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Full Length Research Paper

Brassica rapa L. extract alleviate early hepatic injury in alloxan-induced diabetic rats

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Liver insufficiency is one of the most important consequences of diabetes mellitus. The main objective of this study was to evaluate the protective effect of turnip root ethanolic extract (TREE) on early hepatic injuries in alloxanized-diabetic rats. Eighty male Wistar rats were randomly assigned into 4 equal groups including: normal healthy, normal+TREE, diabetic and 4- diabetic+TREE. Diabetes was induced with a single injection of alloxan (120 mg/kg i.p.). TREE treatment groups received TREE (200 mg/kg) daily for 8 weeks by means of gavage. At the end of the experimental period, levels of functional liver markers [aspartate aminotransaminase (AST), alanine aminotransaminase (ALT) and lactate dehydrogenase (LDH)], TB, albumin and total protein (TP) were assessed in the serum. Product of lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) activity was also assayed in liver homogenates. Finally, the biochemical findings were matched with histopathological verification. Statistically, the quantitative data obtained were compared among the groups by one-way analysis of variance followed by Tukey post-hoc test. Statistical significance was considered as $p<0.05$. In the diabetic rats, TREE significantly decreased the levels of serum biomarkers of hepatic injury. Furthermore, TREE significantly decreased the lipid peroxidation and elevated the decreased values of antioxidant enzymes in diabetic rats. Histopathologically, the changes were parallel to the biochemical findings. The results obtained showed that TREE alleviates early hepatic injuries in the rats with experimentally induced diabetes.

Key words: *Brassica rapa* L., diabetes, hepatic injury, alloxan, rats.

INTRODUCTION

Diabetes mellitus is a metabolic disorder as old as mankind and its incidence (4 to 5%) is considered to be high all over the world (WHO, 1980). This endocrine disorder results from abnormal metabolism of carbohydrates, fats and proteins and causes the increase in blood glucose values. Hepatic and renal failure is the main cause of death in diabetic patients (Pickup et al., 1997). Evidence suggests that oxidative stress and free radicals play an important role in the pathogenesis of diabetes mellitus (Cerielo et al., 1997; Kaneto et al., 2007; Bulter et al., 2000).

The role of free radicals in tissue injuries has been approved in the rat with streptozotocin induced diabetes (Murugana and Pari, 2006). Liver is one of the most important organs that maintains blood glucose levels within normal limits thus enhancement of blood glucose yield to imbalance of oxidation-reduction reactions in hepatocytes, so that, hyperglycemia through increasing in AGEs (advanced glycation end products) facilities free

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Abbreviations: TREE, Turnip root ethanolic extract; **MDA,** lipid peroxidation; **SOD,** superoxide dismutase; **CAT,** catalase; **GPX,** glutathione peroxidase; **GR,** glutathione reductase; **TP,** total protein; **AST,** aspartate aminotransaminase; **ALT,** alanine aminotransaminase; **LDH,** lactate dehydrogenase; **GSSG,** oxide glutathione; **GSH,** reduced glutathione.

radicals production via disturbance in ROS production (reactive oxygen species) such as superoxide dismutase (SOD) and catalase (CAT) (Cameron et al., 2005; Kalia et al., 2004; Jandeleit-Dahm et al., 2005). Hence, it reveals that diabetic hepatic injuries results from several agents and is not controllable only via inhibition of hyperglycemia (Liu et al., 2008).

Although in early stages of diabetes, hepatic injuries are induced via hyperglycemia but its progress in latter stages is not related to hyperglycemia (Vestra and Fioretto, 2003). Therefore, monitoring of blood glucose levels solely is not sufficient in delaying diabetes complications. Cells protect themselves against free radical damages by several mechanisms. Meanwhile, SOD, GPX, CAT and glutathione reductase (GR) have substantial role. Considering the role of free radicals in diabetes mellitus, an important aspect of treatment is to decrease free radicals (Bulter et al., 2000). Meanwhile, during several studies, the effect of antioxidants in prevention or reduction of injuries induced by free radicals in diabetes has been studied (El-Bassiouni et al., 2005; Peerapatdit et al., 2006). Thus, a suitable drug must have both antioxidant and blood glucose decreasing properties (Ramesh and Pugalendi, 2006). Synthetic drugs employed in the treatment of diabetes have several undesirable side effects (Liu et al., 2008; Akhtar and Iqbal, 1991; Holman and Turner, 1991). Also, the use of these drugs in the pregnancy period is harmful (Larner, 1985). Nowadays, medicinal plants have an important role among new pharmaceutical agents (Luo et al., 1998). Use of medicinal plants in the treatment of diabetes dates back to before 1550 BC (Kesari et al., 2005). Several plants from all over the world have been introduced to diabetic patients (Kesari et al., 2005; Gupta et al., 2005; Ivorra et al., 1989; Marles and Farnsworth 1995). Of these, only some of them were approved scientifically and according to WHO recommendation, many researches in this field is necessary (WHO, 1980). Many of these plants were used in traditional medicine including the turnip roots. *Brassica rapa* species has important varieties such as turnip (Sasaki and Takahashi, 2002). Turnip has active biological compounds such as flavonoids (isorhamnetin, kaempferol and quercetin glycosides), phenylpropanoid derivatives (Romani et al., 2006), indole alkaloids and sterol glucosides (Schonhof et al., 2004). Flavonoids have important effects on diabetic patients. For example, isorhamnetin has inhibitory effect on aldose reductase, which has substantial role in complications of diabetes (Lim et al., 2006). Kaempferol has hypoglycemic effect on diabetic rats and is capable of increasing glucose absorption in rat's muscles (Jorge et al., 2004). In other studies, it has been observed that quercetin causes decrease in blood glucose and increase in plasma insulin levels in diabetic rats due to streptozotocin (Vessal et al., 2003). Jung et al. (2008) showed that turnip root ethanolic extract (TREE) through increase in glucose and fat metabolism has antidiabetic effect in diabetes mellitus type II. Most importantly, flavonoids and hydroxycinnamic acid derivatives, which are found frequently in turnip root, have direct and potent antioxidant and free radical removal effects (Bennett et al., 2006). According to various turnip phytochemical effects, it is assumed that extract of the root of this plant is capable of reducing hepatic complication of diabetes. However, there is no study to document this protective effect. Therefore, the present study was undertaken for the first time to evaluate the hepatoprotective activity of TREE against early hepatic injury in alloxanized diabetic rats.

MATERIALS AND METHODS

Experimental plan

This experimental study was carried out in Islamic Azad University Research Center. All procedures were conducted under supervision of Animal Rights Monitoring Committee of Islamic Azad University Research Center.

TREE preparation and maintenance

B. rapa L. was collected from Azerbaijan Province in North of Iran, during April 2010. The plant was identified by Pharmacognosy Department of Islamic Azad University. Fresh roots were cut and their content extracted three times with ethanol. The extracted solutions were filtered and dried using a rotary evaporator under reduced pressure.

The ethanolic extract yields after vacuum evaporation was 10.6 g per 100 g of fresh root material. Dried extract was kept in the refrigerator at 4°C.

Chemicals

All chemicals used in this study were of analytical grade and obtained from Nanjing Jiancheng Bioengineering Institute, Nanjing, China and Ziest Chemi Co., Iran.

Animals

Eighty male Wistar rats, weighing 200±20 g and 9 to 10 weeks old, were obtained from the animal breeding center of Islamic Azad University. The rats were randomly divided into 4 equal groups of 20 animals including: 1- normal control, 2- normal rats treated with TREE, 3- diabetic control, and 4- diabetics treated with TREE. Management and husbandry conditions were identical in all groups with 12/12 h light/dark cycle at 21±2°C. Food and water were provided *ad libitum*. At the beginning of study, prior to induction of diabetes, blood glucose was measured in all experimental rats after 12 h of fasting.

For induction of diabetes, after 15 h fasting, the rats were intraperitoneally injected with alloxan monohydrate at a dose of 120 mg/kg body weight (bw), freshly dissolved in distilled water (5%). Animals with fasting blood glucose of 120 to 250 mg/dl were considered diabetic (Gupta et al., 2005). Blood glucose was estimated by commercially available glucose kit (Ziest Chemi Co., Iran) based on glucose oxidase method. Thereafter, TREE (200 mg/kg in 10 ml/kg normal saline) was gavaged for eight consecutive weeks to groups 2 and 4 (Kim et al., 2006). Simultaneously, groups

1 and 3 were gavaged with similar volume of normal saline solution.

Biochemical factors evaluation

At the end of the experiment, blood samples were collected from the retro-orbital plexus and the sera prepared through centrifuging at 2500 × g for 15 min at 30°C. After 12 h fasting, blood glucose and serum biomarkers of liver function including ALT, AST (Reitman and Frankel, 1957), LDH (Martinek, 1972), albumin, TP (Lowry et al., 1951) and total bilirubin (Malloy and Evelyn, 1937) were measured using commercially available kits.

Measurement of antioxidant activity

All experimental rats were euthanized by cervical dislocation. The rat's Liver were removed immediately and washed in normal saline and homogenate 10% prepared in 1.15% w/v of potassium chloride. The homogenate was centrifuged in 7000 × g for 10 min at 4°C and supernatant were used for measurement of oxidative stress by determination of malondialdehyde (MDA) as well as antioxidant enzymes (AOE) such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX) and glutathione reductase. MDA, SOD, CAT and GSH-PX, GR were measured by using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Liver homogenate MDA levels were expressed as nmol MDA per mg protein and antioxidant activity was expressed as arbitrary units per mg protein.

Degree of lipid peroxidation in kidney tissue homogenates was determined in terms of thiobarbituric acid reactive substances (TBARSs) formation by following the protocol of Esterbauer and Cheesman (Esterbauer and Cheesman, 1990). SOD activity was measured by Nishikimi method (Nishikimi et al., 1972) and was modified by Kakkar method (Kakkar et al., 1984). Each unit of SOD activity was determined as required enzyme concentration for prohibition of creation color at 1 min, under study conditions. CAT activity was measured by Claiborne method (Claiborne, 1985) and was based on hydrogen peroxide breakdown. GPX activity was measured by Rotruck method (Rotruck et al., 1973) and was expressed as micromole of GSSG /minute/milligram of protein, based on blew reaction:

$$2H_2O+GSSG \longrightarrow H_2O_2+2GSH$$

GR activity was measured by Mohandas method (Mohandas et al., 1984), based on blew reaction:

$$NADPH+H^+ +GSSG \longrightarrow NADP^+ +2GSH$$

Microscopic studies

A small piece of hepatic tissue from the anterior portion of the left lateral lobe was removed for histological analysis. The sample was fixed by immersing it in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5 µm sections, and stained with hematoxylin-eosin for blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, hepatocellular necrosis, and inflammatory cell infiltration were evaluated semiquantitatively according to the method reported by Frei et al. (1984). The stained 5 µm sections were graded as follows: 0, absent; I, minimal; II, mild; III, modest; and IV, severe. The histological changes were evaluated in nonconsecutive, randomly chosen × 200 histological fields using light microscope, NIKON ECLIPSE E200 (Xiangchun et al., 2009).

Statistical analysis

The statistical package for social sciences (SPSS Inc., Chicago, IL, USA), version 13.0, was used for statistical analysis. All data are presented as mean ± SEM. Before statistical analysis, all variables were checked for normality and homogeneity of variance by using the Kolmogorov-Smirnoff and Levene tests, respectively. The data obtained were tested by ANOVA followed by Tukey's post-hoc multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

In group 3, alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase serum levels and total serum bilirubin increased significantly ($p<0.001$) and TP and serum albumin decreased significantly compared to group 1 ($p<0.001$ and $p<0.032$, respectively). In group 4, increase in total serum bilirubin and decrease in TP and serum albumin were prevented by TREE so that there was no significant difference between this group and the normal control group. In group 2, TREE did not make a significant difference on liver injury indexes (Table 1).

In group 3, GSH, SOD, CAT, GPX and GR significantly decreased ($p<0.001$) while malondialdehyde values significantly increased ($p<0.001$) compared to group 1. In group 4, increase in malondialdehyde and decrease in GSH, SOD, CAT, GPX and GR are prevented by TREE so that there was not any significant difference among this group and normal control. In group 2, TREE did not make a significant difference on lipid peroxidation of hepatocytes and antioxidant enzymes activity (Table 2). Also, in this group, 8 weeks treatment by TREE significantly decreased blood glucose levels ($p = 0.01$). But in group 4, eight weeks treatment by TREE significantly decreased blood glucose to near normal levels ($p = 0.03$) (Table 1).

Pathologically, liver histological structure was normal and healthy in normal control group (Figure 1A). In group 2 also there were no pathological changes so that hepatic lobular structure seemed quite normal (Figure 1B). In group 3, hepatocytes necrosis concomitant with inflammatory cells infiltration around the central vein and severe inflammation in portal area and also scattered necrotic foci in different portions of hepatic lobules were seen (Figure 2). Finally, in group 4, TREE prevented the pathological changes and only partial hyperemia and degenerative changes were observed in centrilobular portions (Figure 3). Quantitative microscopic results of experimental rats are presented in Table 3.

DISCUSSION

In this study, TREE at the dose rate of 200 mg/kg for 8 weeks reduced blood glucose in normal and alloxanized diabetic rats. Hypoglycemic effects of TREE are compatible with studies undertaken by Jorge et al. (2004),

Table 1. Effect of TREE on serum biochemical parameters and blood glucose in hepatic injuries of diabetic rats.

Groups	Treatment	Biochemical parameter							
		Alanine aminotransferase (U/L)	Aspartat aminotransferase (U/L)	Lactate dehydrogenase (U/L)	Total serum bilirubin (mg/dL)	Albumin (g/dL)	Serum total protein (g/dL)	Glucose	
								Day 0	End of the study
1	Normal control	75.18±1.42[a]	158.17±5.20[a]	681.17±20.52[a]	0.706±0.035[a]	4.60±0.45[a]	7.78±0.64[a]	88.7±4.2	86.8±3.7
2	Normal treated with TREE	77.40±2.20[a]	152.13±4.65[a]	691.11±23.34[a]	0.756±0.067[a]	4.53±0.36[a]	6.76±0.49[a]	86.5±3.4	61.3±2.3*
3	Diabetic control	175.32±3.1[b]	231.39±6.92[b]	1110.72±29.35[b]	1.366±0.070[b]	2.83±0.33[b]	4.32±0.56[b]	156.7±5.4*	154.2±6.1*
4	Diabetic treated with TREE	77.04±1.35[a]	166.95±4.13[a]	710.38±21.51[a]	0.739±0.046[a]	4.57±0.41[a]	6.52±0.61[a]	154.3±4.8*	90.1±3.5
ANOVA		P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P<0.05	P<0.05

Values are presented as mean ± SEM for 20 rats in each group. a,b: Different superscripts indicate significant differences (p<0.05). *Significantly different from the control group (p<0.05).

Table 2. Effect of TREE on antioxidative activity in hepatic injuries of diabetic rats.

Groups	Treatment	Biochemical parameter							
		Alanine aminotransferase (U/L)	Aspartat aminotransferase (U/L)	Lactate dehydrogenase (U/L)	Total serum bilirubin (mg/dL)	Albumin (g/dL)	Serum total protein (g/dL)	Glucose	
								Day 0	End of the study
1	Normal control	75.18±1.42[a]	158.17±5.20[a]	681.17±20.52[a]	0.706±0.035[a]	4.60±0.45[a]	7.78±0.64[a]	88.7±4.2	86.8±3.7
2	Normal treated with TREE	77.40±2.20[a]	152.13±4.65[a]	691.11±23.34[a]	0.756±0.067[a]	4.53±0.36[a]	6.76±0.49[a]	86.5±3.4	61.3±2.3*
3	Diabetic control	175.32±3.1[b]	231.39±6.92[b]	1110.72±29.35[b]	1.366±0.070[b]	2.83±0.33[b]	4.32±0.56[b]	156.7±5.4*	154.2±6.1*
4	Diabetic treated with TREE	77.04±1.35[a]	166.95±4.13[a]	710.38±21.51[a]	0.739±0.046[a]	4.57±0.41[a]	6.52±0.61[a]	154.3±4.8*	90.1±3.5
ANOVA		P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P<0.05	P<0.05

Values are mentioned as mean ± SEM for 20 rats in each group. a, b: Different superscripts indicate significant differences (p<0.05).

Vessal et al. (2003) and Jung et al. (2008).

In the current study, results of biochemical and histopathological assessments, showed liver injuries in rats with alloxan induced diabetes. Significant increase in serum levels of ALT, AST, LDH and bilirubin and significant decrease in TP and serum albumin in alloxanized diabetic rats were observed in comparison with normal control rats. Also, oral administration of TREE (200 mg/kg in 10 ml/kg normal saline) for 8 consecutive weeks conversed liver function biomarkers to normalcy. Serum levels of ALT, AST and LDH are widely used to evaluate liver function. Hepatocellular necrosis causes release of these enzymes into circulation. Increase in serum levels of AST shows hepatic injuries similar to viral hepatitis, infarction and muscular damages. ALT, which mediates conversion of alanine to pyruvate and glutamate, is specific for liver and is a suitable indicator of hepatic injuries. Increased levels of the aforementioned enzymes are an indicator of cellular infiltration and functional disturbance of liver cell membranes (Drotman et al., 1978). On the other hand, bilirubin, albumin and TP serum values are associated with the function of hepatic cells (Muriel et al., 1992).

Return of the previous enzymes to normal serum values following TREE administration, may be due to prevention of intracellular enzyme leakage resulting from cell membrane stability or cellular regeneration (Thabrew and Joice, 1987). Effective control of bilirubin and TP shows early improvement of functional and secretory mechanism of hepatic cells. In this study, widely degenerative changes and central lobular necrosis in diabetic rats were observed. With oral

Table 3. Effect of TREE on hepatic injuries of diabetic rats.

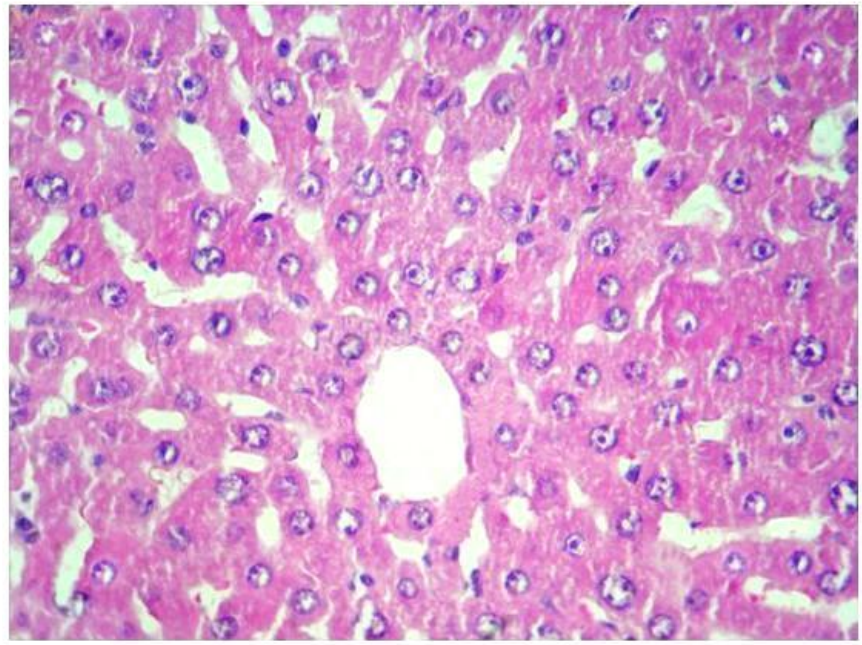
Grades	Groups			
	1	2	3	4
Hyperemia and inflammation in the portal area				
0	20	19	0	12
1	0	1	0	7
2	0	0	0	1
3	0	0	15	0
4	0	0	5	0
Necrosis				
0	20	20	0	15
1	0	0	0	3
2	0	0	3	2
3	0	0	15	0
4	0	0	2	0
Interstitial inflammatory cells infiltration				
0	20	20	0	16
1	0	0	0	3
2	0	0	4	1
3	0	0	14	0
4	0	0	2	0

0 = without injury, 1 = minimum injury, 2 = mild injury, 3 = moderate injury, 4 = sever injury
20 samples were rendered in each group.

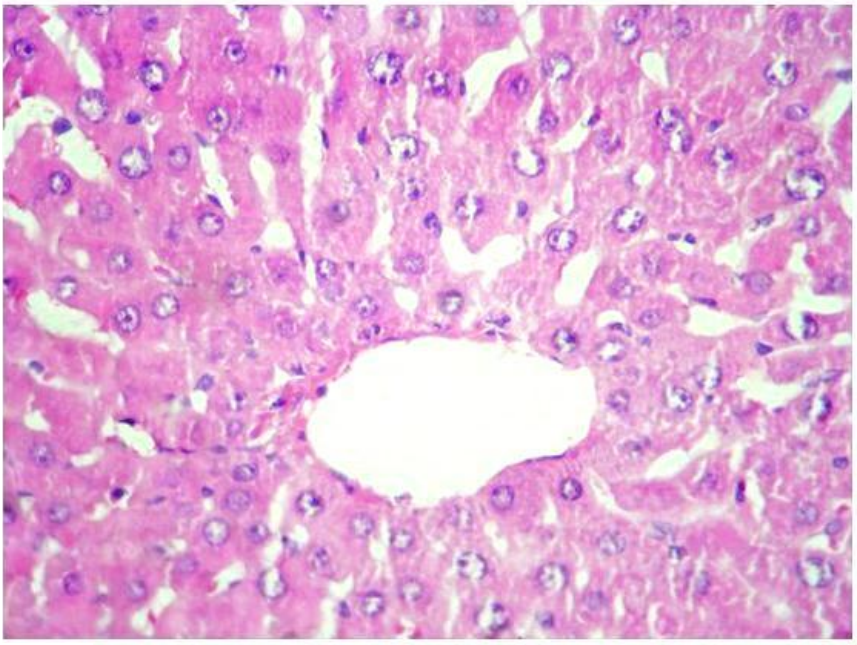
administration of TREE in diabetic rats only mild degenerative changes were observed and there was no necrotic changes indicating the protective effect of TREE against hepatic complications of diabetes. However, pathologic findings are matched with biochemical results. In current study, it seems that free radicals cause membranous lipid peroxidation that leads to production of lipid peroxides (malondialdehyde), loss of cell membrane stability and finally liver injuries. Enhancement of malondialdehyde in diabetic rats is an indicator of peroxidative reactions that causes depression of antioxidants defensive mechanisms. Hence, prevention of free radicals production cannot be sustained (Naik, 2003). In other words, increased amounts of malondialdehyde in liver is an indicator of lipid peroxidation that yields to hepatic injury and antioxidant defensive mechanism disability in prohibition of excessive free radicals production. SOD, CAT and GPX are antioxidant enzymes which establish a defensive system against ROS (Lil et al., 1988).

Reduction in SOD activity is an important index for hepatic injuries. SOD eliminates superoxide anions by converting them to hydrogen peroxide and hence reduces their toxic effects (Curtis et al., 1972). In current study, SOD activity in diabetic rats is significantly decreased because of continual production of superoxide anions. Also, CAT and GPX significantly decreased in these animals. It seems that inactivation of SOD by increased superoxide anions leads to inactivation of CAT and GPX. In this study, oral administration of TREE prevented reduction of SOD, CAT and GPX. This may be due to the active substances of TREE which results in maintenance of these enzymes. CAT is one of the antioxidant enzymes which is diffused widely in animal tissues and has high activity in liver and RBCs. CAT protects tissues from extreme toxic hydroxyl through decomposing of hydrogen peroxide (Chance et al., 1952). GR is one of the cytosolic enzymes which are involved in GSSG reduction, as end product of GPX activity on GSH (Naik and Panda, 2008). Use of TREE in diabetic rats reestablished GR activity that involves GSSG usage to make GSH and enhances detoxification of active metabolites by its conjugation with GSH. With attention to the mentioned reactions, it is revealed that TREE exerts its protective effects on diabetic rats liver injuries by compensation of antioxidant defensive system activity and scavenging of free radicals.

The current study approves other reports about TREE antioxidant and free radicals removing effects. Franciscoa et al. (2009) showed that TREE is full of phenolic antioxidants particularly flavonols and hydroxycinnamic acid. These compounds have strong and direct antioxidant effects and induce expression of different genes involved in metabolic enzymes encoding



A



B

Figure 1. A. Microscopic view from one of the normal control rat's liver. It has normal structure (H&E, /400). B. Microscopic appearance from one of the group 2 rats' liver. Hepatic structure seems normal and has no pathological changes (H&E, /400).

which are effective in reduction of incidence of diseases risks (Bennett et al., 2006). In the study undertaken by Kim et al. (2006), it has been shown that TREE has protective effect against cisplatin-induced nephrotoxicity by reducing oxidative stress. The mechanisms of TREE's role in the study of Kim et al. (2006) are similar with our study. In the study conducted by Rafatullah et al. (2006) it has been shown that TREE prohibits hepatocyte injuries induced by carbon tetrachloride. According to their findings, TREE's protective effect is in association with its antioxidant activities. Choi et al. (2006) showed hepatoprotective and antioxidant effects of TREE both

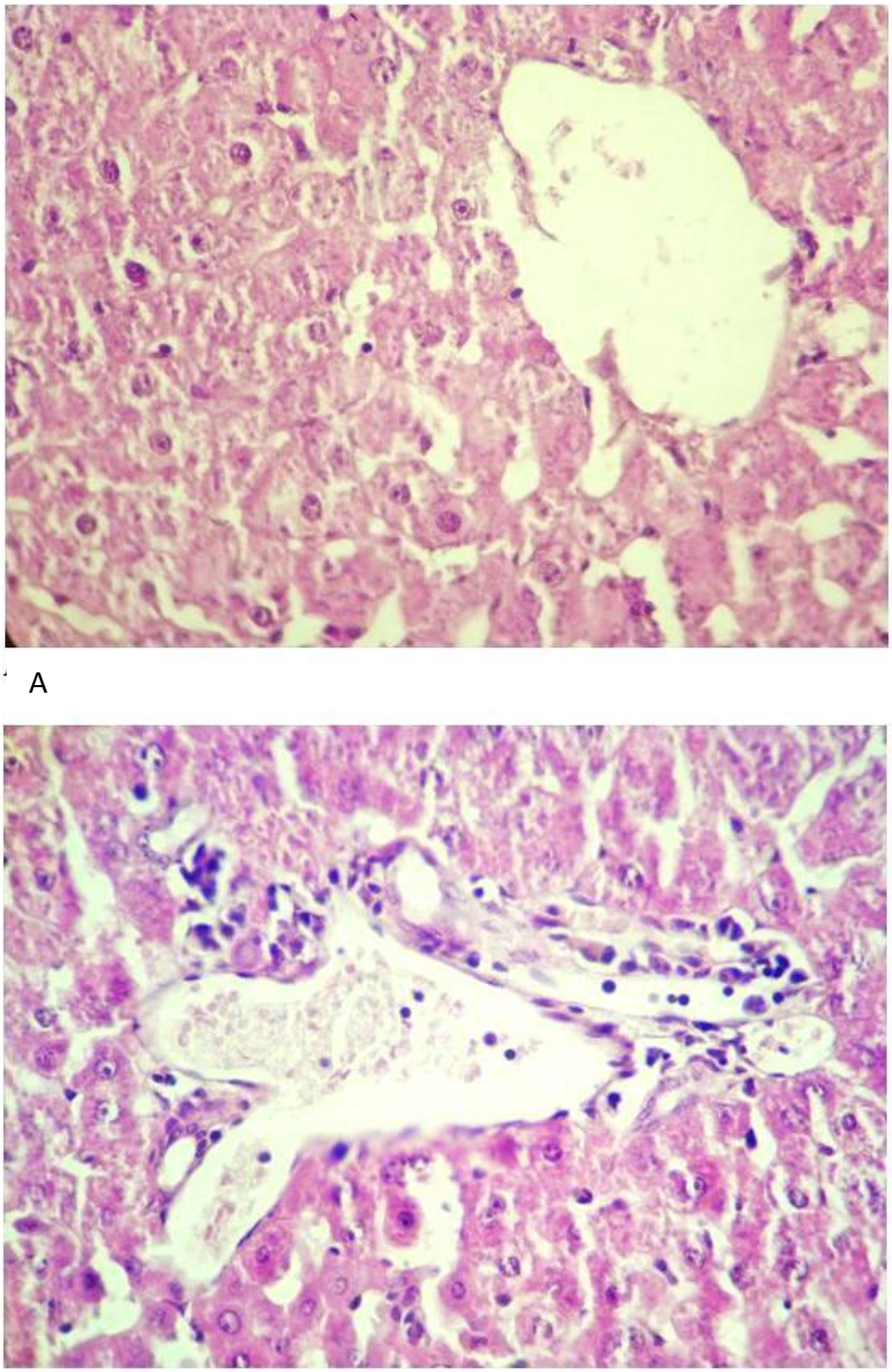


Figure 2. A. Photomicrograph from one of the group 3 rats' liver. Centrilobular hepatic cell necrosis and destruction of central vein wall is seen (H&E, /400). B. Another representative section from one of the group 3 rats' liver. Inflammatory cells infiltration in portal area and hepatocytes necrosis in periportal region is prominent (H&E, /400).

in vivo and *in vitro*. In their study, oral administration of Galactosamine in rats.

The present study indicated pharmacologic effects of TREE in liver complications of diabetes. Therefore, it seems that TREE has positive effects on prevention of TREE improved liver damage induced by D- hepatic

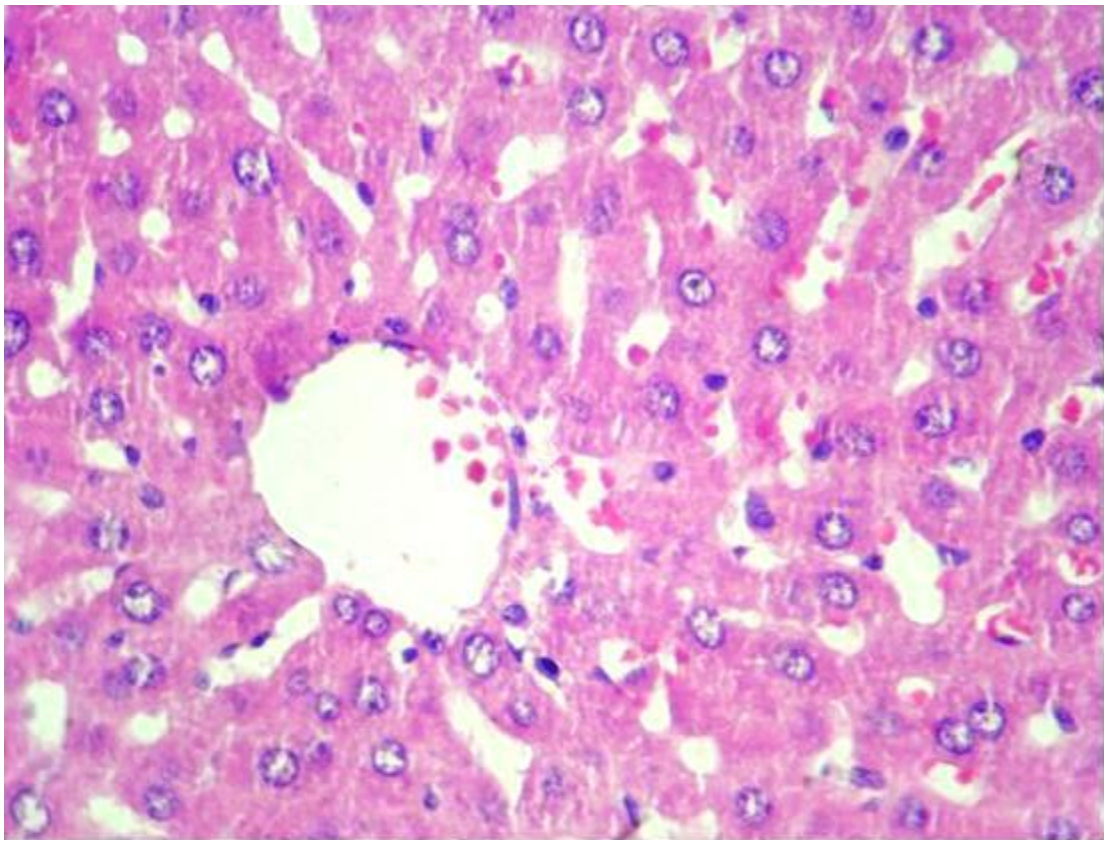


Figure 3. Microscopic view from one of the group 4 rat's liver. Mild central vein and sinusoidal hyperemia is obvious.

injuries due to oxidative stress of diabetes.

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