Rad, USA). The intensity of the bands on gels was converted into a digital image with a gel analyzer.

2.11. Histopathological studies

Pancreases, fixed in Bouins solution, were dehydrated by upgrading from 30% to 100% in alcohol and then xylene each for 1 h, followed by embedding in wax at 60 °C. Paraffin blocks of the tissues were sectioned to 5 µm thickness. The sections were then stained in hematoxylin and eosin following the earlier described methods (Mukherjee, 2003).

2.12. HPLC analysis

Compounds were separated on a 150 mm × 4.6 mm, i.d., 5 µm particle, nova pack column (Waters, USA). The Dual λ absorbance detector (Waters 2487, USA) was operated at 265 nm and the column oven temperature was 30 °C. Each extract was injected in triplicate ($n = 3$). HPLC grade methanol and milli Q water (pH 3.0 adjusted with HPLC grade phosphoric acid) were used as mobile phase in 1:1 ratio at a flow rate of 1 ml/min. The injection volume was 25 µl. Mixtures of authentic samples of rutin, quercitin, myricetin and kaempferol were dissolved in HPLC grade methanol and injected under the same conditions as for the sample.

2.13. Statistical analysis

Values are presented as means ± SEM. The statistical significance was evaluated by one-way ANOVA using the statistical software Origin 6.1 (Origin Lab Corporation, USA).

3. Results

3.1. Fasting blood glucose levels and GTT

Blood glucose levels, estimated in 16 h fasting diabetic mice (FBG), were significantly elevated. However, this level was reduced significantly upon treatment with 300 mg/kg bw of plant extract (Fig. 1, FBG data).

For GTT, overnight-fasted mice were fed with 1 g/kg bw of glucose and the blood glucose level was determined up to 120 min. The blood glucose level had decreased significantly by 90 min (with respect to 30 min level) and this was maintained until 120 min with an effective dose of 300 mg/kg bw of extract (Fig. 1).

3.2. Estimation of lipid profile

Various parameters of blood lipid profiles were tested in streptozotocin-induced diabetic mice before and after the treatment with the plant extract. The levels of TC, LDL, VLDL and TG in diabetic mice were significantly lower in the plant extract-treated animals ($p < 0.05$). About 50%, 65% and 30% falls in TC, LDL cholesterol and TG levels, respectively, were found in diabetic mice treated with plant extract (group III) as compared to diabetic mice which were not treated with the extract (group II). Similarly, HDL levels were found to be increased by 50% in diabetic mice after the treatment with *EJ* seed extract as compared to diabetic mice (not treated with *EJ* extract) (Table 1).

3.3. Glycogen level in tissues

Glycogen content of skeletal muscles, liver, and kidney were estimated on day 15 of the treatment with *EJ seed* extract in control, diabetic and diabetic treated mice as

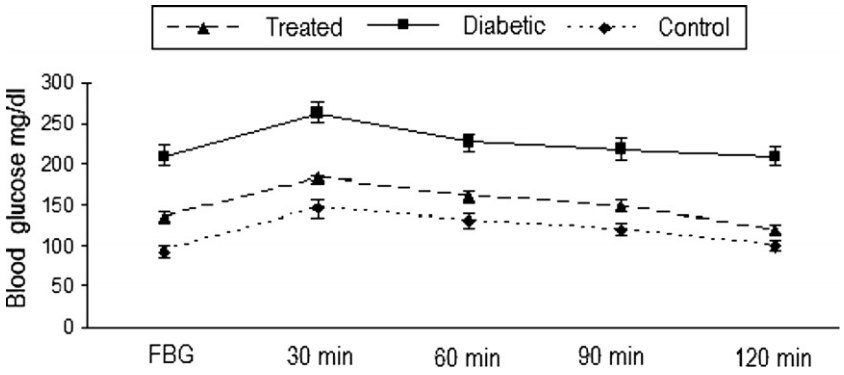

Fig. 1. Oral glucose tolerance test (OGTT) in streptozotocin-induced diabetic mice in response to flavonoid-rich extract of *Eugenia jambolana* treatment. Results are means ± SEM of $n = 5$. FBG, fasting blood glucose.

Table 1
Plasma levels of different lipids before and after the administration of flavonoid-rich fraction of *Eugenia jambolana* seed in streptozotocin-induced diabetic mice

Treatment group	Plasma lipid level (mg/dl)				
	TC	HDLC	LDLC	TG	VLDLC
Control	97 ± 4.6	23 ± 2.8	58 ± 4.2	86 ± 3.8	17 ± 2.0
Diabetic	189 ± 10	19.4 ± 1.9	144 ± 8.1	127 ± 5.6	25.4 ± 1.1
Diabetic	95 ± 6.3*	29 ± 3.2*	46.8 ± 2.1*	89.6 ± 6.5*	17.9 ± 1.3*

Each value represents the mean ± SEM, $n = 5$.*Represents statistically significant result as compared to diabetic group ($p < 0.05$).

shown in (Fig. 2a). In diabetic mice, both liver and muscle glycogen contents decreased significantly, by 49 and 41%, respectively, as compared to non-diabetic control ($p < 0.05$). Treatment with *EJ* extract led to a 45% and 26% increase, respectively in hepatic and muscle glycogen contents in the diabetic treated group. Kidney glycogen level was reduced by 8% in comparison to diabetic mice.

3.4. Enzyme activity in liver

After 15 days of treatment with flavonoid-rich extract of *EJ* there was a significant reduction in hepatic glucose-6-phospahatase activity in the diabetic treated group (group III) as compared to the untreated streptozotocin-induced diabetic mice (group II) (Fig. 2b). By contrast, treatment of the diabetic animals with *EJ* seed extract (300 mg/kg bw) led to a 30% ($p < 0.05$) rise in hepatic hexokinase activity when compared to diabetic mice (group II) where this activity was reduced substantially as compared to that of the non-diabetic control (group I) (Fig. 2b).

3.5. Effect of flavonoid-rich EJ seed extract on insulin release in diabetic rats

Serum insulin level was significantly decreased in diabetic mice with respect to control ($p < 0.05$) (Table 2). After

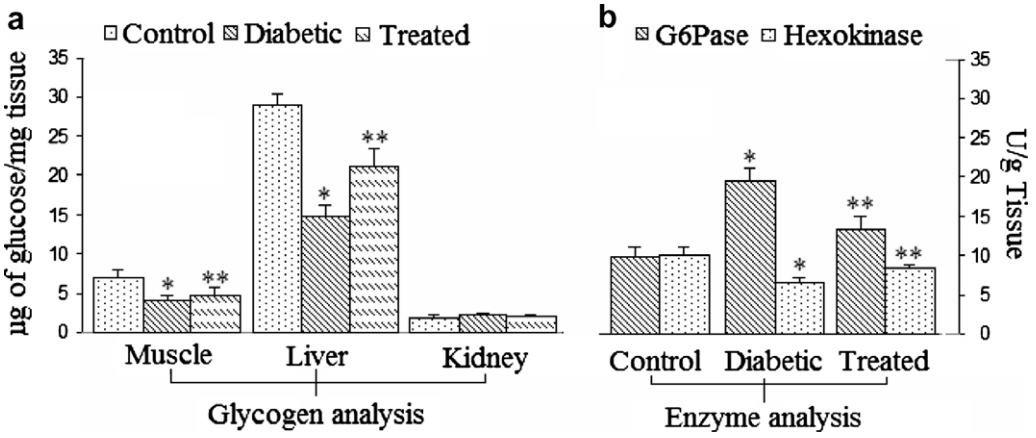

Fig. 2. Effect of flavonoid-rich fraction of *Eugenia jambolana* seed extract on (a) muscle, liver, and kidney glycogen contents and (b) hepatic levels of glucose-6-phosphatase and hexokinase in streptozotocin-induced diabetic male mice. Data are expressed as means ± SEM; $n = 5$. * and ** indicate the significant levels of difference in enzyme levels as compared to control (non-diabetic) and untreated diabetic mice, respectively ($p < 0.05$).

Table 2
Effect of *Eugenia jambolana* seed extract on insulin release in vitro from isolated islets of Langerhans and in vivo in the blood serum

Treatment group	Insulin release (μIU/ten islets/h) (μIU/ml)	Serum insulin level
Control	329 ± 4.7	22.9 ± 1.6
Diabetic	$222 \pm 5.9^{*}$	$14.2 \pm 1.1^{*}$
Diabetic + EJ	$305 \pm 7.1^{**}$	$17.8 \pm 1.9^{**}$

Each value represents the mean ± SEM ($n = 6$). *,**Represents statistically significant ($p < 0.05$) results as compared to normal control and diabetic groups, respectively. *EJ*: *Eujenia jambolana*.

15 days of *EJ* seed extract supplementation to the diabetic mice, there was significant elevation in serum insulin levels as compared to the diabetic group. Treatment with *EJ* seed extract resulted in 29% increase in insulin level. In order to further understand the role of the seed extract as insulinotrophic agent, insulin release *in vitro* from pancreatic islets of Langerhans of diabetic rats was investigated. The incubation of ten islets from diabetic rats with 10 mM glucose in the presence of *EJ* seed extract for 1 h resulted in significant stimulation of insulin ($p < 0.05$), which was about a 37% increase over the diabetic group (Table 2).

3.6. Gene expression profile

As shown in Fig. 3, a marked change in expression profile of mRNA of all the glucose regulatory enzymes studied in liver, muscle, adipose tissue and kidney was observed in diabetic treated groups (group III). The hepatic level of GK was increased by 2.5-fold in diabetic treated mice; at the same time there was a clear reduction in the expression patterns of gluconeogenic enzymes, G6Pase and PEPCK, by almost 2-fold as compared to diabetic mice (group II) (Fig. 3a). Glut-4 is responsible for tissue specific glucose uptake in muscle and adipose tissue. There was a clear increase in the expression level of Glut-4 in the diabetic treated group (about 2-fold as compared to diabetic animals) which was more prominent in adipose tissue than in muscle tissue (Fig. 3b). Further, in kidney, the diabetic treated mice demonstrated a clear reduction of more than 50% in the expression level of aldose reductase, an enzyme which is responsible for diabetes-related complications when compared to diabetic mice (Fig. 3b).

3.7. Histopathological studies

Sections of pancreas from the diabetic (group II) and diabetic treated (group III) mice had clearly shown the protective effect of the flavonoid-rich extract. A clear decrease in the area occupied by the β cells was observed in the pancreatic sections, probably due to the reduction in the number of those cells in streptozotocin-induced diabetic mice (Fig. 4a) which was again normalized in diabetic mice treated with flavonoid-rich extract of *EJ* (Fig. 4b).

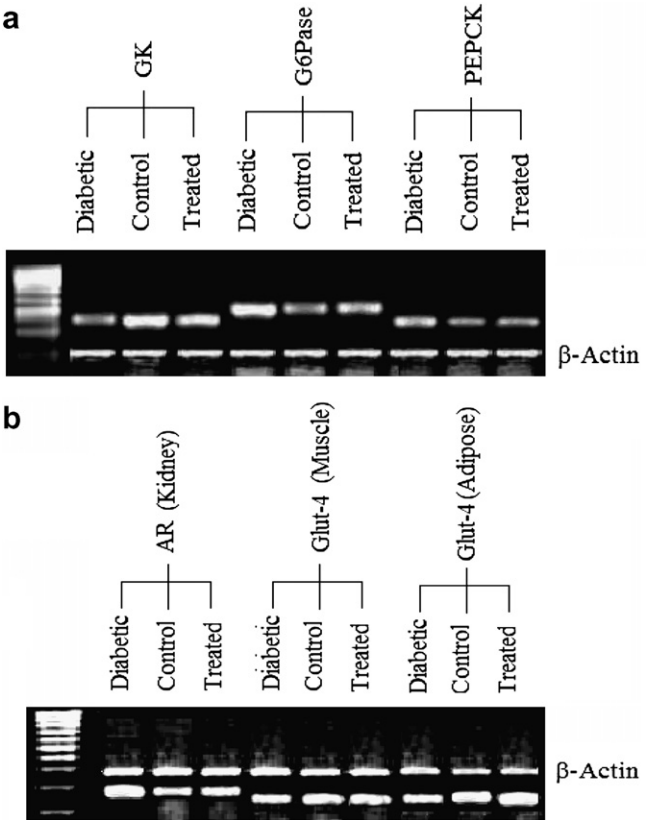

Fig. 3. RT–PCR analysis of (a) liver mRNA expression of glucokinase, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase and (b) kidney, muscle and adipose tissue mRNA expression of aldose reductase and glut-4 genes in diabetic, normal control and diabetic treated animal groups. The total RNA isolated from various tissues (liver, kidney, muscle, and adipocytes) were reverse transcribed and cDNA obtained was subjected to PCR. GK, glucokinase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; AR, aldose reductase.

4. Discussion

Diabetes is a chronic metabolic disorder affecting a major population worldwide. A sustained reduction in hyperglycemia will decrease the risk of developing micro vascular diseases and reduce their complications (Kim, Hyun, & Choung, 2006). The conventional therapies for diabetes have many shortcomings, e.g. side effects and high rate of secondary failure. On the other hand, herbal extracts are expected to have similar efficacy, without side effects, to that of conventional drugs. The present investigation reports the anti-diabetogenic, hypoglycemic and hypolipidemic effects of a flavonoid-rich fraction of *EJ* seeds on streptozotocin-induced diabetic mice. The experimental results presented here could provide a basis for understanding the exact molecular mechanism of action of this plant's active principles.

The significant reduction of peak levels of sugar within 2 h strengthens the anti-diabetogenic potential of this seed extract as previously reported by many authors in rat models (Grover et al., 2002; Sharma et al., 2003; Sridhar, Sheetal, Pai, & Shastri, 2005). Further, the plant extract also

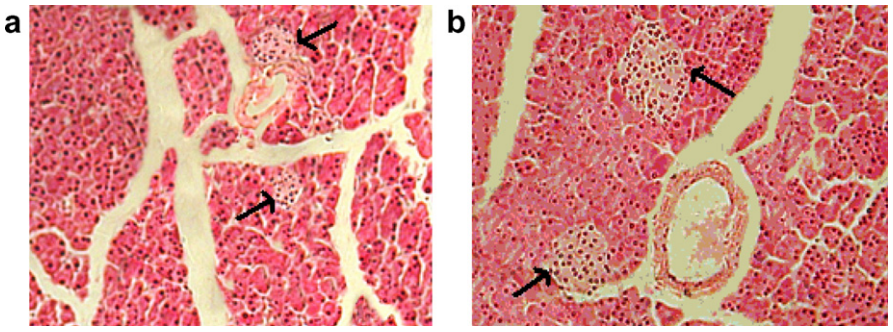

Fig. 4. Photomicrograph of pancreas from (a) streptozotocin-induced diabetic mice and (b) diabetic mice treated with flavonoid-rich extract. Hematoxylin and Eosin, 20 X objectives. The arrow indicates the islet region of the section.

significantly decreased the blood glucose level in glucose loaded mice (GTT) and this fact could be attributed to the potentiation of the insulin effect of plasma by increasing the pancreatic secretion of insulin from existing β-cells or its release from bound insulin. In this context a number of other plants have been observed to have similar patterns of hypoglycemic effects (Eidi, Eidi, & Esmaeili, 2006; Kasiviswanath, Ramesh, & Kumar, 2005). Results on the plasma insulin release from pancreas directly indicate that part of the antihyperglycemic activity of the flavonoid-rich extract is through the release of insulin from the pancreas; i.e. it exerts a direct insulinotropic effect. Earlier studies by Achrekar et al. (1991) and Sharma et al. (2006) demonstrated that the water extract of fruit pulp of *EJ* potentiates insulin release from pancreatic β cells like some of the sulphonylureas, such as tolbutamide. In addition to water extracts, synthetic derivatives of flavonoids have been shown to release insulin from insulinotropic INS-1 cells in culture, which further supports our finding that the flavonoid-rich extract probably acts as an insulinotrophic agent as well (Bozdag, Waheed, Verspohl, & Ertan, 2001). Further studies are needed to identify the exact flavonoids from the extract and their mode of action as insulinotrophic agents. Increased expression of Glut-4 in adipose and muscle tissue is an insulin-dependent process, which is responsible for tissue specific uptake of glucose in these tissues, and might also provide an additional contribution for the effectiveness of *EJ* seed extract.

Hypertriglyceridemia is a common finding in patients with diabetes mellitus and is responsible for vascular complications. It has been reported that the treatment of diabetes with insulin served to lower plasma triglyceride levels by regulating lipoprotein lipase and hydrolysing triglycerides (Shirwaikar, Rajendran, Kumar, & Bodla, 2004; Babu et al., 2007). The administration of *EJ* extract significantly decreased serum triglycerides and cholesterol in diabetic mice. These results are comparable with those of previous researchers (Ravi, Rajasekaran, & Subramanian, 2005). Further our study also demonstrates that the extract increased the serum levels of insulin in the treated group of mice (group III) as compared to diabetic mice (group II). Thus cholesterol-and triglyceride-lowering properties of *EJ* seed extract could be attributed to hypocholesteromic compounds that may act as inhibitors or activators for some enzymes which participate in cholesterol metabolism and also its potentiality to release insulin (Babu et al., 2007; Fan, Yan, Qian, Wo, & Gao, 2006). Further, several authors have also reported that flavonoids have hypolipidemic and hypocholesteromic effects (Rupasinghe et al., 2003; Leontowicz et al., 2002; Guo et al., 2006).

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues, especially in liver and skeletal muscles, are a direct reflection of insulin activity, which regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since streptozotocin causes selective destruction of β-cells of islets of Langerhans, resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin (Golden, Wals, & Okakima, 1979). However, this alteration in muscle and hepatic glycogen content is normalized by insulin treatment (Vats, Yadav, & Grover, 2004). Our study showed that, although the levels of glycogen in *EJ*-treated diabetic mice could not achieve the level same as that of non-diabetic control mice, yet the extract could significantly improve the muscle and hepatic glycogen contents. This indicates one of the possible ways the *EJ* seed extract might act by improving the process of glycogenesis in muscle and liver.

In order to understand the biochemical mechanism of action of *EJ* seed extract as anti-diabetogenic agent, a study was undertaken to estimate the level of hepatic hexokinase and glucose-6-phosphatase in diabetic mice after the treatment with *EJ* seed extract, which was also found to improve significantly as compared to diabetic mice. This effect was in parallel with the changed expression level of GK, G6Pase and PEPCK in diabetic treated mice (group III) when compared to diabetic mice (group II); these enzymes are directly linked to glucose homeostasis and production of glycogen. Reduction in the expression of the latter two enzymes (G6Pase and PEPCK) in response to extract treatment provides an additional clue to its role in carbohydrate metabolism. Moreover, reduction of these

two enzymes has been linked to an improved insulin resistant condition by some authors, indicating this extract's involvement in attenuating the type II diabetic condition. (Wu, Wang, Duan, & Lu, 2007). This result is similar to those reported earlier where several potential herbal plant extracts have been shown to improve the diabetic condition (Gupta, Raju, & Baquer, 1999; Jung, Lee, Park, Kang, & Choi, 2006).

Retinopathy, neuropathy and nephropathy are major complications which make diabetes more severe. Accumulation of sorbitol in different organs due to a higher activity of aldose reductase (AR), is a main cause of these complications (Nishimura, 1998). Therefore, inhibition of aldose reductase activity can provide a better recovery from diabetes-related complications. In this respect AR expression level was checked in kidney and there was a clear decrease in its expression in diabetic treated mice when compared to diabetic mice.

In conclusion, the data obtained from the present study indicates that the flavonoid-rich fraction of *EJ* seeds contains bioactive molecules such as various derivatives of flavonoids and their glycosides which may have beneficial effects as both hypoglycemic and anti-hyperglycemic agents. Some of the flavonoids, as identified by us in the extract by HPLC, e.g. rutin and quercetin (data not shown), are already known to have antidiabetic activity, especially with type II diabetes (Middleton, Kandaswami, & Theoharides, 2000). To the best of our knowledge, this is the first ever report on the role of flavonoids from *EJ* plant seeds in diabetes. It is necessary to identify and isolate the particular compound showing the desired effect from the *EJ* seed extract so as to understand its exact mechanism of action. However, the present study gives a preliminary indication that the flavonoid-rich extract has potential to act at multiple sites of glucose regulatory pathways. Toxicity data have already proved that the dose used in this investigation is far below the LD_{50} dose of the extract and did not show any change in blood parameters. Considering all these facts, it is reasonable to undertake further studies on possible usefulness of flavonoid-rich *EJ* seed extract in the treatment of diabetes mellitus.

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References

Achrekar, B., Kakij, G. S., Pote, M. S., & Kelkar, S. M. (1991). Hypoglycemic activity of *Eugenia jambolana* and *Ficus bengalensis*. *In vivo, 5*(2), 143–148.

Ashok, P., & Daradka, M. M. H. (2001). Antidiabetic activity of *Szygium cumini* seeds extracts in alloxan induced diabetic mice. Hamdard Medicus Vol. XL11.XL1 N No. 4.1.

Babu, P. S., Prabuseenivasan, S., & Ignacimuthu, S. (2007). Cinnamaldehyde-A potential antidibetic agent. *Phytomedicine, 14*(1), 15–22.

Bozdag, D. O., Waheed, A., Verspohl, E. J., & Ertan, R. (2001). Synthesis and hypoglycemic activity of some new flavone derivatives 4th communications: 6 flavonyl-2,4-thiazolidinediones. *Arzneimittelforschung, 51*(8), 623–627.

Brandstrup, N., Kirk, J. E., & Bruni, C. (1957). Determination of hexokinase in tissues. *Journal of Gerontology, 12*, 166–171.

Carroll, K. K., Guthrie, N., So, F. V., & Chambers, A. F. (1998). Anticancer properties of flavonoids, with emphasis on citrus flavonoids. In C. A. Rice-Evans & L. Packer (Eds.), *Flavonoids in health and disease* (pp. 437–446). New York: Marcel Dekker Inc.

Chomczynski, P., & Sacchi, N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry, 162*(1), 156–159.

Eidi, A., Eidi, M., & Esmaeili, E. (2006). Antidiabetic effect of garlic (*Allium sativum* L.) in normal and streptozotocin-induced diabetic rats. *Phytomedicine, 13*(9–10), 624–629.

Fan, C., Yan, J., Qian, Y., Wo, X., & Gao, L. (2006). Regulation of lipoprotein lipase expression by effect of hawthorn flavonoids on peroxisome proliferators response element pathway. *Journal of Pharmacological Sciences, 100*(1), 51–58.

Golden, S., Wals, P. A., & Okakima, F. (1979). Glycogen synthesis by hepatocytes from diabetic rats. *Biochemical Journal, 182*(3), 727–734.

Gomori, G. (1941). Observations with differential stains on human islets of Langerhans. *American Journal of Pathology, 17*(3), 395–406.

Grover, J. K., Yadav, S., & Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology, 81*(1), 81–100.

Guo, L., Hu, W.-R., Lian, J.-H., Ji, W., Deng, T., Qian, M., et al. (2006). Anti-hyperlipidemic properties of CM 108 (a flavone derivative) in vitro and in vivo. *European Jouranl of Pharmacology, 551*(1–3), 80–86.

Gupta, D., Raju, J., & Baquer, N. Z. (1999). Modulation of some gluconeogenic enzyme activities in diabetic rat liver and kidney: Effect of antidiabetic compounds. *Indian Journal of Experimental Biology, 37*(2), 196–199.

Gupta, R. K., Kesari, A. N., Watal, G., Murthy, P. S., Chandra, R., Maithal, K., et al. (2005). Hypoglycemnic and antidiabetic effect of aqueous extract of leaves of *Annona squamosa* (L) in experimental animal. *Current Science, 88*(1), 1244–1254.

Jung, U. J., Lee, M. K., Park, Y. B., Kang, M. A., & Choi, M. S. (2006). Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. *International Journal of Biochemistry and Cell Biology, 38*(7), 1134–1145.

Kasiviswanath, R., Ramesh, A., & Kumar, K. E. (2005). Hypoglycemic and antihyperglycemic effect of *Gmelina asaistica* Linn. In normal and alloxan induced diabetic rats. *Biological and Pharmaceutical Bulletin, 28*(4), 729–732.

Kim, S. H., Hyun, S. H., & Choung, S. Y. (2006). Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice. *Journal of Ethnopharmacology, 104*(1–2), 119–123.

King, H., Aubert, R. E., & Herman, W. H. (1998). Global burden of diabetes 1995–2025 prevalence, numerical estimates and projections. *Diabetes Care, 21*(9), 1414–1431.

Leontowicz, H., Gorinstein, S., Lojek, A., Leontowicz, M., Ciz, M., & Soliva-Fortuny, R. (2002). Comparative content of some bioactive compounds in apples, peaches and pears and their influence on lipids and antioxidant capacity in rats. *Journal of Nutritional Biochemistry, 13*(10), 603–610.

Middleton, E., Kandaswami, C., & Theoharides, C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*(4), 673–751.

Mukherjee, K. L. (2003). Routine biochemical tests. In K. L. Mukherjee (Ed.). *Medical laboratory technology* (Vol. III, pp. 1124–1190). New Delhi: Tata McGraw-Hill Publishing Company Limited.

Nishimura, Y. C. (1998). Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications. *Pharmacological Reviews, 50*(1), 21–23.

Ravi, K., Rajasekaran, S., & Subramanian, S. (2005). Antihyperlipidemic effect of *Eugenia jambolana* seed kernel on streptozotocin-induced diabetes rats. *Food and Chemical Toxicology, 43*(9), 1433–1439.

Rupasinghe, H. P., Jackson, C. J., Poysa, V., DiBerado, C., Bewely, J. D., & Jenkinson, J. (2003). Soyasapogenol A and B distribution in Soybena (Glycine Max LMerr) in relation to seed physiology, genetic variability and growing location. *Journal of Agricultural and Food Chemistry, 51*(20), 5888–5894.

Sadasivam, S., & Manikam, A. (1996). Carbohydrates. In S. Sadasivam & A. Manickam (Eds.), *Methods in biochemistry* (pp. 11–12). New Delhi: New Age International Pvt. Ltd..

Schaftingen, E. V., & Gerin, I. (2002). The glucose-6-phosphatase system. *Journal of Biochemistry, 362*, 513–532.

Sharma, S. B., Nasir, A., Prabhu, K. M., Dev, G., & Murthy, P. S. (2003). Hypoglycemic and hypolipidemic effect of ethanolic extracts of seeds of *E. Jambolana* in alloxan-induced diabetic model of rabbits. *Journal of Ethnopharmacology, 85*(2–3), 201–206.

Sharma, S. B., Nasir, A., Prabhu, K. M., & Murthy, P. S. (2006). Antihyperglycemic effect of the fruit-pulp of *Eugenia jambolana* in experimental diabetes mellitus. *Journal of Ethnopharmacology, 104*(3), 367–373.

Shirwaikar, A., Rajendran, K., Kumar, C. D., & Bodla, R. (2004). Antidiabetic activity of aqueous leaf extract of *Annona squamosa* in streptozotocin-nicotinamide type 2 diabetic rats. *Journal of Ethnopharmacology, 91*(1), 171–175.

Sridhar, S. B., Sheetal, U. D., Pai, M. R. S. M., & Shastri, M. S. (2005). Preclinical evaluation of the antidiabetic effect of *Eugenia jambolana* seed powder in streptozotocin-diabetic rats. *Brazilian Journal of Medical and Biological Research, 38*(3), 463–468.

Vats, V., Yadav, S. P., & Grover, J. K. (2004). Ethanolic extract of *Ocimum sanctum* leaves partially attenuates streptozotocin-induced alterations in glycogen content and carbohydrate metabolism in rats. *Journal of Ethnopharmacology, 90*(1), 155–160.

Wang, H. X., & Ng, T. B. (1999). Natural products with hypoglycemic, hypotensive, hypocholesterolemic, antiatherosclerotic and antithrombitic activites. *Life Sciences, 65*(25), 2663–2677.

Welton, A. R., Hurley, I., & Will, P. (1988). Flavonoids and arachidonic acid metabolism. In V. Cody, E. Middleton, J. B. Harborne, & A. Beretz (Eds.), *Plant flavonoids in biology and medicine ii: biochemical cellular and medicinal properties* (pp. 301–312). New York: Alan R. Liss, Inc.

Wu, M., Wang, X., Duan, Q., & Lu, T. (2007). Arachidonic acid can significantly prevent early insulin resistance induced by a high-fat diet. *Annals of Nutrition and. Metabolism, 51*(3), 270–276.

Xia, M., & Laychok, S. C. (1993). Insulin secretion myoinositol transport and Na^{+}-K ATPase in glucose desensitized rat islets. *Diabetes, 42*(10), 1392–1400.

Yadav, S., Vats, V., Dhunnoo, Y., & Grover, J. K. (2002). Hypoglycemic and antihyperglycemic activity of *Murraya koenigii* leaves in diabetic rats. *Journal of Ethnopharmacology, 82*(2), 111–116.