Food and Chemical Toxicology 46 (2008) 2376–2383

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Hypoglycemic and hypolipidemic effects of flavonoid rich extract from *Eugenia jambolana* seeds on streptozotocin induced diabetic rats

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ARTICLE INFO

Article history:
Received 11 September 2007
Accepted 19 March 2008

Keywords:
Eugenia jambolana
Flavonoid rich extract
Streptozotocin-diabetic rats
Serum lipids
Peroxisome proliferator activated receptor

ABSTRACT

The hypoglycemic and hypolipidemic effects of flavonoid rich extract obtained from seeds of *Eugenia jambolana* (EJ) was analyzed in streptozotocin induced diabetic rats. Hypoglycemic activity was assessed by reduction in fasting blood glucose (FBG) and peak blood glucose level within 60 min of glucose tolerance test (GTT) in mild and severe diabetic (MD and SD respectively) rats. Different biochemical parameters like glycogen biosynthesis, glucose homeostatic enzyme (glucose-6-phosphatase, hexokinase) activities demonstrated significant ($p < 0.05$) improvement as compared to diabetic counter parts. Further, the flavonoids also stimulated 16% increase in insulin release in vitro from pancreatic islets. The hypolipidemic action after this extract supplementation was confirmed by significant ($p < 0.05$) decrease in the levels of LDL (27% MD, 29% SD), triglycerides (about 35% MD, 37% SD) and increase in HDL (21% MD, 34% SD) over untreated diabetic rats. The above mentioned action of this plant extract was found to be through dual up regulation of both the peroxisome proliferators-activated receptors (PPARα and PPARγ) up to about 3–4 folds (over control) and their capacity to differentiate 3T3-L1 preadipocytes. The present data suggests that the flavonoid rich extract from EJ plant has both hypoglycemic and hypolipidemic effects which can help the cure and management of diabetes.

1. Introduction

Diabetes mellitus is a group of metabolic alterations characterized by hyperglycemia resulting from defects in insulin secretion, action or both. It has already been established that chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and eventually the failure of organs, especially the eyes, kidneys, nerves, heart and blood vessels (Huang et al., 2005). The use of medicinal plants for the treatment of diabetes mellitus dates back from the Ebers papyrus of about 1550 B.C. A multitude of herbs, spices and other plant materials have been described for the treatment of diabetes throughout the world (Marles and Fransworth, 1995; Kesari et al., 2005, 2006). The medicinal plants provide a useful source of oral hypoglycemic compounds for the development of new pharmaceutical leads as well as a dietary supplement to existing therapies (Bailey and Day, 1989). Some of the plants which are being used for the treatment of diabetes have received scientific or medicinal scrutiny and even the WHO expert committee on diabetes recommends that this area warrant further attention (WHO, 1980).

India has about 45,000 plant species and many of them have medicinal properties. Out of a large number of herbal drugs stated to possess anti-diabetic activity in the Ayurvedic system of medicine of India, *Eugenia jambolana* (EJ) of family Myrtaceae (called black plum in English and Jamun in Hindi in India) is being widely used to treat diabetes by the traditional practitioners over many centuries (Nadkarni, 1954; Sharma et al., 2006). It is a large evergreen tree growing up to 30 m high found widely in India. It is also found in Thailand and Philippines. Its fruits are oval to elliptical 1.5–3.5 cm long, dark purple or nearly black, luscious, fleshy and edible (Sharma et al., 2006).

The anti-hyperglycemic activity of seeds of EJ is well established (Shrotri et al., 1963; Bansal et al., 1981; Kohli, 1983; Achrekar et al., 1991; Grover et al., 2000; Vikrant et al., 2001; Sharma et al., 2003, 2006). However, there is little information on the effect of this plant extract in different types of diabetes and its role in improving lipid profiles except for the one by Sharma et al. (2003). They compared the ethanolic extract's effect on severe diabetic (SD) rabbits (type-I or IDDM) where pancreas was near totally to destroyed and when the mildly diabetic (MD) rabbits (type 2 or NIDDM) still had functional β cells.

The aim of this present work was to explore the scientific basis of the utility of the flavonoid rich extract of EJ seed on biochemical

Abbreviations: EJ, *Eugenia jambolana*; MD, mild diabetic; SD, severe diabetic; PPAR, peroxisome proliferator-activated receptors; GTT, glucose tolerance test; FBG, fasting blood glucose; STZ, streptozotocin.

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doi:10.1016/j.fct.2008.03.020

and enzymatic parameters of SD and MD diabetic rats for correction of hyperglycemia and hyperlipidemia of diabetes. Though there are several reports on anti-hyperglycemic action of aqueous and ethanol extracts of the EJ seeds extracts but almost no information is available on the effects of the flavonoid rich extract from EJ plants on diabetic animal models. Flavonoids are low molecular weight substances, found in all vascular plants, with phenyl-benzopyrones (phenylchromones) with an assortment of structures based on a common three-ring nucleus. This basic structure is comprised of two benzene rings linked through a heterocyclic pyran or pyrone (with a double bond) ring to the third ring in the middle. Over 4000 structurally unique flavonoids have been identified in plant sources (Harborne et al., 1975; Harborne, 1986). The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities. The flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers. We extracted the flavonoid rich extract from EJ and tested their efficacy on SD and MD rats representing two distinct categories of diabetes namely type-I and type-II respectively.

2. Materials and methods

2.1. Plant material

The seeds of EJ plants were collected from in and around the campus of Indian Institute of Technology Roorkee during of month of July–October. The plant materials were identified as per the literature of Ayurveda and by local in-charge of herbal garden and also confirmed by Dr. H.S. Dhaliwal, Professor of Plant Biotechnology, Indian Institute of Technology Roorkee.

2.2. Preparation of flavonoid rich extract of the seed

EJ seeds were thoroughly washed with water, and dried in shade. One hundred grams of air dried seeds were ground in 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 was then centrifuged at 10,000 rpm at room temperature (25 °C) and the pellet was discarded. The supernatant part of the extract 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. Residual layer was extracted three times with ethyl acetate. All the extracts were then pooled together and concentrated using rotavapor. Ethyl acetate extract was redissolved in 20 ml of water and chromatographed over polyamide column eluting with water followed by increasing concentrations of methanol to yield flavonoid rich main fractions.

2.3. Selection of animal and induction of diabetes mellitus

The study was conducted on twenty-five matured Wistar strain male albino rats purchased from Jamia Hamdard University, 3 months of age, weighing 130 ± 5 g, which were housed in colony cages (four rats per cage) at an ambient temperature of 25 ± 2 °C with 12 h-light and 12 h-dark cycle. The rats were fed normal diets purchased commercially from vendors and also had free access to water *ad libitum*. The animals were allowed to acclimatize to the laboratory environment for 1 week and then randomly divided into various groups. All the procedures regarding injection and other treatments of the experimental animals were approved by the Institutional Animal Ethics Committee and conformed to the UFAW Handbook on the Care and Management of Laboratory Animals.

2.4. Chemicals

Streptozotocin (STZ) was purchased from Sigma Chemical Company (USA) and all other chemicals were purchased locally and were of analytical grade.

2.5. Induction of diabetes mellitus

Induction of diabetes was carried out as per the method described by Maiti et al. (2005). Briefly, the rats were fasted for 24 h before the induction of diabetes by STZ injection. Normoglycemic male rats (fasting blood glucose level of 75 ± 5 mg/dl) were injected with STZ at a dose of 4 mg/0.5 ml and 7 mg/0.5 ml of physiological saline/100 g body weight (bw)/ rat for developing MD and SD conditions respectively. The single dose of STZ produced SD or type-I diabetes (having fasting blood glucose level more than 250 mg/dl) and MD or type-II diabetes mellitus (having fasting blood glucose level more than 90 mg/dl but less than 250 mg/dl) after 24 h of STZ injection and this diabetic condition was maintained throughout the experimental schedule.

2.6. Experimental design

Twenty five rats were divided into five equal groups as follows:

(i) Control group: received single intramuscular injection of physiological saline (0.5 ml/100 g body weight, bw).
(ii) Mild diabetic group: received intramuscular injection of STZ (4 mg/0.5 ml physiological saline/100 g bw).
(iii) Severe diabetic group: received intramuscular injection of STZ (7 mg/0.5 ml physiological saline/100 g bw).
(iv) Mild diabetic + EJ supplement group: MD rats were forcefully gavaged with flavonoid rich extract of EJ seed at the dose of 50 mg/0.5 ml of 0.01% ethanol/100 g bw/day/rat after 24 h of STZ injection for next 21 days.
(v) Severe diabetic + EJ supplement group: SD rats were forcefully gavaged with flavonoid rich extract of EJ seed at the dose of 100 mg/0.5 ml of 0.01% ethanol/ 100 g bw/day/rat after 24 h of STZ injection for next 21 days.

Animals of control group (i), MD (ii) and SD (iii) were subjected with forceful feeding of 0.5 ml of 0.01% ethanol/ 100 g bw/day/rat after saline or STZ injection (according to case) for the next 21 days. On starting day of extract supplementation to MD and SD rats, fasting blood glucose (FBG) was monitored of all the animals in each group.

On the 22nd day of the experiment, 4 h before animal sacrifice, intravenous glucose tolerance test (GTT) was performed as per standard protocol (see below) on all the animals and then they were sacrificed under light ether anesthesia for collection of blood and organs like liver and muscles.

2.7. Effect of fasting blood glucose level

Fasting blood glucose was measured after 21 days of treatment with EJ flavonoid rich extract, during which the animals were fed with normal diets. For the determination of fasting blood glucose, on completion of the 21 days of treatment with EJ seed extract and vehicle control, the rats were fasted overnight. The blood was collected from the tip of the tail vein of the over night fasted rats and the blood glucose was measured using GOD –POD glucose estimation kit (Excel Diagnostics Pvt. Ltd. India). The results were expressed in terms of milligrams per deciliter (mg/dl) of blood.

2.8. Glucose tolerance test (GTT)

After overnight fasting, on the day of animal sacrifice, 0-min blood was taken from tip of the tail vein from all the rats in control, MD, SD, MD + EJ and SD + EJ supplemented groups. Glucose solution at a dose of 1 g/kg bw in 0.1 ml water was administered to over night fasted rats. 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.

2.9. Estimation of lipid profile in blood samples

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

2.10. Biochemical estimation of enzyme activities and tissue glycogen content

Glucose-6-phosphatase catalyzes the conversion of glucose-6-phosphate to glucose. The estimation of this enzyme activity was performed as per the method described earlier (Schaftingen and Gerin, 2002). The hexokinase activity was tested based on the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase as per the method described earlier (Brandstrup et al., 1957). Glycogen content of liver and skeletal muscles were measured according to earlier established methods using anthrone reagent (Maiti et al., 2004; Sadasivam and Manickam, 1996). The enzyme activities were expressed as unit per gram wet weight of tissue. The amount of glycogen in tissue sample was expressed in microgram of glucose per milligram tissue.

2.11. Cell culture and reporter gene bioassay for peroxisome proliferator activated receptor (PPAR)

The cells kindly provided by Dr. R.K. Tyagi of Jawaharlal Nehru University, New Delhi, India were originally collected from National Center for Cell Sciences, Pune, India. The HepG2 cells and preadipocyte 3T3-L1 cells were cultured in DMEM (Himedia, Mumbai, India). All the media in addition to the regular antibiotics (penicillin and streptomycin) were also supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate as per the culture condition described by Shen et al. (2006).

The plasmids pcMX-mPPARα, pcMX-mPPARγ and PPREx3-tk-Luc were a kind gift of Dr. Ronald M. Evans of The Salk Institute for Biological Studies, California, USA. The reporter gene bioassays were performed as previously described (Shen et al., 2006). Briefly, cells were seeded at 40,000 cells/well in 24-well microtiter plates and incubated for 24 h before transfection. Twenty-five nanograms of full length PPAR expression plasmids and/or and 250 ng of reporter-gene plasmid (PPREx3-tk-Luc) were cotransfected in HepG2 cells or differentiated 3T3-L1 preadipocytes using Fugene (Roche, USA). Transfected cells were exposed to test samples in charcoal-stripped medium for 24–36 h. On completion of the treatment the cells were lysed with the lysis buffer (0.6 M NaCl, 0.1 M EDTA, 0.2 M $MgSO_4$, 0.2 M DTT, Triton X-100, 0.08 M Tricine) and the luminescence was measured using luciferin as substrate. Luciferase inductions in response to each treatment group were expressed as percent transactivation with respect to reference drugs.

2.12. Adipocyte differentiation assay

The adipocyte differentiation assays were performed according the method described earlier by Shen et al. (2006). Briefly, murine fibroblast or preadipocyte 3T3-L1 cells were seeded at a density of 40,000 cells/well in 24-well plates and cultured to confluency for two days in DMEM with 10% heat inactivated fetal calf serum. Post confluent preadipocytes were exposed to induction medium containing 10% charcoal-stripped serum, 5 mg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. Induction medium was removed after two days and the cells were washed two times with PBS and exposed to varying concentrations of extracts and standards for the next eight days. Medium was replenished with appropriate ligands every two days. On completion of incubation, the cells were washed with PBS, lysed with the lysis buffer and cellular triglyceride content was determined using the commercially available kit (Transasia Biomedical Limited, Mumbai India).

2.13. Effect of flavonoid rich extract on the release of insulin

2.13.1. In vivo studies

In order to analyze if the flavonoid rich extract could stimulate the release of insulin, serum insulin levels were measured before and after treatment for 21 days to diabetic rats with extract. Serum insulin levels were estimated in each sample of blood using enzyme linked immunoabsorbent assay kits (Boehringer Manheim Diagnostic, Manheim, Germany).

2.13.2. In vitro studies

Isolation of pancreatic cells was made according to method reported earlier (Xia and Laychok, 1993; Gupta et al., 2005) with slight modification. Briefly, after removing the pancreas from the rats, they were perfused with Hank's balanced salt solution (HBSS) (pH 7.4) followed by collgenase treatment (4 mg/ml). The islet cells were then separated from acinar cells using varying gradients of Ficoll (Type-400) (Himedia, Mumbai, India) followed by centrifugation. The purity of the islet cells was checked by Gomori's chromium hematoxylin phloxin stain (Gomori, 1941). After dividing 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 CO_2 incubator. The incubation was continued for another 1 h after adding 30 mg/l of plant extract or buffer for controls. Aliquots of 50 μl were removed from the incubation mixture at the end of incubation (i.e. 1 h) and stored at −20 °C till insulin assay.

2.14. Western blot analysis

Immunoblotting of total cell lysates was performed according the method described earlier (Roy et al., 2004). Briefly, differentiated preadipocyte 3T3-L1 cells were cultured on 10 mm dishes and after incubation with rosiglitazone or EJ extract (as appropriate), the cells were lysed directly in the dish with 50 μl of lysis buffer (160 mM Tris, pH 6.9, 200 mM DTT, 4% SDS, 20% glycerol, 0.04% bromophenol blue) in the presence of a protease inhibitor cocktail (Sigma, USA). The lysate was boiled for 10 min and cellular debris pelleted at 13,000xg for 10 min. The extracts were immunoblotted with a polyclonal antibody for PPAR$_\gamma$ (kindly donated by Dr. A. Bandyopadhyaya, Indian Institute of Chemical Biology, Kolkata, India). The signal was detected by using the ECL kit from Amersham.

2.15. Statistical analysis

Values are presented as means ± S.E.M. 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

There was a significant elevation in fasting blood glucose in SD and MD rats as compared to control group. However, supplementation of aqueous extract of seed of EJ to MD and SD rats for 21 days resulted in significant recovery of fasting blood glucose level and resettling to the control level (Fig. 1, zero min data).

3.2. Glucose tolerance test

The levels of blood glucose in control, MD, SD, MD plus EJ and SD plus EJ supplemented groups demonstrated a significant change in blood glucose level after administration of glucose (1 g/kg bw). The rats of MD and SD diabetic groups had a significant elevation in blood glucose level throughout the total measurement period i.e. for 150 min with respect to control (Fig. 1) also it did not come back to the initial value (0 min level) even at the end of the period tested by us (150 min). However, in both the supplemented groups, blood glucose level although reached the peak level within 30 min of administration of glucose but it almost resettled to the control level by 60 min (Fig. 1).

3.3. Glycogen level in tissues

Glycogen content of the liver and muscle tissues in control, diabetic (MD and SD) and diabetic supplemented with EJ extract groups are shown in Fig. 2. Hepatic and skeletal muscles glycogen contents were significantly decreased in both MD and SD rats with respect to control ($p < 0.05$). However, treatment with flavonoid rich extract from EJ *seeds* led to increase in liver (MD 35%, SD 67%) and muscle glycogen contents (MD 42%, SD 67%) over control.

3.4. Enzyme activity in liver

After 21 days of treatment with flavonoid rich extract of EJ there was a significant ($p < 0.05$) reduction in liver glucose-6-phospahatese activity in both the diabetic groups compared to the untreated

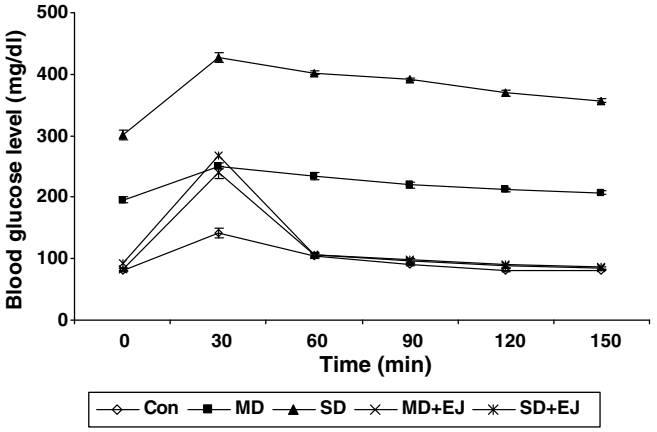

Fig. 1. Oral glucose tolerance test (OGTT) in streptozotocin induced diabetic MD and SD male albino rats in response to flavonoid rich extract of *Eugenia jambolana* treatment. Results are mean ± S.E.M. of $n = 5$. Zero min glucose level indicates fasting blood glucose. MD, mild diabetic; SD, severe diabetic.

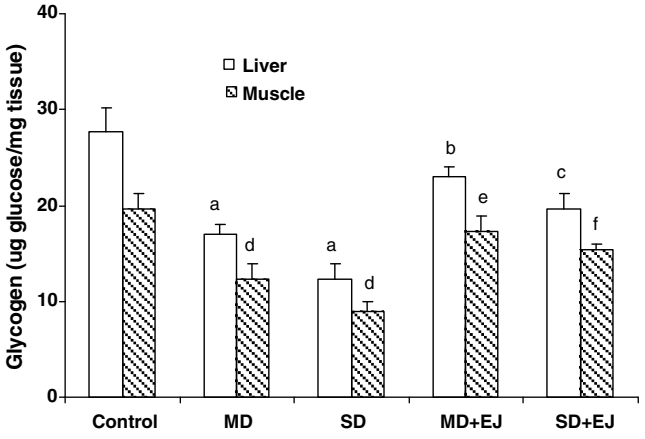

Fig. 2. Effect of flavonoid rich fraction of *Eugenia jambolana* seed extract on liver and muscle glycogen contents in streptozotocin induced diabetic male MD and SD rats. Data are expressed as mean ± S.E.M; $n = 5$. a, b, and c indicates the significant level of differences in hepatic glycogen contents as compared to control (non-diabetic), MD and SD respectively ($p < 0.05$); d, e, and f indicates the significant level of difference in muscular glycogen contents as compared to control (non-diabetic), MD and SD respectively ($p < 0.05$). MD, mild diabetic; SD, severe diabetic.

streptozotocin induced diabetic rats. The reduced level of enzyme concentrations (MD 26%, SD 29%) was almost the same as that of the non-diabetic control rats (Fig. 3).

As compared to non-diabetic control, the mean values of hepatic hexokinase activity decreased significantly ($p < 0.05$) in diabetic rats (Fig. 3). Treatment of these diabetic (MD and SD) animals with EJ seed extract led to a rise in percentage of these enzyme by 36% and 50% for MD and SD groups respectively ($p < 0.05$), as compared to diabetic controls.

3.5. Estimation of serum lipid profile

Serum TC and TG levels were significantly elevated ($p < 0.05$) in both diabetic groups in comparison to controls (Table 1). Supplementation of EJ seed extract for 21 days to the MD and SD rats resulted a significant diminution of these parameters ($p < 0.05$) and the levels of these parameters were resettled towards the control level (Table 1).

Other hyperlipidemic parameters like serum LDL and VLDL cholesterol, both were elevated in MD (72% and 29% respectively) and SD (102% and 83% respectively) diabetic groups in comparison to control (Table 1). However, both these parameters were decreased significantly ($p < 0.05$) in the extract supplemented groups of MD (27% and 21% respectively) and SD (29% and 31% respectively), and were resettled towards the control level.

HDL cholesterol, a friendly lipoprotein, was decreased in both the diabetic groups in respect to the control (Table 1). After 21 days of treatment with the EJ seed extract there was a significant elevation ($p < 0.05$) of this lipoprotein level in both MD and SD group of rats (Table 1).

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

Serum insulin level was significantly decreased in MD and SD rats with respect to control ($p < 0.05$) (Fig. 4). After 21 days of EJ seed extract supplementation to the MD and SD groups of diabetic animals, though there was a significant elevation in serum insulin levels in SD rats as compared to their diabetic counterpart ($p < 0.05$), the improvement was only marginal in case of MD rats (statistically not significant). 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 were investigated. The incubation of ten islets from diabetic rats with 10 mM glucose in presence of EJ seed extract for 1 h resulted in significant stimulation of insulin ($p < 0.05$), which was about 16% increase over the diabetic control group (Table 2).

3.7. Identification of PPAR activity by EJ seed extracts

Flavonoid rich extracts of EJ seeds were tested for PPAR activity using PPAR reporter-gene bioassay (Fig. 5). The extracts significantly stimulated both PPARα and PPARγ activities in a dose dependent manner ($p < 0.05$). At a dose of 30 mg/l, the extract increased PPARα and PPARγ activity up to 3 and 4 folds over the vehicle control respectively (Fig. 5). Thereafter, though there was no signifi-

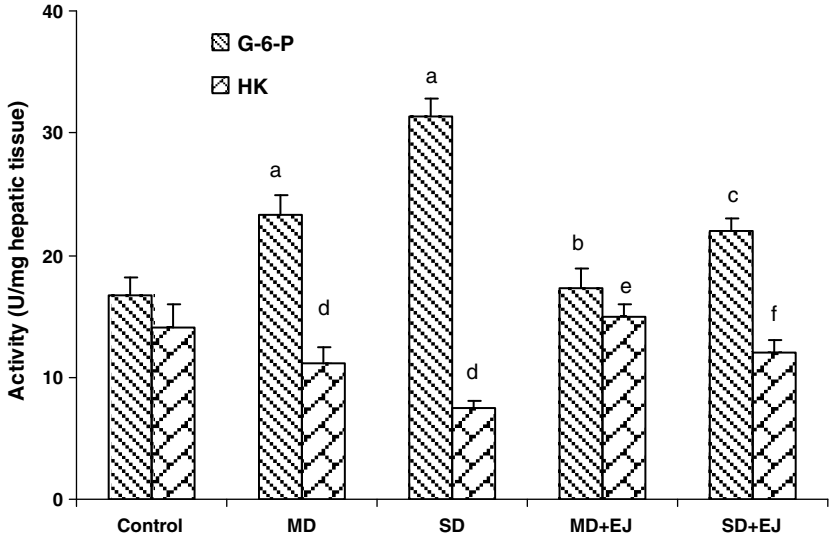

Fig. 3. Effect of flavonoid rich fraction of *Eugenia jambolana seed extract* on hepatic glucose 6 phosphatase and hexokinase activity in streptozotocin induced diabetic MD and SD rats. Data are expressed as mean ± S.E.M; $n = 5$. a, b, and c indicates the significant level of differences in hepatic glucose 6 phosphatase activity as compared to control (non-diabetic), MD and SD respectively ($p < 0.05$); d, e, and f indicates the significant level of difference in hepatic hexokinase activity as compared to control (non-diabetic), MD and SD respectively ($p < 0.05$). MD, mild diabetic; SD, severe diabetic.

Table 1
Serum level of different lipids before and after the administration of flavonoid rich fraction of *Eugenia jambolana* seed in streptozotocin induced diabetic rats

Treatment group	Serum lipid level (mg/dl)				
	TC	HDL	LDL	VLDL	TG
Control	75.2 ± 5.1	69.2 ± 4.0	64.3 ± 6.0	29.1 ± 4.0	97.5 ± 3.6
MD	97.7 ± 2.5[a]	48.5 ± 4.5[a]	110.5 ± 5.9[a]	37.6 ± 2.7[a]	164.7 ± 5.3[a]
SD	120.3 ± 5.0[a]	38.0 ± 2.6[a]	129.7 ± 3.5[a]	53.3 ± 3.5[a]	191.6 ± 7.4[a]
MD + EJ	81.9 ± 3.6[b]	58.0 ± 2.9[b]	80.3 ± 4.7[b]	29.7 ± 1.5[b]	107.2 ± 2.6[b]
SD + EJ	84.8 ± 4.0[c]	51.6 ± 4.7[c]	92.1 ± 3.0[c]	36.7 ± 2.0[c]	120.4 ± 5.7[c]

Each value represents the mean ± S.E.M ($n = 6$).
a, b, c represents statistically significant ($p < 0.05$) as compared to normal control, MD and SD groups respectively among each class of lipids. MD: Mild diabetic; SD: Severe diabetic.

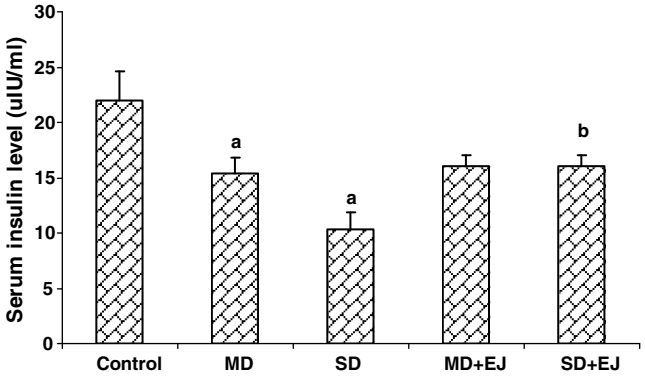

Fig. 4. Effect of flavonoid rich fraction of *Eugenia jambolana* seed extract on serum insulin levels in streptozotocin induced MD and SD male rats. Data are expressed as mean ± S.E.M; $n = 5$. a and b indicates the significant level of difference in serum insulin levels as compared to non-diabetic control and SD respectively ($p < 0.05$). MD, mild diabetic, SD, severe diabetic.

Table 2
Effect of *Eugenia jambolana* seed extract on insulin release in vitro from isolated islet of Langerhans of normal and diabetic rats

Treatment Group	Insulin release (μIU/ten islets/h)
Control	344.5 ± 14.2
Control + EJ	385.6 ± 11.4[a]
MD	280.3 ± 10.2[a]
MD + EJ	325.0 ± 9.5[b]

Each value represents the mean ± S.E.M ($n = 6$).
a, b represents statistitically significant ($p < 0.05$) as compared to normal control and MD groups respectively. MD: Mild diabetic.

cant increase in the EJ induced activity in PPARγ, that of PPARα activity kept increasing which stabilized at 500 mg/l concentrations (data not shown). Thus flavoind rich EJ seed extract seems to demonstrate dual PPARα and PPARγ activity in a dose dependent manner.

3.8. Effects of flavonoid rich extract on PPAR dependent transcriptional activities and differentiation of preadipocytes

In order to investigate the relevance of flavonoid rich EJ seed extract on lipid metabolism, their effects on differentiation of 3T3-L1 preadipocytes was analyzed, in which PPARγ is thought to have a major role. The PPAR-driven reporter gene was transfected into differentiated preadipocytes to measure PPARγ-driven responses. As reported earlier by many authors, rosiglitazone induced a significant rise in PPARγ activity (6 folds over vehicle control) (Fig. 6). When tested for various doses of EJ extracts, all of them demonstrated an increase in PPARγ activity. At doses of 3, 10 and 30 mg/l of EJ extract treatment, resulted in 2, 3.3 and 4 folds over vehicle control respectively for PPARγ activity (Fig. 6).

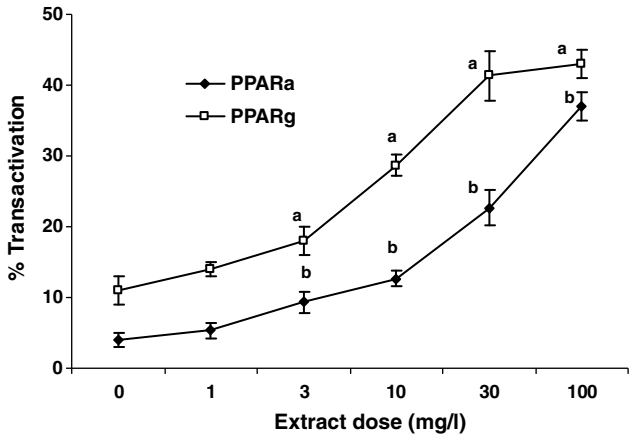

Fig. 5. Flavonoid rich extract activated PPARs as determined by lucifersae assays using full length PPAR constructs. tk-PPREX3-Luc, pRL-CMV and pCMX-mPPARα or pCMX-mPPARγ were transfected to HepG2 cells and after 24 h of transfection, the cells were treated with flavonoid rich EJ extract of varying concentrations starting from 1 mg/l to 100 mg/l for 24 h. Values are means ± S.E.M of five independent experiments each performed in quadruplicates and are expressed as percentage of the positive controls for PPARα (Wy 14643, 50 μM) and PPARα (Rosiglitazone 5 μM). a and b indicates the significant level of difference in values as compared to vehicle treated control incubation for PPARγ and PPARα respectively ($p < 0.05$).

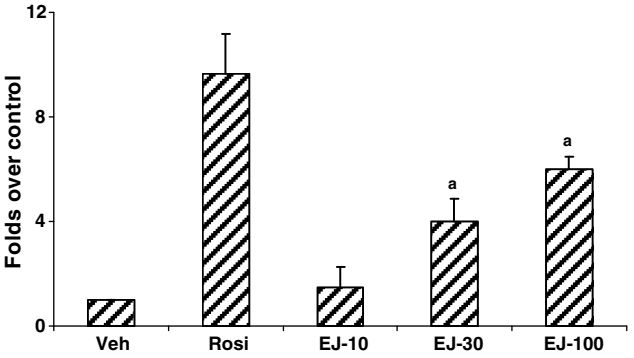

Fig. 6. Effect of EJ seed extract on endogenous PPARγ function in adipocytes. Differentiated 3T3-L1 cells were transfected with tk-PPREX3-Luc reporter only, and then exposed to varying concentrations of extract (3, 10 and 30 mg/l). Rosiglitazone (20 μM was used as positive control. Data are expressed as folds over vehicle control which is taken as 1. Values are means ± S.E.M of five independent experiments each performed in quadruplicates. a represents significant levels of difference in values as compared to vehicle treated control. Veh, vehicle treated control; Rosi, Rosiglitazone treated; EJ-10, treated with 10 mg/l of EJ extract; EJ-30, treated with 30 - mg/l of EJ extract; EJ-100, treated with 100 mg/l of EJ extract.

This was further authenticated by immunoblotting analysis of PPARγ in 3T3-L1 adipocytes. Western blot analysis with specific antibody for PPARγ indicated that the extremely low levels of endogenously expressing PPARγ in 3T3-L1 adipocytes are

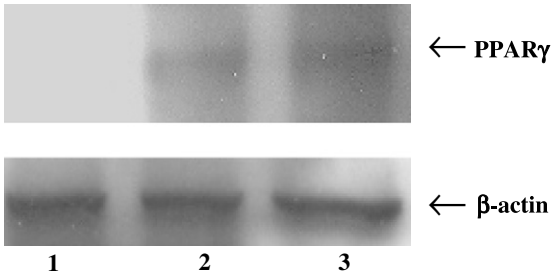

Fig. 7. Immunoblot showing PPARγ protein of differentiated adipocytes in response to EJ extract and rosiglitazone (positive control). 3T3-L1 cells were exposed to EJ extract (100 mg/l) and PPARγ protein was measured with an anti-PPARγ polyclonal antibody. 1, vehicle treated; 2, treated with 100 mg/l of EJ extract; 3, treated with 20 μM of rosiglitazone.

significantly elevated in response to rosiglitazone (as positive control) and flavonoid rich extract of EJ seed (Fig. 7).

Since adipocyte differentiation is marked by accumulation of lipid droplets and rise in the cellular triglyceride content, we estimated the cellular triglyceride levels in differentiated 3T3-L1 preadipocytes after treating them with increasing concentration of EJ seed extract. Cellular triglyceride levels showed significant rise at the concentration of 3 mg/l and this continued further till 30 mg/l ($p < 0.05$). There after the levels of triglycerides starts declining (Fig. 8). Maximum amount of triglyceride accumulation (approximately 3 folds over vehicle control) was observed after the addition of 30 mg/l of EJ seed extract.

4. Discussion

Diabetes is a chronic metabolic disorder affecting a major proportion of the population worldwide. A sustained reduction in hyperglycemia will decrease the risk of developing microvascular diseases and reduce their complications (Kim et al., 2006). The conventional therapies for diabetes have many shortcomings like side effects and high rate of secondary failure. On the other hand herbal extracts are expected to have similar efficacy without side effects as that of conventional drugs. The present investigation reports the anti-diabetogenic and hypoglycemic effects of flavonoid rich fraction of *E. jambolana* seed on STZ induced diabetic rats. STZ injection resulted in diabetes mellitus, which is probably due to the destruction of β cells of islets of Langerhans as proposed by many authors (Maiti et al., 2004; Beppu et al., 2006). It is generally accepted that SD is of IDDM type and MD is of NIDDM type (Vessal et al., 2003; Sharma et al., 2003; Maiti et al., 2005). This effect is being depicted by the high level of blood glucose in animals. The main aim of this study was to assess the multiple roles of flavonoid rich extract from the seeds of EJ plant as anti-diabetic agent for correction of SD or IDDM where β cells degeneration is dramatic and MD or NIDDM where β cell degeneration is partial.

The flavonoid rich extract resulted in the significant reduction of peak level of sugar within 2 h time and this fact further strengthens the anti-diabetogenic potentiality of EJ flavonoids as reported by many authors earlier for other extracts (Sharma et al., 2003; Sridhar et al., 2005; Grover et al., 2002). Anti-hyperglycemic potency of the flavonoid rich extract of EJ seeds in SD and MD rats has been indicated here by improvement in FBG levels which is also an important parameter for monitoring diabetes (Maiti et al., 2005). Further the plant extract also significantly decreased the blood glucose level in glucose loaded rats (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 pattern of hypoglycemic effects (Kasiviswanath et al., 2005). Results on the insulin release from pancreas (both in vitro and in vivo) directly indicates that part of the anti-hyperglycemic activity of the flavonoid rich extract is through the release of insulin from the pancreas i.e. it exerts a direct insulinotropic effect. However, surprisingly the insulinotropic efficacy of this extract is lower in MD rats in vivo probably due to higher level of serum insulin as compared to SD rats. A similar effect has also been reported earlier by *Tarmarindus indica* seed extract in vivo (Maiti et al., 2005). Earlier studies by Achrekar et al. (1991) and Sharma et al. (2006) demonstrated that the water extract of pulp of EJ potentiates insulin release from pancreatic β cells like some of the sulphonylureas such as tolbutamide. Further, synthetic derivates of flavonoids has been shown to release insulin from insulinotropic INS-1 cells in culture which further supports our finding that flavonoid rich extract probably act as an insulinotrophic agent as well (Bozdag-Dundar et al., 2001). Bansal et al. (1981) reported that the increase in plasma insulin brought by seeds of EJ may be attributed to pro-insulin to insulin conversions, possibly by pancreatic cathapsin B, and/or its secretion.

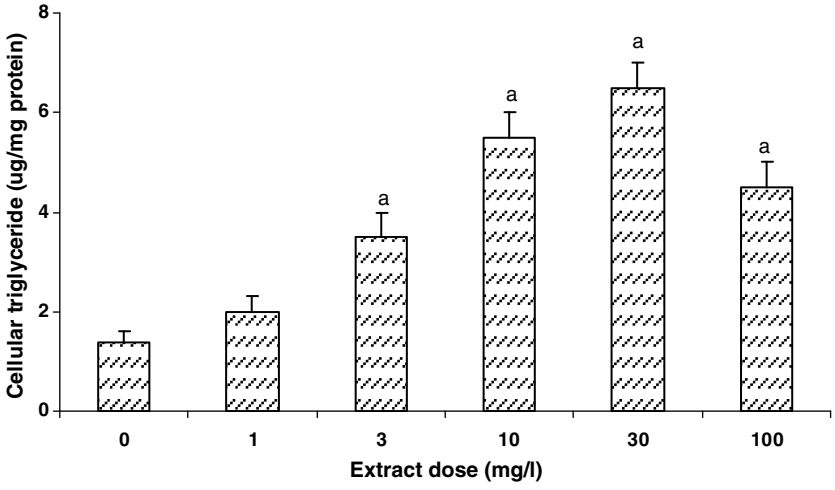

Fig. 8. Dose dependent effect of EJ seed extract on cellular triglyceride content in 3T3-L1 cells. Various concentrations of EJ extract was added from the induction stage. On day 8, the cellular triglyceride content was determined as described in Section 2 and expressed as μg/mg of protein. Values represent means ± S.E.M. of five independent experiments each performed in quadruplicates. a indicates $p < 0.05$ as compared vehicle treated control.

The administration of flavonoid rich extract significantly decreased serum triglycerides and cholesterol in diabetic rats. In consistence with the present data, other workers have also reported that the administration of crude EJ plant extract to streptozotocin induced diabetic rats improved the serum cholesterol and triglyceride levels as compared to control and this effect was also similar to insulin treatment (Lopes-Virella et al., 1983; Ravi et al., 2005). Cholesterol lowering property of EJ seed extract could be attributed to several factors like: presence of hypocholesterolemic compounds that may act as inhibitors for some enzymes such as hydroxyl methyl glutaryl CoA reductase, which participates in cholesterol synthesis or reduces the absorption of cholesterol from intestine (Sharma et al., 2003) or the extract might stimulate the production of insulin which in turn inhibits lipoprotein lipase activity (Ravi et al., 2005) or reduces lipid peroxidation (Ravi et al., 2004).

Glycogen is the primary intracellular storable form of glucose and its level in various tissues especially in liver and skeletal muscles indicates direct reflection of insulin activity since it regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since STZ 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 and recovers on insulin treatment (Golden et al., 1979; Weber et al., 1966; Vats et al., 2004). Our results showed that upon supplementation of diabetic rats with EJ flavonoid rich extract there is significant elevation in both muscle and hepatic glycogen content. Further, the flavonoids were also found to improve hepatic hexokinase and glucose-6 phosphatase enzyme levels, two major enzymes involved in glucose homeostasis, in both MD and SD rats. On treatment with flavonoids from EJ plants to diabetic rats, the levels of hexokinase and glucose-6 phosphatase increased and decreased respectively almost to control level in those animals. This result is similar to that of reported earlier where several potential herbal plant extract and pure flavonoids like quercetin have been shown to improve the diabetic condition (Gupta et al., 1999; Vessal et al., 2003; Maiti et al., 2005). Extract did not show any significant improvement in LDH activity (data not shown).

Till this point, the possible mechanism of action of this extract appears to be both pancreatic and extra pancreatic that has been supported by several assays as described earlier. For example, in serum insulin assay though the increase in serum insulin level in MD rats supplemented with EJ extract did not increase significantly as compared to untreated MD group, yet these supplemented rats demonstrated a significant improvement in its glucose-6 phosphatase activity and glycogen storage capacity. The extra pancreatic activity may be by the sensitization of insulin receptor in target organ or by inhibiting insulinase activity in both liver and kidney as has been reported earlier for some other plants (Maiti et al., 2005). Water extract of pulp of EJ seeds has already been reported to have insulinase inhibiting activity (Achrekar et al., 1991). However, to the best of our knowledge there is no report on the effect of EJ plant extract on sensitization of insulin receptors. PPAR, a sub-family of the 48-member steroid and nuclear receptor superfamily, are ligand dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism (Shen et al., 2006). They exists in three different subtypes: PPARα, PPARβ/δ and PPARγ out of which PPARα is expressed in liver, kidney, heart, muscles and are involved in lipoprotein metabolism while PPARγ are predominantly expressed in adipose tissue and are involved in controlling insulin resistance, adipocyte differentiation and lipid storage. Due to their insulin receptor sensitizing activity the agonist for these receptors (thiazolidinediones) are used in treatment of type-II diabetes mellitus. It has been reported earlier that small phenolic molecules presenting some "anti-diabetic" botanical foods may activate the PPAR-signaling system (Shen et al., 2006). The flavonoid rich extract from EJ seeds also demonstrated a dual regulator function for PPARα/γ which could be attributed to some polyphenolic constituents within the extract. Although there have been reports on the hypolipidemic constituents in the EJ plant extract, there was almost no information on the activation of the PPAR dependent action of the constituents (Sharma et al., 2003; Ravi et al., 2005). A similar pattern of PPAR activation and anti-hyperlipidemic effect has been reported earlier from ethanolic extracts of licorise (Mae et al., 2003), purified isoflavones from *Astragalus membranaceus* and *Pueraria thomosonii* (Shen et al., 2006), carotenoids, terpenoids and fenofibrates (Takahashi et al., 2002), ethyl acetate extract of *Punica granatum* flowers (Huang et al., 2005), CM 108, a flavone derivative, (Guo et al., 2006), flavonoids isolated from Hawthorn leaves (Fan et al., 2006). In our study the EJ extract was also found to be involved in the differentiation of adipocytes from preadipocytes which was shown by cellular accumulation of triglycerides on treating the cells with rosiglitazone (as positive control) and the extract. Further the extract could also up regulate the expression of PPARγ protein which has also been shown earlier in response to various other plant extracts (Park et al., 2005; Shen et al., 2006).

The data obtained from the present study indicates that the flavonoid rich fraction of *E. jambolana* seed contains some bioactive molecules which may have beneficial effects as both hypoglycemic, anti-hyperglycemic and anti-hyperlidemic agents. The exact mechanism of action needs further detailed studies using isolated individual compounds. EJ plant extract has already been reported to be rich in substances like triterpenoids, anthocianins, essential oils, oleic acid, saponins, glycosides, several members of flavonoids like rutin, quercetin, myricetin, myricitrin, myricetin 3-O-(4″-acetyl)-α-L-rhamnopyranosidase (Timbola et al., 2002; our unpublished data). Hence it may be concluded that the hypolipidemic effect produced by the extract may be due to the presence of these, any of these, or a combination of them having direct or indirect effects on this mechanism (Ravi et al., 2005). However, to the best of our knowledge the present study for the first times puts some light on the fact that the flavonoid rich EJ seed extract has the potential to act at multiple sites not only in NIDDM but also in IDDM. Toxicity studies (data not shown) have already proven that the used dose in this investigation is far below the LD_{50} dose of the extract and did not show any change in the blood parameters as is shown in case of toxicity. Further investigations are in progress to isolate the individual compounds in this flavonoid rich extract and also to elucidate the detailed mechanism of hypoglycemic and hypolipidemic effects in IDDM and NIDDM by these molecules.

Conflict of interest statement

We do hereby state that we do not have any conflicts of interest in the present work.

Acknowledgements

Authors gratefully acknowledge the Department of Science and Technology, Government of India for funding to carry out this project (Sanction No. SR/FT/L-57/2004) to PR and University Grants Commission and Ministry of Human Resources and Development (MHRD) for providing research support to B.S. and CBM respectively.

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