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Original article

Study of effect of *Stevia rebaudiana* bertoni on oxidative stress in type-2 diabetic rat models

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ABSTRACT

A clinical condition "Diabetes" is more often associated with the release of a massive amount of toxic free radicals, which significantly decrease the level of antioxidant enzymes, increase lipid peroxidation, and worsen the disease state by causing further hyperglycemia. Many plant extracts and plant-derived natural compounds have been reported to possess antioxidant activities, and useful in preventing the deleterious effects of oxidative stress. Here, we have demonstrated the free-radical scavenging effects of a natural sweetener or a dietary supplement *Stevia rebaudiana* bertoni standardized extract on diabetes-induced oxidative stress animal model. The present study was also aimed to investigate the effect of this extract on hyperglycemia and hepatic antioxidant enzymes of animal models of type 2, non-insulin dependent diabetes mellitus (NIDDM). The hyperglycemic state was induced by alloxan monohydrate with a dose of 150 mg/kg/b.w., i.p. Animals of normal treated and diabetic treated groups were administered with a dose of 250 mg/kg/b.w., p.o., of stevia extract for 28 days and blood sugar levels were measured intermittently. At the end of the experiment, rat liver tissues were collected and the tissue homogenate was assayed for various oxidative scavenging enzymes. The results of hepatic antioxidant enzyme assays and lipid peroxidation clearly indicates that stevia extract have significant antioxidant effect on diabetes pathology. Administration of the extract also reduced the abnormal blood sugar levels significantly ($P < 0.001$) and improved the hyperglycemic condition.

1. Introduction

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage [1,2]. All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA [3,4]. In humans, oxidative stress is involved in many diseases, such as diabetes mellitus, atherosclerosis, Parkinson's disease, myocardial infarction, Alzheimer's disease and chronic fatigue syndrome.

Diabetes mellitus is probably the fastest growing metabolic disease in the world. Because of its heterogenic nature, diabetes makes more challenging task and it needs more appropriate therapies [5]. Traditional plant remedies have been used for very long time in the treatment of diabetes, but only a few of them have been significantly evaluated. Therefore, the present work aimed to evaluate the effect of purified standard *Stevia rebaudiana* extract on blood glucose profile and biomarkers of oxidative stress, in liver tissue of alloxan-induced diabetic rats. Members of stevia comprise mostly of herbs but also shrubs and trees. Originally it is said to be native to subtropical South America (Paraguay and Brazil) and Central America. It had been used for centuries as both a sweetener and a medicine. It is now grown and consumed in many other parts of the world for these same properties. The leaf is many times sweeter than refined sugar, but contains no carbohydrates or calories [6].

A detailed review of literature afforded no information on the in vivo studies of antioxidant potential of *Stevia rebaudiana* Bertoni [7–9]. Therefore, we have investigated the effect of commercially available purified standard *Stevia rebaudiana* extract on various antioxidant enzymes, viz., catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) activities in vivo, and in liver tissues. The effect on lipid peroxidation has also been evaluated by measuring amount of thiobarbituric acid reactive substances (TBARS). The hypoglycemic activity was monitored by fasting blood glucose level (FBG) and oral glucose tolerance test (OGTT).

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2. Materials and methods

2.1. Plant extract

Purified standard stevia extract *HERBOCAL* (Batch no. 0101) manufactured by India Foods Pvt. Ltd, Mathura Arcade, Vile Parle (E), Mumbai, India was used as supplied.

2.2. Animals

Male Wistar rats weighing about 150 to 200 g were used in the present study. Animals were obtained from the Central Animal House facility, Devi Ahilya Vishwavidyalaya, Indore, and housed in individual cages under natural light and dark cycles at a temperature of 28 ± 4 °C, given a standard pellet diet and water ad libitum and were acclimatized for 7 days before the experiment. The animals were fasted for 12 hours prior to experiment, with water ad libitum.

2.3. Experimental design

The rats were segregated into four groups with minimum six rats in each group. Animals of normal treated (group III, NT) and diabetic treated (group IV, DT) groups were administered with a dose of 250 mg/kg, b.w., p.o., of stevia extract for 28 days. Control (group I, CL) and diabetic control (group II, DC) groups received same amount of distilled water for 28 days.

2.4. Induction of experimental diabetes [10,11]

Rats of group II and IV were starved overnight and each rat received a single intraperitonial injection of freshly prepared ice-chilled solution of alloxan monohydrate (150 mg/kg, b.w.) dissolved in 0.9% w/v saline solution. The control and normal treated received same amount of 0.9% w/v saline solution.

After 1 hour of alloxan administration, animals were given free access to feed ad libitum. Fasting blood glucose levels of rats were checked after 72 hours with help of accu-chek sensor. As a result of alloxan injection, about 80% of rats became diabetic with blood glucose value 3 to 4 times the normal fasting blood glucose level. Animals with fasting blood glucose > 200 mg/dl as well as with polyuria, polydipsia and polyphagia were selected for study. Stevia extract treatment was started 1 week after the administration of alloxan, when stable hyperglycemia development was assured.

2.5. Treatment protocol

Animals described as above were fasted overnight but allowed free access to water before extract administration for 28 days. Normal treated and diabetic treated group received 250 mg/kg b.w. p.o. Stevia extract powder dissolved in distilled water for 28 days. Control and diabetics control groups received only distilled water in place of stevia extract [12].

2.6. Estimation of fasting blood glucose level (FBD)

Fasting blood glucose level of each rat was estimated by accu-chek sensor strips based on glucose oxidase method, intermittently.

2.7. Oral glucose tolerance test (OGTT) [13,14]

The Oral glucose tolerance test was performed at the end of experiment on 28th day on overnight fasted rats. Rats were administered a high dose of glucose (1.5 gm/kg b.wt.) p.o. and glucose level was measured at interval of 0, 30, 60, 120 min. respectively.

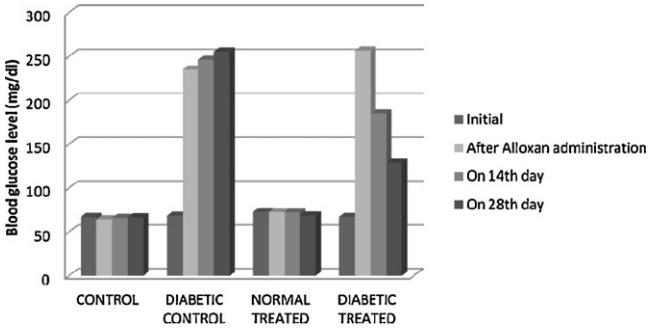

Fig. 1. Effect of stevia extract on blood glucose level (mg/dl).

2.8. Biochemical assays [15]

After performing OGTT test, all rats were sacrificed by cervical dislocation method and their livers excised, rinsed twice in ice-cold 0.9% normal saline solution and blotted dry and transferred in eppendorf's tube and stored at -80^0C for enzyme assay. One gram of liver was weighted and transferred to homogenizing tube having 10 ml of ice-chilled 50 mM Tris-Hcl buffer. Homogenization of liver tissue was done at 4^0C to obtain a 10% w/v solution. A 10% w/v homogenate was centrifuged at 16000 rpm at 4^0C for 25 minutes to obtain post-mitochondrial supernatant and utilized for estimation of various biochemical parameters namely lipid peroxidation (Ohkawa et al.) [16], superoxide dismutase (Kakkar et al., 1984) [17], reduced glutathione (Ellman's et al. 1959) [18,19], catalase (Aebi, 1974) [20] and protein content by method of Lowry et al. (1951).

2.9. Histopathological study

The small part of each liver tissue were preserved in 20% formalin immediately after removal from the animals of each group for 1 hour to rectify shrinkage due to high concentration of formalin, processed and embedded in paraffin wax to obtain 5 to 6 μm thick hematoxylin stained sections. Sections were analyzed under microscope for comparing histological changes at 100 X.

2.10. Statistical analysis

The data are expressed as mean ± S.D. Statistical comparison were performed by one-way analysis of variance (Anova). The results were considered statistically significant if the *P*-value were 0.05 or less.

3. Result and discussion

It has been established that oxidative stress lies at the root of a number of pathological processes and diseases such as cancer, atherosclerosis, rheumatic arthritis, hematological and neurodegenerative disorders are not exempt with more making the list among which is diabetes mellitus. Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodent and many other animal species. This causes an insulin dependent diabetes mellitus in these animals, which characteristically similar to type I diabetes in human. Alloxan preferentially accumulates in beta-cells through uptake via the GLUT2 glucose transporter. Alloxan, in the presence of intracellular thiol, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid. The beta-cell toxic action of alloxan is initiated by free radicals formed in this redox reaction [21]. *Stevia rebaudiana* bertoni not only decreases the increased level of blood glucose (Fig. 1) but also increases the

Table 1
Comparison of effect of stevia extract on blood glucose level of each group.

Groups	Blood glucose level (mg/dl)			
	Day			
	0th (initial)	After alloxan administration	14th	28th
Control	67.16 ± 3.71	64.66 ± 4.03	66.16 ± 2.87	66.83 ± 3.76
Diabetic control	68.66 ± 2.87	235.16 ± 34.26	246.33 ± 33.19	255.66 ± 35.02[a]
Normal treated	73 ± 4.28	72.83 ± 5.1	72.33 ± 3.50	68.83 ± 5.98
Diabetic treated	67.33 ± 4.88	256.83 ± 46.74	185.16 ± 32.46[c]	128.83 ± 16.21[b]

[a] $P < 0.001$ (as compared to control).
[b] $P < 0.001$.
[c] $P < 0.01$ (as compared to diabetic control).

Table 2
Oral glucose tolerance test (OGTT) in rat (mg/dl).

Groups	0 min	30 min	60 min	120 min
Control	66.83	207.83	147.83	95.93
Diabetic control	256	472	389.83	340.5
Normal treated	66.83	198.5	147.50	99.17
Diabetic treated	123.83	240.33	191.83	159.66[a]

[a] $P < 0.001$ as compare to diabetic control group.

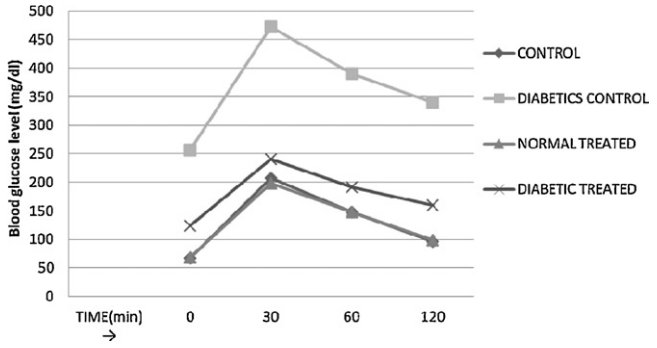

Fig. 2. Comparison of oral glucose tolerance test (OGTT) of each group on 28th day.

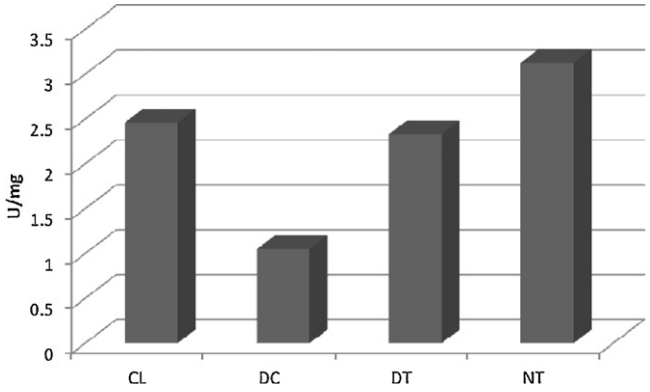

Fig. 3. Effect of stevia extract on superoxide dismutase enzyme.

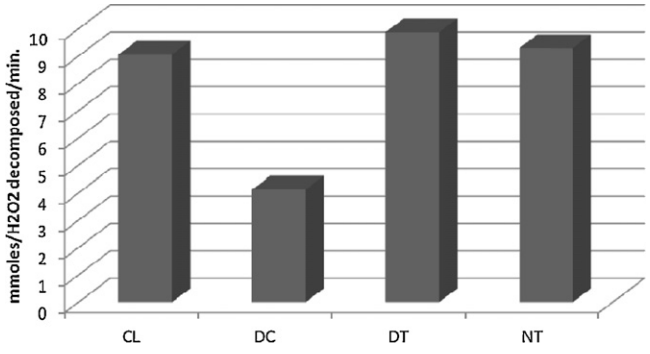

Fig. 4. Effect of stevia extract on catalase enzyme.

decreased levels of antioxidant enzymes (CAT, SOD, GSH) in liver and maintains the cells integrity by inhibiting the lipid peroxidation in stressed condition. It is evident from Table 1 that treatment for 4 weeks with the stevia extract powder brought down fasting blood glucose (FBG) to significantly ($P < 0.01$) low level as compared to diabetic control group in just a 2 weeks. At the end of 4 weeks, glucose level reduced more significantly ($P < 0.001$) as compared to diabetic control group. However, there was no any significant change noted in FBG level at the end of 2nd and 4th week of treatment in normal treated groups as compared to control group, which shows that stevia is also safe for use in food and beverages as natural sweetening agent.

A similar improvement was seen in glucose tolerance studies also (Table 2). Blood glucose level were in normal range at the end of 2 hours in diabetic treated and normal treated group, whereas in case of diabetic control glucose level were much higher as compared to rest of groups (Fig. 2). From the result of glucose tolerance test it concluded that stevia improved the normal functioning of pancreatic ß-cells, which resulted in improving metabolism and glucose transport to cells.

Superoxide dismutase (SOD) protects tissue against reactive oxygen species (ROS) by catalyzing the removal of superoxide

Table 3
Comparison of effect of stevia extract on biochemical parameters (enzyme) of each group after 28 days.

Groups	Antioxidative enzymes			
	LPO	CAT	SOD	GSH
Control	144.2 ± 15.99	9.055 ± 1.92	2.46 ± 0.64	37.38 ± 9.64
Diabetic control	363.1 ± 34.14[a]	4.12 ± 1.97[a*]	1.049 ± 0.49[a*]	25.71 ± 5.04[a*]
Normal treated	121.7 ± 26.97	9.29 ± 2.2	3.127 ± 0.68	70.76 ± 8.55[a*]
Diabetic treated	168.39 ± 28.35[b]	9.878 ± 2.22[b*]	2.33 ± 0.61[b*]	49.31 ± 7.33[b*]

LPO in nanomoles/mg protein; CATALASE in mmoleH_2O_2 decomposed/min/mg protein; GSH in μM/mg protein; SOD in U/mg protein.
[a] $P < 0.001$ as compare to diabetic control group, [a*] $P < 0.01$ as compare to diabetic control group.
[b] $P < 0.001$ as compare to diabetic treated group, [b*] $P < 0.01$ as compare to diabetic treated group.

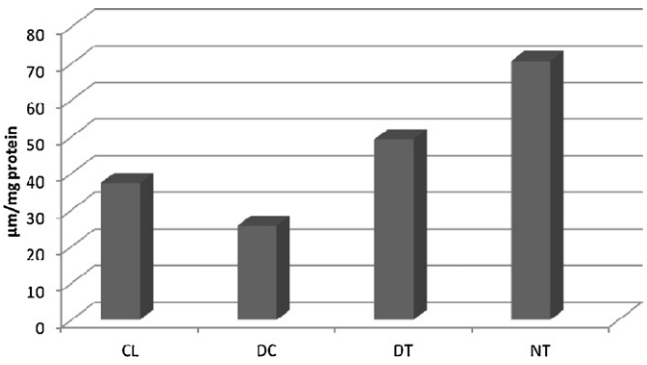

Fig. 5. Effect of stevia extract on reduced glutathione (GSH).

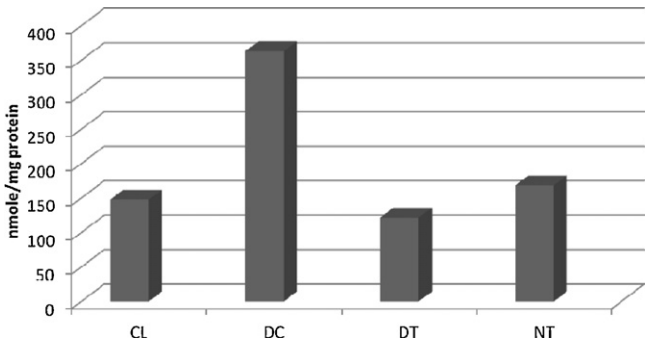

Fig. 6. Effect of stevia extract on lipid peroxidation.

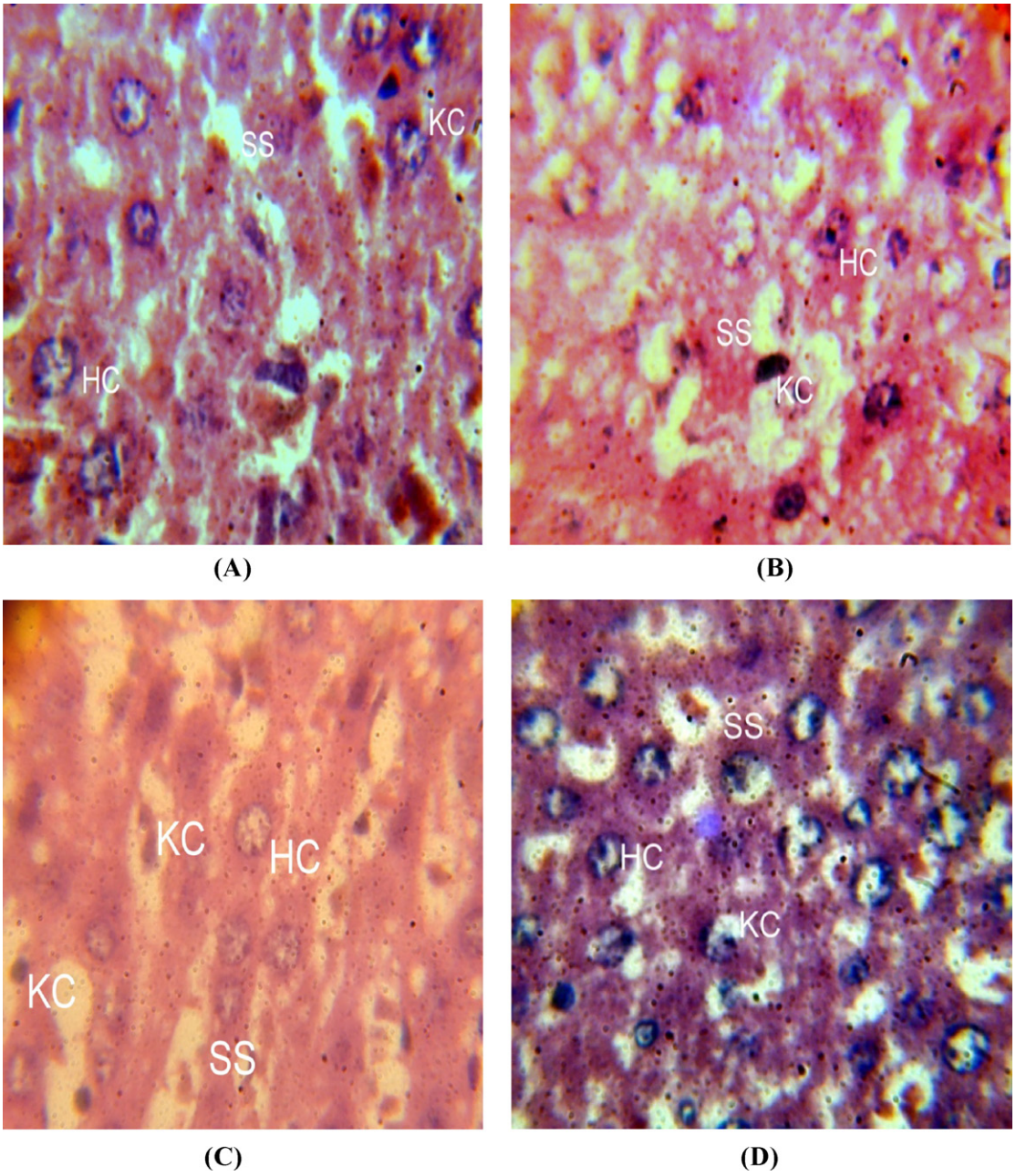

Fig. 7. Photographs of liver section stained in hematoxyline (100 X). (A) Normal histology of normal liver with polygonal hepatocytes (HC) with prominent nucleus, necrosis (NC), sinusoidal space (SS) and Kupffer cell (KC) in control group. (B) i.p. administration of alloxan-induced deformation in hepatocytes architecture (HC) with pycnosis of nuclei, ballooning of sinusoidal spaces in diabetic control group. (C) Hepatocytes with prominent nucleus (HC), slight enlarged sinusoidal space (SS) and kupffer cells (KC) in normal treated group. (D). Well-formed hepatocytes with prominent nucleus (HC) and maintained sinusoidal space (SS) in diabetic treated group.

radical ($O^{2.-}$) which damages the membrane and biological structure. Catalase has been shown to be responsible for detoxification of significant amount of H_2O_2. SOD and catalase are the two major scavenging enzymes that remove the toxic free radicals in vivo. Reduced activities of SOD and catalase in liver resulted in number of deleterious effects due to accumulation of superoxide radicles ($O^{2.-}$) and hydrogen peroxide. The present study clearly showed that the stevia extract increased the amount of SOD in diabetic rats as shown in Table 3. At the end of 28-day treatment concentration of SOD in diabetic treated rats increased ($P < 0.05$) as compared to diabetic control group (Fig. 3). Twenty-eight-day treatment of stevia in normal treated group also caused slight increase in amount of SOD as compared to control group but elevation was statistically not significant.

The catalase activity was found to increase significantly ($P < 0.01$) in diabetic treated rats as compared to diabetic control group (Fig. 4), whereas in case of normal treated group there was no significant change noted as compared to control groups as shown in Table 3. All the above observations revealed that the normal by functioning of hepatic catalase enzyme of diabetic treated group was caused the decomposition of H_2O_2.

Glutathione is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants in their reduced (active) forms. Through direct conjugation, it detoxifies many xenobiotics (foreign compounds) and carcinogens. It plays a fundamental role in numerous metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system. In our study, it was found that stevia extract powder increased the decreased level of GSH (Fig. 5) in diabetic treated group ($P < 0.01$) as compared with diabetic control group and also with control group (Table 3). A very significant increase in reduced glutathione (GSH) was found in case of normal treated group ($P < 0.01$) as compared with control group.

Lipid peroxidation is a free radical-induced process leading to oxidative deterioration of polyunsaturated fatty acids under physiological condition, low concentration of lipid peroxides are found in tissue. Elevated level of peroxides in plasma in diabetic rat is a characteristic feature of chronic diabetes. Lipid peroxides mediated tissue damage has been observed in development of both Type 1 and Type 2 diabetes. In the present study it was observed that the stevia extract powder decreased the increased level of lipid peroxide in diabetic treated group very significantly ($P < 0.001$) as compared with diabetic control group as shown in Table 3. There was a slight reduction in LPO value in normal treated group as compared to control group but statistically it was not significant (Fig. 6).

3.1. Histopathological observations

Light microscopic examination (Fig. 7) of stained slides of control animals showed normal architecture of the liver depicting normal pentagonal hepatocytes, sinusoidal space responsible for blood supply having high concentration of nutrient and few normal scattered kupffer cells were also appreciated. Intraperitonial administration of alloxan resulted in lipid infiltration and ballooning of hepatocytes and sinusoidal space. Also a pycnosis of hepatocyte's nuclei, cloudy swelling and mild fatty infiltration has been observed in liver section of diabetic control rat, were characteristic alterations occurred due to intoxication of alloxan in diabetes.

After 4-week treatment of stevia extract in diabetic treated rats, hepatocytes with prominent nucleus and sinusoidal space having improved architecture was observed. Necrosis and lipid infiltration around hepatocytes also decreased which shows the hepatoprotective effect of stevia extract. In normal treated groups, hepatocytes with prominent nuclei, kupffer cells and slight enlargement of sinusoidal space was observed. Thus, it can be concluded that stevia extract is useful in ameliorating the oxidative stress in type-2 diabetes mellitus and maintains the integrity of liver cells.

4. Conclusion

Recently, the use of herbal medicines for the treatment of diabetes mellitus is increasing and most patients consider herbal medicines are more safe as they are of natural origin. These herbal medicines have a tremendous potential to combat the oxidative free radical generated during disease with least potential to cause any adverse effects as compared to allopathic drugs. To date the literature describing these herbal drugs with their oxidative potential is limited and most are in preclinical studies. Hence, more research is required to explore their beneficial therapeutics effect. *Stevaia rebaudiana* bertoni is a most commonly used natural sweetener in food and beverages, so its potential activity should be evaluated. In present work it was found that stevia extract powder have a beneficial activity in diabetes as it reduced the enhanced blood glucose level and improved the functioning of oxidative scavenging enzymes significantly and maintains the integrity of cells in diabetic condition. Therefore, the present study concluded that *Stevia rebaudiana* bertoni is useful in ameliorating the oxidative stress in type-2 diabetic rat model. However, more mechanism based research work is required to seek out the effect of this herbal medicine on other enzymes which involves in the pathophysiology of type-2 diabetes mellitus.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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