African Journal of Pharmacy and Pharmacology Vol. 5(19), pp. 2166-2173, 22 November, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.624
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Attenuation of oxidative stress of hepatic tissue by ethanolic extract of saffron (dried stigmas of *Crocus sativus* L.) in streptozotocin (STZ)-induced diabetic rats

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Accepted 2 November, 2011

Oxidative stress is an underlying mechanism of diabetes mellitus complications. The aim of this study was to evaluate the effect of saffron ethanolic extract on oxidative stress of hepatic tissue in streptozotocin (STZ)-induced diabetic rats. Malondialdehyde (MDA) and reduced glutathione (GSH) contents were measured to assess free radical activity in the liver tissue. Hepatic antioxidant activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) were also determined. Wistar rats were made diabetic through single injection of STZ (75 mg/kg i.p). The rats were randomly divided into four groups of 10 animals each: Group 1, healthy control; Group 2, non-diabetics treated with 40 mg/kg intraperitoneal injection of saffron body weight (b.w.)/day extract; Group 3, diabetics; Group 4, diabetics treated with saffron extract (40 mg/kg b.w./day, i.p.) for 8 weeks. Liver MDA content in Groups 3 significantly increased as compared to Group 1 ($P < 0.05$) and significantly decreased in Group 4 as compared to Group 3 ($P < 0.05$). The GSH, SOD, CAT and GSH-Px contents of the liver in Group 3 significantly decreased as compared to Groups 1 ($P < 0.05$) and significantly increased in Group 4 as compared to Group 3 ($P < 0.05$). This study showed that saffron has antioxidant properties and alleviates oxidative stress of hepatic tissue in experimentally induced diabetes.

Key words: *Crocus* (Saffron), oxidative stress, diabetes mellitus, liver, rats.

INTRODUCTION

Diabetes mellitus is a serious metabolic disorder which is a major source of ill health all over the world and its incidence is expected to increase by 5.4% in 2025 (Kim et al., 2006). Diabetes mellitus is characterized by hyperglycemia and is associated with disturbances in carbohydrate, protein and fat metabolism which occurs secondary to an absolute (type I) or relative (type II) lack of insulin (Alberti and Zimmet, 1998). Oxidative stress occurs when the balance between oxidant and antioxidant systems shifts in favor of the former leading to the generation of free oxygen radicals. Reactive oxygen species are involved in the pathogenesis of many diseases including hypoxia, hypercholesterolemia, atherosclerosis, hypertension, ischemia reperfusion injury and heart failure (Taniyama and Griendling, 2003; Wilcox and Gutterman, 2005). It has been shown that patients with diabetes mellitus have increased oxidative stress and impaired antioxidant defense systems, which appear to contribute to the initiation and progression of diabetes-associated complications (Maritim et al., 2003). There is convincing experimental and clinical evidence that the generation of reactive oxygen species increased in both types of diabetes. Normally, the level of oxidative stress is modulated by antioxidant defense systems (Saxena et al., 1993). Diabetes-linked alterations in antioxidant defense system enzymes, such as catalase, glutathione peroxidase and superoxide dismutase have been demonstrated (Maritim et al., 2003). The negative impact

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of diabetes on the retinal, renal, nervous and cardiovascular systems is well recognized, yet little is known about its effect on the liver (Lipscombe and Hux, 2007; Orasanu and Plutzky, 2009). However, Lipid peroxidation and antioxidant status of hepatic tissue were studied in experimental diabetes (Feillet-Coudray et al., 1999). Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. More attention has been paid to the protective effects of natural antioxidants against chemically induced toxicities (Frei and Higdon, 2003). Herbal formulations with a simultaneous antioxidant effect would thus be more useful in the management of diabetes mellitus. Their use alone or in combination with oral hypoglycemic agents or insulin may help in the better control of blood glucose level in diabetic subjects. Kazi et al. (2009) proved the beneficial effect of freshly prepared aqueous extracts of *Psidium guajava*, *Momordica charantia* and *Coccinia* indica leaves and their combination in STZ-induced diabetes rats. Similarly, antidiabetic activity of hydro-ethanolic extracts of *Nymphaea stellata* flowers has been documented in alloxan-induced diabetic rats (Rajagopal and Sasikala, 2008). Natural antioxidants strengthen the endogenous antioxidant defenses from reactive oxygen species (ROS) and restore and optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in disease prevention. In this context, saffron can rightly be mentioned as a plant of considerable interest.

Saffron (dried stigmas of *Crocus sativus* L.) is the world's most expensive spice, and genuine saffron is worth its weight in gold. This plant belongs to the Iridaceae family and is widely cultivated in Iran and other countries, such as India and Greece. As a therapeutic plant, saffron is considered an excellent aid for stomach ailments and an antispasmodic, it helps digestion and increases appetite. It has been reported that *C. sativus* has hypolipaemic, anti-inflammatory, antioxidant and anticancer effects. Moreover, according to Commission E, *C. sativus*, is applicable for the treatment of nervous disorders, spasms and asthma (Hosseinpour Chermahini et al., 2010; Abdullaev, 2002; Abe and Saito, 2000; Ríos et al., 1996). Aqueous saffron extract and its active constituent, crocin, are useful agents for the prevention of renal Ischemia-Reperfusion (IR)-induced oxidative injury in rats (Hosseinzadeh et al., 2005). Furthermore, saffron extract protects against oxidative damage in rat primary hepatocytes. It also suppresses aflatoxin B1-induced hepatotoxic lesions and has a modulatory effect on aflatoxin B1, cytotoxicity. It also has a protective effect on the bladder toxicity, induced by cyclophosphamide (Giaccio, 2004). Recently, it was reported that the saffron extract, crocin and safranal exhibited significant radical scavenging activity and thus antioxidant activity (Assimopoulou et al., 2005).

Considering the antioxidant effects of saffron, this study was designed to evaluate the antioxidant activity of saffron ethanolic extract in hepatic tissue of streptozotocin-induced diabetes in rats. To our knowledge, this is the first investigation on the effect of saffron extract on the antioxidant status of liver tissue in experimental diabetic rats. We reported the effect of saffron ethanolic extract on liver tissue oxidative parameters in rats with streptozotocin-induced diabetes.

MATERIALS AND METHODS

Chemicals

Streptozotocin was from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade. All chemicals used in this study were of analytical grade and were obtained from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

Plant

The saffron used in this study was dedicated by Novin Zaferan Co. (Mashhad, Iran) and was identified by the Department of Cultivation and Development of Institute of Medicinal Plants, Tehran, Iran.

Preparation of the extract

In the maceration method, 10 g of stigmas were macerated in 500 ml ethanol (80 v/v) for three days. The mixture was subsequently filtered and concentrated under reduced pressure at 40°C. The extract yield was 51% w/w.

Induction of diabetes mellitus

Diabetes was induced by intravenous injection of streptozotocin (Sigma, St. Louis, Mo, USA) into the tail vein at a dose of 65 mg/kg body weight. STZ was extemporaneously dissolved in 0.1 M cold sodium citrate buffer, pH 4.5. After 18 h, animals with fasting blood glucose levels greater than 120 to 250 mg/dl were considered diabetic and then included in this study (Gupta et al., 2005). Fasting blood glucose was estimated by using one touch glucometer (Accuchek sensor) of Roche Diagnostics, Germany.

Animal treatment

This experimental study was carried out in Islamic Azad University Research Center and all procedures and works on animals was conducted under Animal Rights Monitoring Committee of Islamic Azad University Research Center.

Forty healthy male Wistar rats (about 180 to 200 g body weight) were purchased from Animal House, Islamic Azad University. All animals were conditioned at room temperature at a natural photoperiod for 1 week before experiment execution. A commercial balanced diet and tap water *ad libitum* were provided. The duration of experiment was 8 weeks. The rats were randomly divided into 4 groups (10 rats each) as the following: Group 1, healthy control rats received isotonic saline solution (ISS, 10 ml/kg1) intraperitoneally; Group 2, non-diabetic rats were treated with 40 mg/kg b.w./day intraperitoneal (i.p.) injection of saffron extract; in Group 3, diabetic rats administered ISS (10 ml/kg1) were given through intraperitoneal route; Group 4, diabetic rats were treated with saffron extract (40 mg/kg b.w./day, i.p.) for a period of 8 weeks. The animals of different groups were sacrificed under light anesthesia

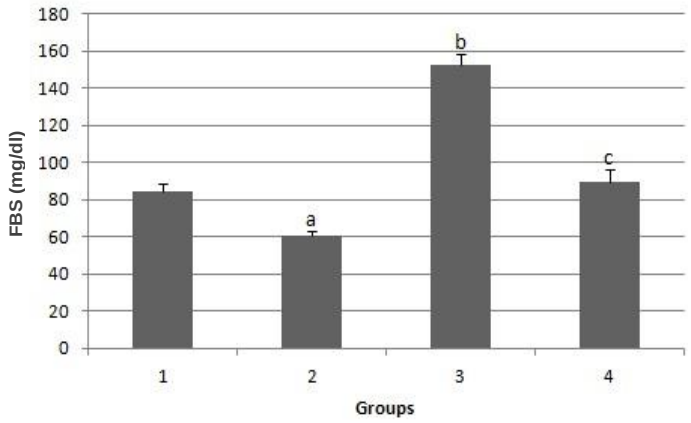

Figure 1. Comparison of the effect of saffron extract on blood glucose levels among the experimental groups (Mean ± SEM). *P < 0.05, [a,b]Compared to Group 1 and [c]compared to Group 3.

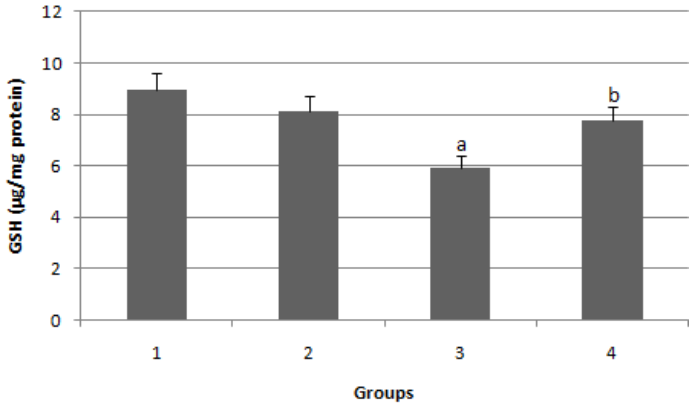

Figure 2. Comparison of the effect of saffron extract on liver GSH content among the experimental groups (Mean ± SEM). *P < 0.05, [a]compared to Group 1, [b]compared to Group 3.

(diethyl ether) 1 day after the end of the treatment.

Measurement of antioxidant activity

The rat's liver were removed immediately and were 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 estimation of reduced glutathione (GSH) and determination of malondialdehyde (MDA) as well as antioxidant enzymes (AOE) such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). GSH, MDA, SOD, CAT and GSH-Px were measured by using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Reduced glutathione (GSH) content was determined according to Sedlak and Lindsay (1968). GSH reacts with 5,5′-dithiobis-2-nitrobenzoic acid, and the absorbance spectra of the product have a maximum absorbance at 410 nm. The results were expressed as µmol/gwt. 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 liver tissue homogenates was determined in terms of thiobarbituric acid reactive substances (TBARSs) formation by following the protocol of Esterbauer and Cheesman (1990). SOD activity was measured by Nishikimi et al. (1972) method and was modified by Kakkar et al. (1984) method. 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 (1985) method and was based on hydrogen peroxide breakdown. GSH-Px activity was measured by Rotruck et al. (1973) method and was expressed as micromole of GSSG/minute/ milligram of protein, based on the following reaction:

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

Microscopic studies

The animals of different groups were sacrificed under light anesthesia (diethyl ether) 1 day after the end of the treatment. 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 was stained with hematoxylin-eosin for blinded histological assessment. The degree of liver tissue injury was evaluated semiquantitatively according to the method reported by Jamshidzadeh et al. (2008). The stained 5 µm sections were graded as follows: 0, absent; 1, minimal; 2, mild; 3, modest; 4, severe. The histological changes were evaluated in nonconsecutive, randomly chosen ×200 histological fields using light microscope, NIKON ECLIPSE E200.

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

Results of the effect, of daily treatment of saffron ethanolic extract with the dose of 40 mg/kg for 8 weeks on blood glucose levels of experimental rats are presented in Figure 1. The saffron extract produced significant hypoglycemic effect in normal (P < 0.05) and diabetic (P < 0.01) rats after 8 weeks of administration.

Figures 2 to 6 show the effects of saffron ethanolic extract on antioxidative activity of liver tissue in diabetic rats.

MDA contents of the liver tissue in groups 3 was found to significantly increased as compared to group 1 (P < 0.05) and liver MDA level in group 4 significantly decreased as compared to group 3 (P < 0.05).

The GSH, SOD, CAT and GSH-Px contents of the liver in group 3 significantly decreased as compared to groups 1 (P < 0.05) and GSH, SOD, CAT and GSH-Px activity

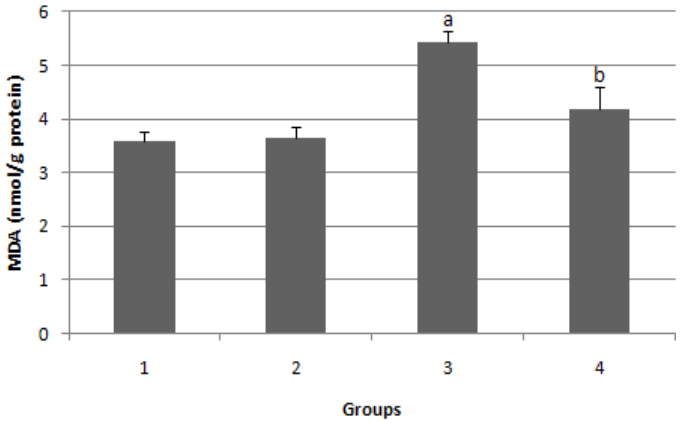

Figure 3. Comparison of the effect of saffron extract on liver MDA content among the experimental groups (Mean ± SEM). *P < 0.05, [a]compared to Group 1 and [b]compared to Group 3.

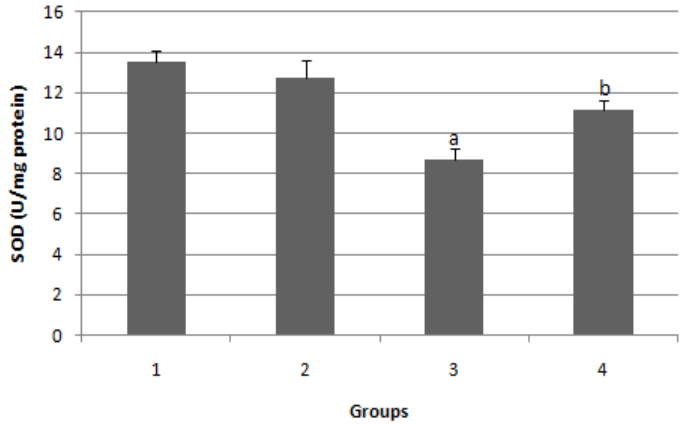

Figure 4. Comparison of the effect of saffron extract on liver SOD activity among the experimental groups (Mean ± SEM). *P < 0.05, [a]compared to Group 1 and [b]compared to Group 3.

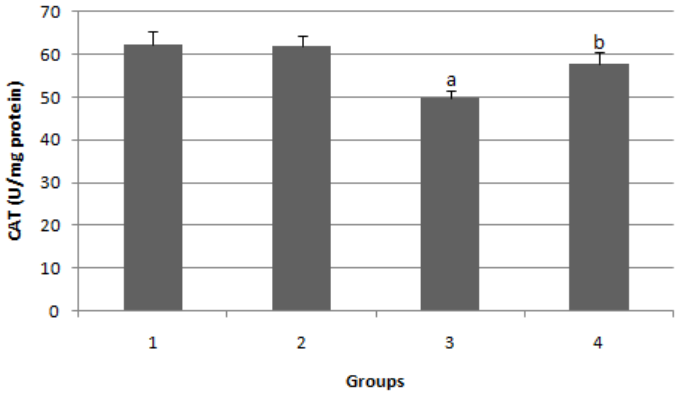

Figure 5. Comparison of the effect of saffron extract on liver CAT activity among the experimental groups (Mean ± SEM). *P < 0.05, [a]compared to Group 1 and [b]compared to Group 3.

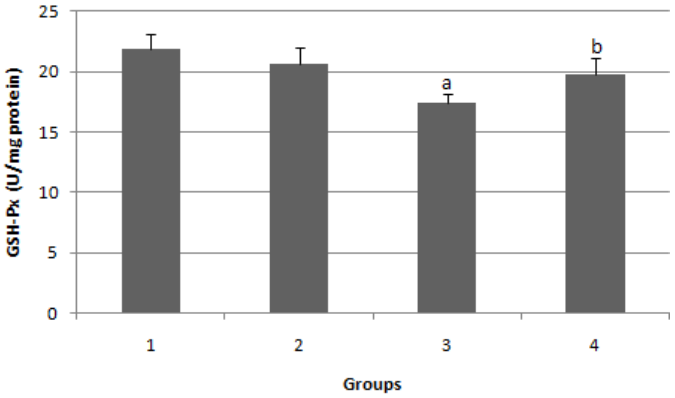

Figure 6. Comparison of the effect of saffron extract on liver GSH-Px activity among the experimental groups (Mean ± SEM). *P < 0.05, [a]compared to Group 1 and [b]compared to Group 3.

increased in Group 4 as compared to group 3 (P < 0.05).

Pathologically, liver histological structure was normal in healthy control group (Figure 7A). In Group 2 also, there were no pathological changes so that hepatic lobular structure seemed quite normal (Figure 7B). In Group 3, diabetic rats showed fatty changes in centrilobular portions of the livers (Figure 7C). Finally, in Group 4, saffron extract prevented the pathologic changes and no considerable fatty change was observed (Figure 7D). Quantitative microscopic results of experimental rats are presented in Table 1.

DISCUSSION

In the current study, intraperitoneal injection of ethanolic extract of *C. sativus* L. (saffron) stigma significantly produced reduction (28.18%) of blood glucose level in healthy normal rats. In addition, saffron ethanolic extract caused significant hypoglycemic effect (40.86% reduction in blood glucose level) in diabetic rats. Such a phenomenon of hypoglycemic response with saffron ethanolic extracts has been reported (Mohajeri et al., 2008, 2009).

In the present study, significant (34.18%) decline in GSH level and antioxidant enzymes activity including SOD (36.08%), CAT (19.83%) and GSH-Px (20.00%) as well as increased lipid peroxidation (50.97%) in the liver tissue of rats reflects oxidative stress of the liver in experimental diabetes. These results are in line with the findings reported by Feillet-Coudray et al. (1999), who observed that STZ-induced diabetes in rat accompanied with an increase in the susceptibility to lipid peroxidation. In the current study, results of histopathological assessments, reflects liver injuries in rats with streptozotocin-induced diabetes. The data of our study also revealed that daily treatment of saffron extract markedly improves antioxidant status of liver tissue of rats with streptozotocin-

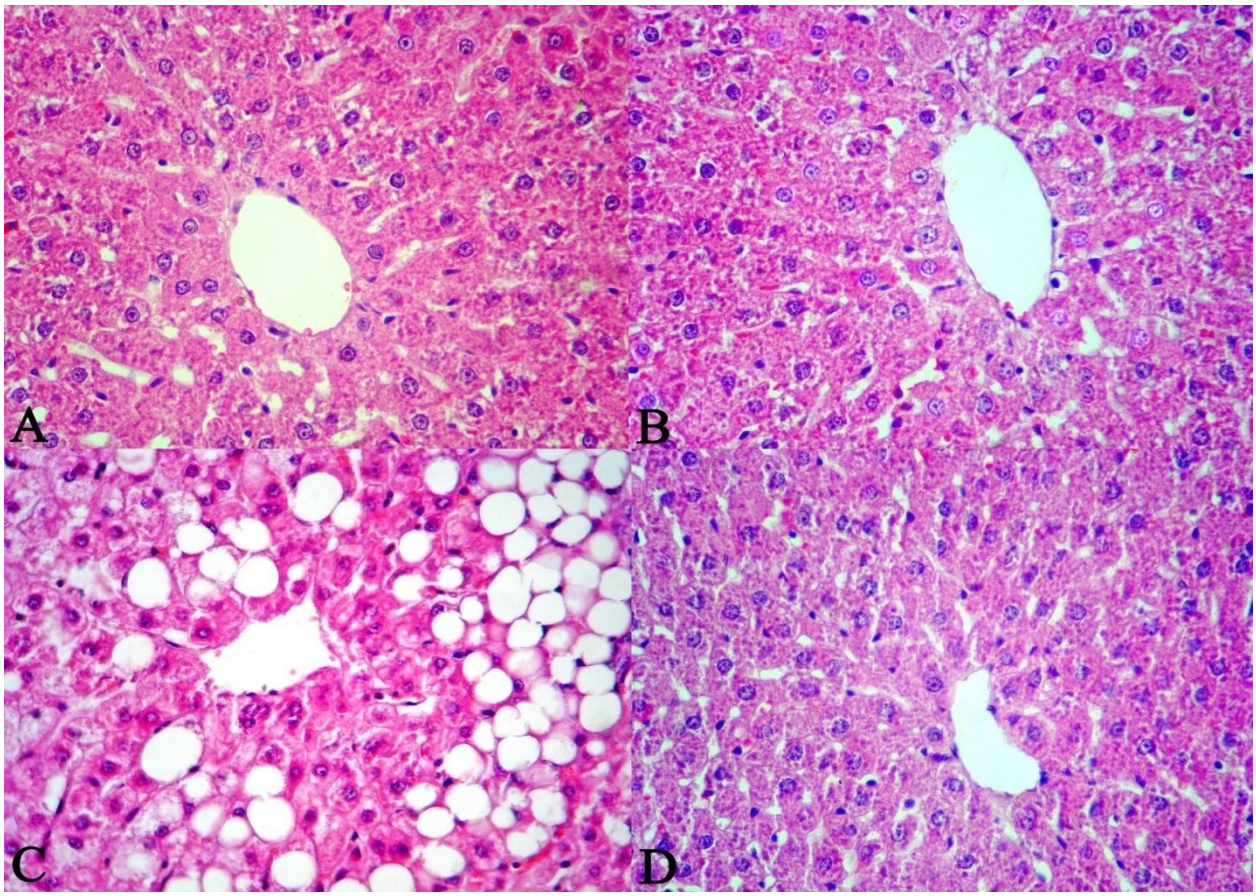

Figure 7. Microscopic appearance from liver tissues of the experimental rats (H and E, 100×). A- Healthy control rat liver showing normal hepatocytes. B- Non-diabetic + saffron extract (40 mg/kg b.w./day, i.p.) treated rat liver shows normal appearance. C- Diabetic rat liver showing macrovesicular fatty change in centrilobular portions. D- Diabetic + saffron extract (40 mg/kg b.w./day, i.p.) treated rat liver showing no fatty change.

Table 1. Effect of saffron extract (40 mg/kg b.w./day, i.p.) on hepatic injuries of diabetic rats (mean ± SEM).

Group	Degree of liver tissue injury	The Kruskal-Wallis test
1 (healthy control rats)	0.0 ± 0.0	P < 0.001
2 (non-diabetic rats + saffron extract)	0.0 ± 0.0	
3 (diabetic rats)	2.38 ± 0.25[a]	
4 (diabetic rats + saffron extract)	0.45 ± 0.12[b]	

0 = without injury, 1 = minimum injury, 2 = mild injury, 3 = moderate injury, 4 = sever injury (n = 10).
[a]Compared to Group 1, [b] Compared to Group 3.

induced diabetes as GSH level and antioxidant enzymes activities comprising SOD, CAT and GSH-Px significantly (31.57, 28.75, 16.23 and 13.05%, respectively) increased and MDA level markedly (22.87%) decreased. Our histopathological examinations also revealed that daily treatment of saffron extract markedly improves histopathological status of rats with streptozotocin-induced diabetes.

GSH (an important part of the non-enzymatic antioxidant system) is a major non-protein thiol in living organisms, which plays a central role in coordinating the body's antioxidant defense processes. Perturbation of GSH status of a biological system can lead to serious consequences. Elevation in MDA level and reduction in GSH stores of liver tissue of diabetic rats suggest that oxidative stress due to free-radical damage is one of the possible mechanisms in the pathophysiology of diabetic hepatopathy. On administration of saffron extract, the MDA

levels have decreased and the GSH levels have increased. This indicates that in the presence of saffron extract there is an improvement in the oxidative stress. Increased oxidative stress in the tissues of streptozotocin diabetic rats was similarly reported. This was said to be a contributory factor in the development of the complications of diabetes (Kakkar et al., 1995; Curcio et al., 1995).

Free radicals are the chemically most reactive substances in the human or animal organism (Halliwell and Gutteridge, 2002; Fridovich, 1995). The unbalance between formation and detoxification of free radical species results in the progression of oxidative stress (Sies, 1991). Oxidative stress causes serious damage in biomolecules, such as proteins (Goldstein et al., 1994; Berlett and Stadtman, 1997; Hazen et al., 1997; Leeuwenburgh et al., 1999; Stadtman, 1990), lipids (Nacítarhan et al., 1995; Ziouzenkova et al., 1998) and nucleic acids (Murata et al., 1998; Marnett, 2000), and leads to the development of a wide spectrum of serious diseases (Halliwell, 1992), e.g. some types of neoplasia (Toyokumi, 1996; Mitchell et al., 2003), inflammation processes (Pavlick et al, 2002), ischaemic and reperfusion states (Ghoneim et al, 2002), acute pancreatitis (Uruñuela et al., 2002), atherosclerosis (Noguchi, 2002) or diabetes mellitus (Vozár, 1998; Pekiner et al., 2002) and diabetic complications (Halliwell and Gutteridge, 1989). The findings of Kakkar et al. (1998) study suggest that oxidative stress starts at early onset of diabetes mellitus and increases progressively. Therefore, the structural damage to hepatic tissue or other complications of diabetes mellitus may be due to oxidative stress.

SOD, CAT and GPx constitute a mutually supportive team of defense against ROS. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O_2^-. In hyperglycaemia, glucose undergoes autooxidation and produces superoxide, and it produces free radicals that in turn lead to lipid peroxidation in lipoproteins. CAT is localized in the peroxisomes or the microperoxisomes, which catalyses the decomposition of H_2O_2 to water and oxygen, and thus protects the cell from oxidative damage produced by H_2O_2. GPx catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. In our study, decline in the activities of these enzymes in liver tissue of streptozotocin-induced animals and attainment of near normalcy in saffron extract treated rats indicate oxidative stress elicited in hepatic tissue of diabetic rats had been nullified due to the effect of the extract. This observation perfectly agrees with those of Krishnakumar et al. (1999) who demonstrated hypoglycaemic and antioxidant activity of *Salacia oblonga* extract in streptozotocin induced diabetic rats. Meanwhile, Gohil et al. (2010) showed hypoglycaemic and hypolipidemic effects of *Eugenia jambolana* seed and *Aegle marmelos* leaf extracts in alloxan- induced diabetic rats (Gohil et al., 2010).

A multitude of herbs, spices and other plant materials as useful source of natural antioxidants have been described for the treatment of diabetic complications throughout the world. In this manner, Saradha Devi et al. (2011), declared that *Cynodon dactylon* has very good antioxidant and hepatic protective effect of normal oxidative stress in Balb/c mice. Similar results were obtained by earlier researchers Veena et al. (2002) in dry stem crude extraction of *Tinospora cordifolia*, and polyphenols extracts in tea (Frei and Higdon, 2003).

Present study showed pharmacologic effect of saffron (*C. sativus* Linn.) extract in liver complications of diabetes. Therefore, it seems that saffron extract has positive effects in prevention of early hepatic injury in diabetes mellitus due to oxidative stress, and can be recommended in diabetic humans as herbal drug after randomized clinical trials. Taken in all, the use of this plant in diabetes is then supported, but the precise active substance(s) of saffron, site(s) and cellular and molecular mechanism(s) of its pharmacological effect are still to be determined. In addition, the possible long-term toxic effects of ethanolic saffron extract and protective effects of different doses of that also remain to be clarified.

Conclusion

This study demonstrated that *C. sativus* extract reduced the blood glucose and attenuated oxidative stress of hepatic tissue in streptozotocin-induced diabetic rats. Many question related to antihyperglycemic and antioxidant effect of *C. sativus* extract remain unanswered. Much more work is clearly needed before phytotherapy for diabetic hepatopathy can be advanced to clinic.

ACKNOWLEDGEMENT

This work was supported by a Research Fund of the Ahar Branch, Islamic Azad University.

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