

## Protective Effect of *Eugenia jambolana* Seed Kernel on Tissue Antioxidants in Streptozotocin-Induced Diabetic Rats

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**Oxidative stress plays an important role in chronic complications of diabetes. In the present study the antioxidant effect of oral administration of ethanolic extract of *Eugenia jambolana* seed kernel on tissue antioxidant enzymes and lipid peroxidation in liver and kidney of streptozotocin-induced diabetic rats was evaluated. Administration of seed kernel to diabetic rats significantly decreased the levels of blood glucose, glycosylated hemoglobin and increased body weight gain, plasma insulin and hemoglobin. The diabetic rats showed the low activities of superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione content in liver and kidney, which were restored to near normal levels by treatment with the seed kernel extract. The increased levels of lipid peroxidation and hydroperoxides in diabetic rats were reverted back to near normal levels after the treatment with seed kernel extract. Diabetic rats treated with seed kernel extract restored almost normal architecture of liver and kidney and were confirmed by histopathological examination. The present study reveals the efficacy of *Eugenia jambolana* seed kernel in the amelioration of diabetes, which may be attributed to its hypoglycemic property along with its antioxidant potential. The antioxidant effect of *Eugenia jambolana* seed kernel was also compared with glibenclamide, a standard hypoglycemic drug.**

**Key words** antioxidant; *Eugenia jambolana* seed kernel; free radical; oxidative stress; streptozotocin diabetes

Oxidative stress plays an important role in chronic complications of diabetes and it is postulated to be associated with increased lipid peroxidation.<sup>1)</sup> Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the etiology of diabetic complications.<sup>2)</sup> Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycated end products (AGEs) and protein kinase C. Elevated glucose level causes slow but significant non-enzymatic glycosylation of proteins in diabetes.<sup>3)</sup> The oxidatively modified proteins may be recognized as 'foreign' by the immune system, triggering the antibody formation.<sup>4)</sup> There are several potential sources of increased free radical production in diabetes including auto-oxidation of plasma glucose, activation of leucocytes and increased transition metal bioavailability.<sup>5)</sup> Several *in vivo* and *in vitro* studies have demonstrated that reactive oxygen metabolites including free radicals like superoxide radical, hydroxyl radical and hydrogen peroxide are important mediators of tissue injury.<sup>6)</sup> The concentration of the reactive oxygen species are modulated by antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and non-enzymatic scavengers like reduced glutathione (GSH).<sup>7)</sup> Marked reductions in antioxidant enzyme activities and tissue GSH concentrations have been reported in diabetes.<sup>8,9)</sup>

Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes.<sup>10–12)</sup> Despite the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major problem in the world population. *Eugenia jambolana* LAM (Fam: Myrtaceae) is commonly called as Jamun, Black plum or Indian Black berry. *Eugenia jambolana* seeds have hypoglycemic,<sup>13)</sup> anti-inflammatory,<sup>14)</sup> neuropsychopharmacological,<sup>15)</sup> anti-bacterial,<sup>16)</sup> anti-HIV<sup>17)</sup> and anti-diarrheal<sup>18)</sup> effects. Bhatia *et al.* have reported that the *Eugenia jambolana* seed contains several active con-

stituents such as flavonoids, gallic acid, ellagic acid and tannins.<sup>19)</sup> We have reported that the antidiabetic activity of *Eugenia jambolana* seed kernels (EJs-kernels) on streptozotocin-induced diabetic rats.<sup>20)</sup> The present study was aimed to investigate the effect of EJs-kernel on tissue lipid peroxides and enzymic antioxidants in streptozotocin-induced diabetic rats. The efficacy was compared with a standard hypoglycemic drug, glibenclamide.

### MATERIALS AND METHODS

**Chemicals** Streptozotocin was procured from Sigma Chemical Co., St. Louis, MO, U.S.A. RIA kit for plasma insulin assay was purchased from Linco research Inc., U.S.A. All other chemicals used were of analytical grade.

**Plant Material** Fresh, mature *Eugenia jambolana* fruits were collected from a tree in Kolli Hills, Tamil Nadu, India. The plant was identified at the Herbarium of Botany, Centre for Advanced Studies in Botany, University of Madras. A voucher specimen (No. 1283) was deposited in the department herbarium.

**Preparation of Plant Extract** The fruits of jambolana were first washed well and pulp was removed from the seeds. Seeds were washed several times with distilled water to remove the traces of pulp from the seeds. The seeds were dried at room temperature. The kernel of the seeds was selectively separated from the seed coat. The kernel was powdered in an electrical grinder and stored at 5 °C until further use. Kernel powder (100 g) was extracted with petroleum ether (60–80 °C) to remove lipids. It was then filtered and the residue was extracted with 95% ethanol by Soxhlation. Ethanol was evaporated in a rotary evaporator at 40–50 °C under reduced pressure. The yield of kernel was –3.2 g/100 g.

**Animals** Male albino Wistar rats, weighing about 150–180 g obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India were used for the present investigations. The animals were maintained on standard rat

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feed supplied by Hindustan Lever Ltd., Bangalore, India. The experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines.

**Experimental Induction of Diabetes** The animals were fasted overnight and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ) (55 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5).<sup>21)</sup> The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. Control rats were injected with citrate buffer alone. The animals were considered as diabetic, if their blood glucose values were above 250 mg/dl on the 3rd day after STZ injection. The treatment was started on the 4th day after STZ injection and this was considered as 1st day of treatment. The treatment was continued for 30 d.

**Experimental Design** The rats were divided into four groups comprising of six animals in each group as follows:

Group I: Control rats receiving 0.1 M citrate buffer (pH 4.5)

Group II: Diabetic controls

Group III: Diabetic rats given EJs-kernel extract (100 mg/kg b.w/d) in aqueous solution orally for 30 d

Group IV: Diabetic rats given glibenclamide (600 µg/kg b.w/d) in aqueous solution orally for 30 d.<sup>22)</sup>

At the end of the experimental period, the rats were anaesthetized and sacrificed by cervical dislocation. Blood was collected in tubes containing potassium oxalate and sodium fluoride. Plasma was used for the estimation of glucose by O-Toluidine method of Sasaki *et al.*<sup>23)</sup> The levels of hemoglobin and glycosylated hemoglobin were estimated according to methods of Drabkin *et al.*<sup>24)</sup> and Nayak *et al.*,<sup>25)</sup> and insulin was estimated by using radioimmunoassay kit.

The liver and kidney tissues were excised, rinsed in ice-cold saline and then homogenized in Tris-HCl buffer (pH 7.4). The tissue homogenates were used for the following estimations: thiobarbituric acid reactive substances (TBARS) (Lipid peroxides) and hydroperoxides were estimated according to method of Ohkawa *et al.*<sup>26)</sup> and Jiang *et al.*<sup>27)</sup> respectively. Reduced glutathione (GSH) was estimated by the method of Sedlak *et al.*<sup>28)</sup> Protein was estimated by the method of Lowry *et al.*<sup>29)</sup> The activity of superoxide dismutase (SOD) was assayed by the method of Misra *et al.*<sup>30)</sup> The activity of glutathione peroxidase (GPx) was assayed according to the method of Rotruck *et al.*<sup>31)</sup> Catalase (CAT) activity was assayed by the method of Takahara *et al.*<sup>32)</sup>

**Histopathological Studies** A portion of the liver or kidney tissues was fixed in 10% buffered neutral formal saline solution for histological studies. After fixation, tissues were embedded in paraffin, solid sections were cut at 5 µm and stained with haematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.

**Statistical Analysis** All the grouped data were statistically evaluated with SPSS/7.5 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. *p* values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean±S.D. for

six animals in each group.

## RESULTS

Figures 1a,b demonstrate the levels of blood glucose and plasma insulin in control and experimental groups of rats. There was a significant increase in the level of blood glucose and a concomitant decrease in the level of insulin in diabetic rats. Administration of EJs-kernel or glibenclamide to diabetic rats significantly decreased the level of blood glucose and increased the level of insulin.

Table 1 shows the levels of total hemoglobin, glycosylated hemoglobin (HbA<sub>1c</sub>) and change in body weight of control and experimental groups of rats. The diabetic rats showed a significant decrease in the levels of total hemoglobin and a significant increase in the level of HbA<sub>1c</sub>. Administration of EJs-kernel or glibenclamide to diabetic rats restored the

Table 1. Levels of Body Weight, Total Hemoglobin and Glycosylated Haemoglobin (HbA<sub>1c</sub>) in Control and Experimental Groups of Rats

Groups	Body weight (g)		Total hemoglobin (g/dl)	Glycosylated hemoglobin (% Hb)
	Initial	Final		
Control	190±15.2	208±11.5	13.20±0.92	5.6±1.12
Diabetic control	183±10.5*	151±12.5*	10.42±0.76*	12.5±2.1*
Diabetic+EJs-kernel	182±17.5*	189±19.8*	12.42±1.07*	6.8±1.09*
Diabetic+glibenclamide	185±14.5*	190±12.2*	12.10±0.85*	7.2±0.7

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \**p*<0.05. Diabetic control was compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control.

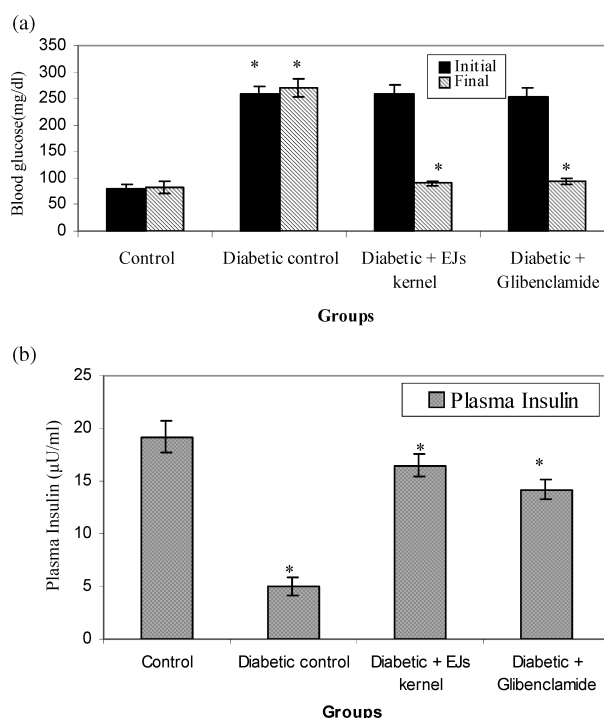


Fig. 1. (a) Blood Glucose Levels in Control and Experimental Groups of Rats on Initial (1st Day) and Final (30th Day) of Treatment Period and (b) Plasma Insulin Level of Control and Experimental Groups of Rats

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \**p*<0.05. Diabetic control rats were compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control.

Table 2. Levels of TBARS and Hydroperoxides in Liver and Kidney of Control and Experimental Groups of Rats

Groups	TBARS (mm TBARS/100 g of wet tissue)		Hydroperoxides (mm hydroperoxides/100 g of wet tissue)	
	Liver	Kidney	Liver	Kidney
Control	0.92±0.02	1.31±0.22	71.12±1.5	57.03±1.1
Diabetic control	1.71±0.26*	2.19±0.20*	97.23±0.6*	75.48±0.6*
Diabetic+EJs-kernel	0.92±0.03*	1.58±0.15*	72.62±1.3*	60.50±1.5*
Diabetic+glibenclamide	1.05±0.13*	1.80±0.12*	74.12±0.9*	62.40±0.8*

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \* $p<0.05$ . Diabetic control was compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control.

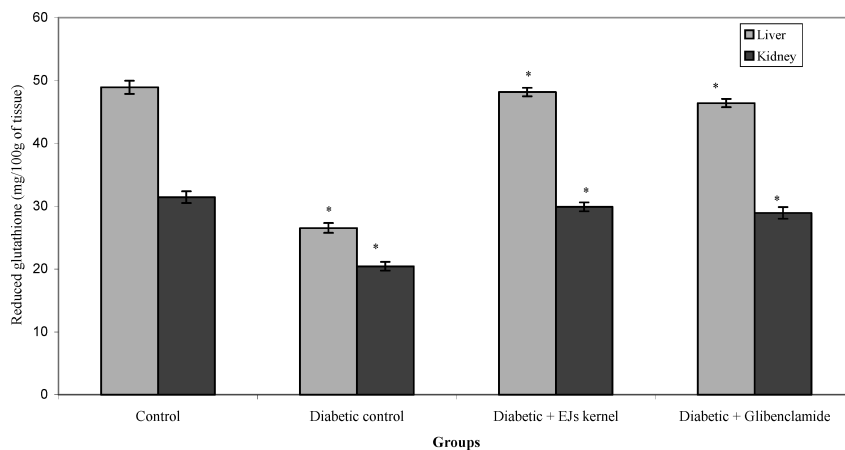


Fig. 2. Level of Reduced Glutathione (GSH) in Liver and Kidney of Control and Experimental Groups of Rats

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \* $p<0.05$ . Diabetic control rats were compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control.

Table 3. Activities of Superoxide Dismutase, Catalase and Glutathione Peroxidase in Liver of Control and Experimental Groups of Rats

Groups	SOD	CAT	GPx
Control	10.60±0.36	77.55±0.40	9.35±0.51
Diabetic control	4.56±0.19*	40.60±0.29*	5.56±0.28*
Diabetic+EJs-kernel	9.47±0.35*	76.13±0.61*	8.35±0.42*
Diabetic+glibenclamide	8.45±0.32*	75.32±0.44*	7.83±0.49*

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \* $p<0.05$ . Diabetic control was compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control. Units: 50% of inhibition of epinephrine auto oxidation per min for SOD;  $\mu\text{mol}$  of hydrogen peroxide decomposed per min per mg of protein for catalase;  $\mu\text{mol}$  of glutathione oxidized per min per mg of protein for GPx.

Table 4. Activities of Superoxide Dismutase, Catalase and Glutathione Peroxidase in Kidney of Control and Experimental Groups of Rats

Groups	SOD	CAT	GPx
Control	15.62±0.25	41.25±0.72	7.60±0.22
Diabetic control	8.52±0.32*	26.27±0.45*	4.45±0.42*
Diabetic+EJs-kernel	14.38±0.35*	37.15±0.48*	6.70±0.23*
Diabetic+glibenclamide	13.92±0.28*	35.43±0.63*	5.93±0.45*

Values are given as mean±S.D. for groups of six animals each. Values are statistically significant at \* $p<0.05$ . Diabetic control rats were compared with control rats. Diabetic+EJs-kernel and diabetic+glibenclamide were compared with diabetic control. Units: 50% of inhibition of epinephrine auto oxidation per min for SOD;  $\mu\text{mol}$  of hydrogen peroxide decomposed per min per mg of protein for catalase;  $\mu\text{mol}$  of glutathione oxidized per min per mg of protein for GPx.

changes in the level of body weight, total hemoglobin and HbA1c to almost control levels.

Table 2 shows the concentration of TBARS and hydroperoxides in liver and kidney of control and experimental groups of rats. There was a significant elevation in tissue TBARS and hydroperoxides in diabetic rats. Administration of EJs-kernel or glibenclamide to diabetic rats decreased the levels of tissue TBARS and hydroperoxides to near normal levels.

Figure 2 shows the concentration of GSH in liver and kidney of control and experimental groups of rats. A significant decrease in the concentration of GSH was observed in diabetic rats when compared to control group of rats. Administration of EJs-kernel or glibenclamide to diabetic rats tends to bring the concentration of GSH to near normal level.

Tables 3 and 4 demonstrate the activities of SOD, CAT and GPx in liver and kidney of control and experimental groups of rats. A significant decrease was observed in the activities of SOD, CAT and GPx in liver and kidney of diabetic rats. Administration of EJs-kernel or glibenclamide recovered the activities of these enzymes to near normal in diabetic rats.

The histopathological examination revealed extensive alterations in liver and kidney of STZ-induced diabetic rats. The liver of control rat (Fig. 3a) shows normal architecture. The liver of diabetic rat (Fig. 3b) shows perivenular inflammatory infiltration filling over the sinusoidal vacuolation of the hepatocyte nuclei. The pathomorphological changes observed in STZ-induced diabetes become apparently normal after treatment with EJs-kernel extract (Fig. 3c) or glibenclamide (Fig. 3d).

The kidney of control rat (Fig. 4a) shows normal glomeruli and tubules. The kidney of diabetic rat shows (Fig. 4b) thickening of vesicles, glomeruli show some cellular proliferation

with fibrosis. EJs-kernel extract (Fig. 4c) or glibenclamide (Fig. 4d) treated diabetic rat show glomeruli, which appear normal with mild dilated tubules.

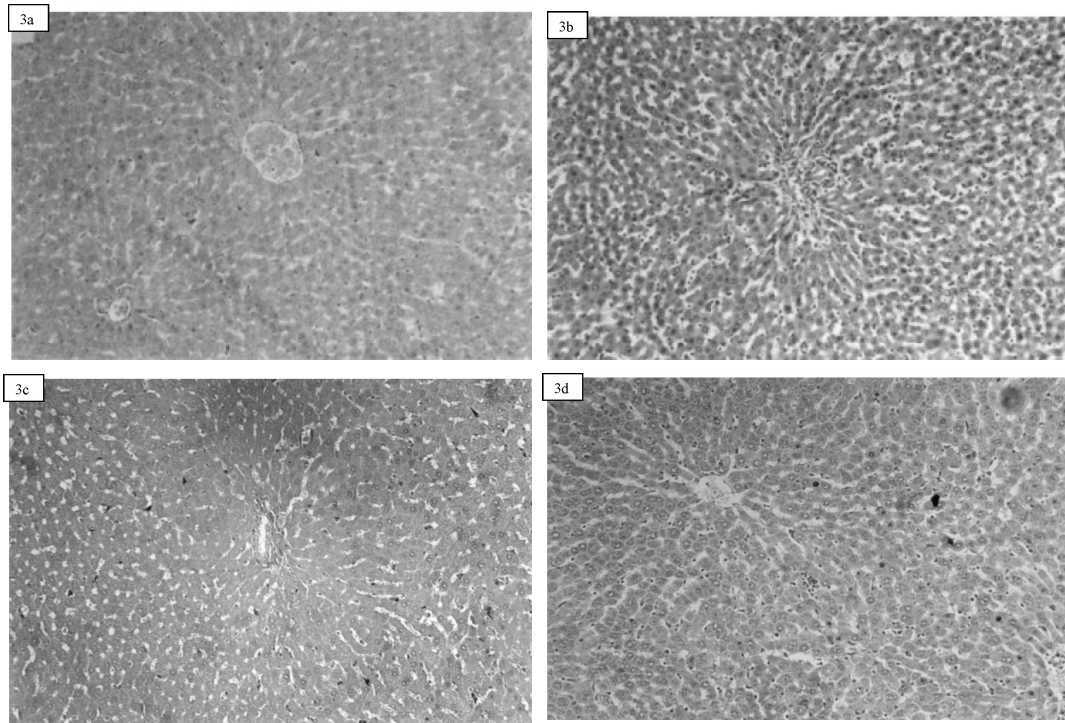


Fig. 3. Histopathological Studies of the Liver Tissue of Control and Experimental Groups of Rats (HE 100×)

(a) Section of liver tissue from control rat showing normal architecture. (b) STZ-induced diabetic rat showing inflammatory infiltration filling over the sinusoidal vacuolation of hepatocytic nuclei. (c) Liver tissue from EJs-kernel treated diabetic rat with normal architecture. (d) Glibenclamide treated diabetic rat showing more or less normal architecture with mild changes in hepatocytes.

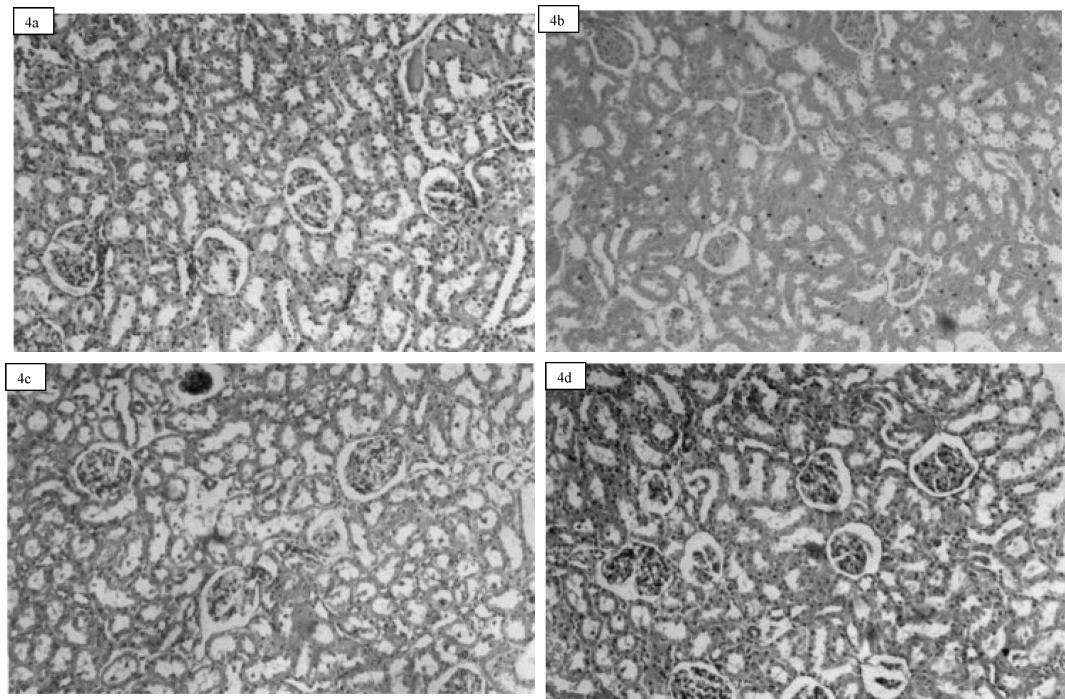


Fig. 4. Histopathological Studies of the Kidney Tissue of Control and Experimental Groups of Rats (HE 100×)

(a) Section of kidney tissue from control rat showing normal glomeruli and tubules. (b) STZ-induced diabetic rat which shows thickening of vesicles and fibrosis in glomeruli. (c) Kidney tissue from EJs-kernel treated diabetic rat with normal glomeruli and mild dilation of tubules. (d) Glibenclamide treated diabetic rat showing mild changes in glomeruli and tubules.

## DISCUSSION

In the present study oral administration of EJs-kernel extract decreased the blood glucose level in diabetic rats. Administration of medicinal plant extract to mildly STZ-diabetic rats resulted in activation of  $\beta$ -cells and granulation returns to normal giving insulinogenic effect.<sup>33)</sup> EJs-kernel may bring about its hypoglycemic action through stimulation of surviving  $\beta$ -cells of islets of langerhans to release more insulin. This was clearly evidenced by the increased level of plasma insulin in diabetic rats treated with EJs-kernel. Since the percentage fall in plasma glucose levels was different in models with varying intensity of hyperglycemia it implies that the anti-hyperglycemic effect of that plant is dependent upon the dose of diabetogenic agent and therefore on the degree of  $\beta$ -cell destruction.<sup>34)</sup> A number of other plants have also been observed to exert hypoglycemic activity through insulin-release stimulatory effects.<sup>22,35)</sup>

The hypoglycemic activity of EJs-kernel was compared with glibenclamide, a standard hypoglycemic drug. Sulfonylureas such as glibenclamide have been used for many years to treat diabetes, to stimulate insulin secretion from pancreatic  $\beta$ -cells principally by inhibiting ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels in the plasma membrane.<sup>36)</sup> Further, it was concluded that sulfonylureas have a direct effect on  $\beta$ -cell exocytosis and that effect is mediated by a mechanism that does not involve direct activation of protein kinase C, which plays a major role in controlling the  $\beta$ -cell potential.<sup>37)</sup> The inhibition of ATP sensitive channels leads to membrane depolarization, activation of voltage gated  $Ca^{2+}$  channels, increased  $Ca^{2+}$  influx, a rise in cytosolic  $[Ca^{2+}]$  and thereby insulin release.<sup>38)</sup> Courtois *et al.*, have reported that oral administration of glibenclamide to the STZ-induced diabetic rats, decrease the blood glucose level.<sup>39)</sup> From the results of the present study, it may be suggested that the mechanism of action of EJs-kernel mechanism is similar to glibenclamide action.

The decreased body weight in diabetic rats is due to excessive breakdown of tissue proteins.<sup>40)</sup> Treatment with EJs-kernel improved body weight significantly indicating prevention of muscle wasting due to hyperglycemic condition.

The decreased level of total hemoglobin in diabetic rats is mainly due to the increased formation of glycosylated hemoglobin (HbA<sub>1c</sub>). HbA<sub>1c</sub> was found to increase in patients with diabetes mellitus and the amount of increase is directly proportional to the fasting blood glucose level.<sup>41)</sup> During diabetes, the excess glucose present in the blood reacts with hemoglobin to form HbA<sub>1c</sub>.<sup>42)</sup> HbA<sub>1c</sub> is used as a marker for estimating the degree of protein glycation in diabetes. HbA<sub>1c</sub> alters the structure and function of antioxidant enzymes such that they are unable to detoxify free radicals, exacerbating oxidative stress in diabetes.<sup>43)</sup> Administration of EJs-kernel to diabetic rats reduced the glycosylation of hemoglobin by virtue of its normoglycemic activity and thus decreases the level of glycosylated hemoglobin in diabetic rats. This normalization of glycosylated hemoglobin indicates decreased glycation of proteins. This suggests amelioration of oxidative stress due to hyperglycemia by the treatment of EJs-kernel extract.

Oxygen derived free radicals generated in excess in response to various stimuli can be cytotoxic to several tissues. Most of the tissue damage is considered to be mediated by

these free radicals by attacking membranes through peroxidation of unsaturated fatty acids.<sup>44)</sup> Induction of diabetes in rats with STZ uniformly results in an increase in lipid peroxidation (TBARS), an indirect evidence of intensified free radical production.<sup>43)</sup> In the present study the concentrations of lipid peroxides and hydroperoxides were increased in liver and kidney of diabetic rats, indicating an increase in the generation of free radicals. Increased lipid peroxidation in diabetes can be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems. The present finding indicates significantly increased lipid peroxidation of rats exposed to STZ and its attenuation by EJs-kernel treatment. This suggests protective role of seed kernels, which could be due to the antioxidative effect of flavonoids<sup>19)</sup> present in the seeds which act as strong superoxide radical and singlet oxygen quenchers.

Reduced glutathione (GSH) is known to protect the cellular system against the toxic effects of lipid peroxidation.<sup>45)</sup> GSH functions as a direct free radical scavenger, as a cosubstrate for GPx activity and as a cofactor for many enzymes and forms conjugates in endo and xenobiotic reactions.<sup>46)</sup> A marked decrease in the level of GSH in liver and kidney during diabetes were observed. Several studies support the hypothesis that in diabetes, chronic hyperglycemia increases the polyol pathway as well as advanced glycation end products (AGEs) formation and free radical generation rates, leading to increased GSH oxidation. A relative depletion of NADPH due to aldose reductase activation and secondary to reduced production through the pentose cycle impairs GSH generation and leads to depletion of this free radical scavenger.<sup>47)</sup> GPx metabolizes hydrogen peroxide to water by using GSH as a hydrogen donor.<sup>48)</sup> The reduced activity of GPx may result in the accumulation of toxic products due to oxidative damage. The significant recovery of GSH content and GSH dependent enzyme GPx by treatment with EJs-kernel indicates the protective effect of EJs-kernel on antioxidants.

Reduced activities of SOD and CAT in liver and kidney of diabetic rats have been observed in our study. The decreased activities of SOD and CAT in both liver and kidney during diabetes may be due to increased production of reactive oxygen radicals that can themselves reduce the activity of these enzymes.<sup>8)</sup> SOD is an important defense enzyme, which converts superoxide radicals to hydrogen peroxide.<sup>49)</sup> CAT is a hemeprotein, which decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals.<sup>50)</sup> The reduction in the activity of these enzymes may result in a number of deleterious effects. Administration of EJs-kernel increased the activity of enzymes and may help to avoid the free radicals generated during diabetes.

In conclusion, the antioxidant potential of *Eugenia jambolana* seed kernel may be attributed mainly due to the amelioration of hyperglycemia induced oxidative stress by its normoglycemic effect. The presence of flavonoids, further strengthens the efficacy of *Eugenia jambolana* seed kernel in protecting the tissue defense system against oxidative damage in streptozotocin-induced diabetes.

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