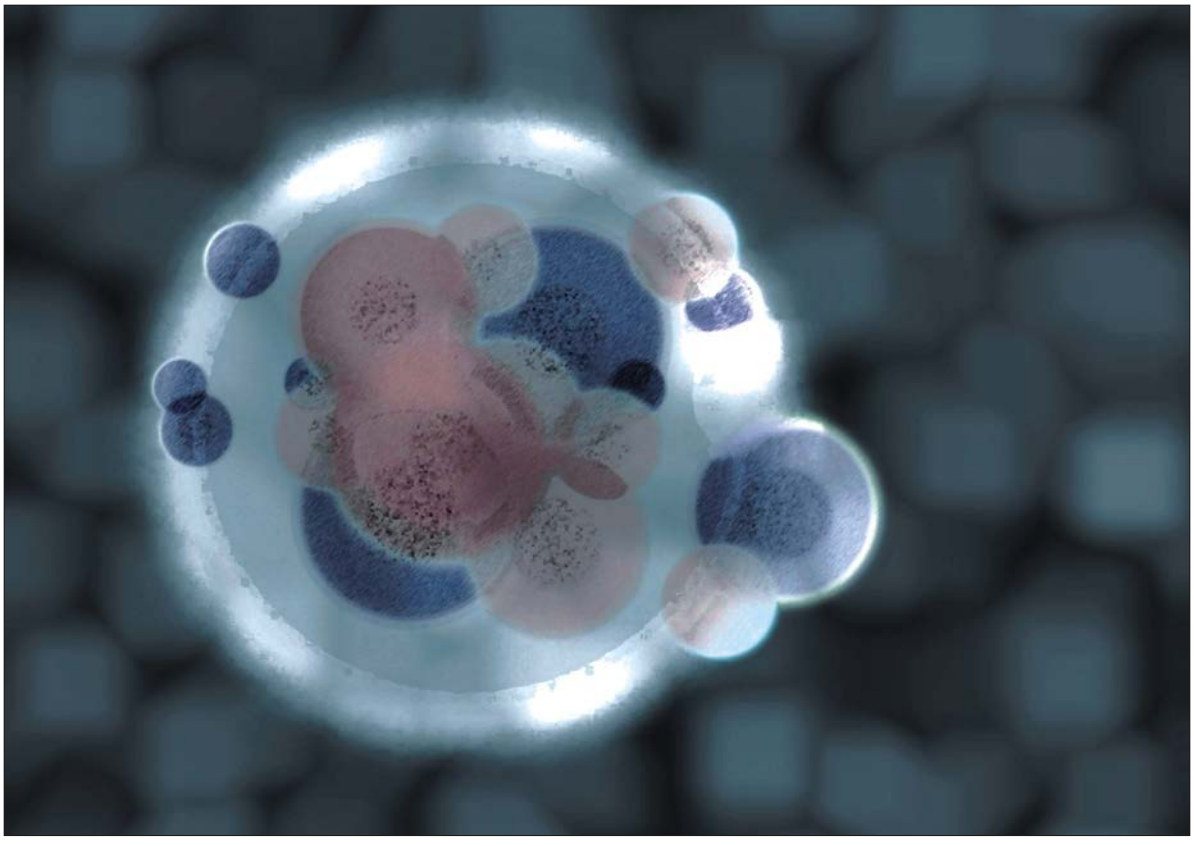

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Antihyperglycemic and Pancreas-Protective Effects of *Crocus sativus* L. (Saffron) Stigma Ethanolic Extract on Rats with Alloxan-Induced Diabetes

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Abstract: Adequate characterization of hypoglycemic effect of ethanolic saffron extract has not been yet done, though the activity has been reported. The scientific evaluation of its hypoglycemic activity was, therefore, explored and compared with the effect of a standard hypoglycemic drug, tolbutamide. In this study, we also report on alteration in patterns of pancreatic islet cells using histopathology and immunohistochemistry of alloxanized diabetic rats treated with ethanolic saffron extract. The ethanolic extract of *Crocus sativus* L. stigma was administered orally and intraperitoneally at different doses (20, 40 and 80 mg kg^{-1}) to normal rats for finding the more effective hypoglycemic dose and administration route. Acute hypoglycemic effects produced by more effective dose of ethanolic saffron extract on the Fasting Blood Glucose (FBG) levels and effects of the same dose of ethanolic saffron extract on the FBG and plasma insulin levels in alloxanized Mild Diabetic (MD) and Severely Diabetic (SD) rats were assayed. Histopathological and immunohistochemical studies were also carried out on pancreatic islet cells of control and diabetic rats. The dose of 40 mg kg^{-1} was found to be more effective dose in intraperitoneally (i.p.) route for decreasing Blood Glucose Level (BGL). The extract administered by i.p. route at more effective dose showed an acute hypoglycemic effect in MD and SD rats. Treatment of MD and SD rats for 14 days with the more effective dose significantly reduced the FBG levels in these animals (41.4% MD, 30.7% SD). Serum insulin level showed significant increase in diabetic rats (33.3% MD, 27.3% SD) after 14 days. The histopathological studies of pancreas in ethanolic extract treated diabetic groups showed a reversed damage caused by alloxan to the pancreatic islets as almost normal appearance. In addition, diabetic (MD and SD) rats showed obvious decreases in insulin immunoreactivity and the number of β-cells in pancreas, but the pancreas of extract-treated diabetic rats was improved and the number of immunoreactive β-cells was significantly increased. The control group given saffron extract was not different from the other intact control group considering the insulin immunoreactivity in β-cells. The findings of present study indicate the hypoglycemic and potential antihyperglycemic nature of the extract, helping in regeneration of damaged pancreas in experimental diabetes. Thus, after randomized clinical trials, saffron extract may be implicated as a preventive or therapeutic agent against diabetes mellitus.

Key words: *Crocus sativus* L. stigmas, alloxan, diabetic rats, pancreas

INTRODUCTION

Diabetes mellitus is a serious, complex chronic condition, which is a major source of ill health all over the world (Kim *et al*., 2006). There are approximately 143 million people in the world with diabetes and this number will probably double by the year 2030 (Boyle *et al*., 2001). Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by elevation of both fasting and post-paradinal blood sugar levels. The synthetic oral hypoglycemic agents can produce serious side effects (Akhtar and Iqbal, 1991; Holman and Turner, 1991). In addition, they are not considered safe for use during pregnancy (Larner, 1985). Furthermore, after the recommendation made by WHO on diabetes mellitus investigation on hypoglycemic agents from medicinal plants have become more important (Alarcon-Aguilara *et al*., 1998). Plants have played a major role in the introduction of new therapeutic agents. A medicinal plant, *Galega officinalis*, led to the discovery and synthesis of metformin (Luo *et al*., 1998) but it is still an extensive demand for new oral antidiabetic drugs without

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side effect in human. A multitude of herbs, spices and other plant materials have been described for the treatment of diabetes throughout the world (Gupta *et al*., 2005; Ivorra *et al*., 1989; Marles and Farnsworth, 1995). The medicinal plants might provide a useful source of new oral hypoglycemic compounds for development of pharmaceutical entities or as a dietary adjunct to existing therapies (Bailey and Day, 1989). Few of the plants used for the treatment of diabetes have received scientific or medical scrutiny and even the WHO expert committee on diabetes recommends that this area warrant further attention (Alarcon-Aguilara *et al*., 1998). Despite the presence of known antidiabetic medicines in the pharmaceutical market, screening for new antidiabetic sources from natural plants is still attractive because they contain substances that have an alternative and safe effect on diabetes mellitus. 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 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, 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 treatment of nervous disorders, spasms and asthma (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). *Crocus sativus* is a promising compound for reducing cisplatin-toxic side effects including nephrotoxicity, but the exact mechanism by which the saffron extract exert its protective effect against cisplatin-induced toxicity is not yet known (El-Daly, 1998). Besides, saffron extract and its active constituent (Crocin) inhibits neuronal cell death induced by both internal and external apoptotic stimuli (Soeda *et al*., 2001). *Crocus sativus* stigmas given together with cisplatin lead to an even greater decrease in blood glucose than that seen with cisplatin alone (El-Daly, 1998). However, adequate characterization of hypoglycemic activity has not yet been done on *Crocus sativus* L. (Saffron) stigma. Therefore, there are no available reports on the pharmacological action of *Crocus sativus* L. (Saffron) stigma until date. This research was performed to characterize the: (1) hypoglycemic effect of the ethanolic extract of *Crocus sativus* L. (Saffron) stigma in normal and alloxanized diabetic rats and (2) pancreas-protective effect of ethanolic extract of *Crocus sativus* L. (Saffron) stigma on rats with alloxan-induced diabetes.

MATERIALS AND METHODS

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 3 days. The mixture was subsequently filtered and concentrated under reduced pressure at 40°C. The extract yield was 51% w/w.

Animals: Male Wistar rats, 200-250 g obtained from Pasteur Institute of Iran were housed in colony rooms with 12/12 h light/dark cycle at 21±2°C and fed with laboratory pellet chow and given water *ad libitum*. Animals were acclimatized to their environment for one week prior to experimentation. Investigations using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the United States guidelines (United States National Institutes for Health, Publication no. 85-23, revised in 1985) and our Ethical Committee on Animal care approved the protocol.

Induction of diabetes in rats: After 15 h fasting, rats were intraperitoneally treated daily with alloxan monohydrate (Sigma chemicals, USA) at a dose of 120 mg kg^{-1} body weight (b.wt.), freshly dissolved in distilled water (5%) for 2 or 3 consecutive days.

To confirm diabetes, glycemia was daily determined after the administration of the last alloxan dose. Depending on their Fasting Blood Glucose (FBG) level the animals were divided arbitrarily in to two groups (Gupta *et al*., 2005): (1) Mild Diabetic (MD) animals with FBG of 120-250 mg dL^{-1} and (2) Severely Diabetic (SD) animals showing FBG of 250-300 mg dL^{-1}.

Estimations: Blood glucose was estimated by using one touch glucometer (Accu-chek sensor) of Roche Diagnostics, Germany for regular checkup and the glycose oxidase method of Biomerieux Laboratory (France) was used for weekly estimations. Blood samples were collected from the retro-orbital plexus. Basal plasma

insulin concentrations were determined by radioimmunoassay kit (Pharmacia, Uppsala, Sweden) with a Beta matic counter (Cronex, Dupont, France). The kit included human insulin as standard and ^{125}I-labelled human insulin antibody, which cross-reacts with rat insulin.

Biological assays

Effect in normoglycemic rats using two routes of administration: Healthy rats fasted for 18 h were used. The animals were separated in two groups of 30 rats each. In the first group the treatments were administered orally (p.o) and in the other, they were administered through intraperitoneal (i.p.) route. Thirty rat in the first group were separated in 5 sub groups of 6 animals each. Subgroup 1 received Isotonic Saline Solution (ISS, 10 mL kg^{-1}) as control; sub groups 2 received tolbutamide, purchased from Sigma-Aldrich Co., (200 mg kg^{-1}) as positive control and sub groups 3-5 received orally the variable single doses of 20, 40 and 80 mg kg^{-1} of ethanolic saffron extract. In this manner, the other 30 rats in the second group were divided in five sub groups of six animals each. Sub groups 6 received Isotonic ISS (10 mL kg^{-1}) as control; sub groups 7 received tolbutamide (200 mg kg^{-1}) as positive control and in other sub groups (8-10), ethanolic extract of *Crocus sativus* L. (Saffron) stigma (20, 40 and 80 mg kg^{-1}) was i.p. administered. In all the cases, ethanolic extract and tolbutamide were dissolved in 10 mL kg^{-1} b.wt. of ISS. Blood glucose levels were estimated before and after 2, 4, 6 and 8 h of extract administration.

Acute effects produced by i.p. administration of more effective dose of ethanolic saffron extract on the fasted blood glucose levels in mild and severely diabetic rats: Mild and severely diabetic rats were randomly assigned to three different groups (n = 6 in each group). Control groups received ISS (10 mL kg^{-1}); positive control groups received tolbutamide (200 mg kg^{-1}) and extract treated groups received a single dose of ethanolic extract of *Crocus sativus* L. (Saffron) stigma (40 mg kg^{-1}). All treatments were administered by i.p. route. Glycemic levels were determined in fasted animals (t = 0) and at intervals of 120 min for 4 h.

Effects of the i.p. daily administration of more effective dose of ethanolic saffron extract on the FBG and plasma insulin levels in MD and SD rats: Diabetic rats were randomly assigned to three different groups (n = 12 in each group). Normal rats were separated in 2 groups of 12 rats each and treated with ethanolic extract of saffron stigma (40 mg kg^{-1}) or ISS (10 mL kg^{-1}). Diabetic groups received ethanolic extracts of *Crocus sativus* at a dose of 40 mg kg^{-1} b.wt.; ISS (10 mL kg^{-1}) or tolbutamide at a dose of 200 mg kg^{-1}. All experiments were performed in overnight fasted rats. All treatments were administered by i.p. route daily for 2 weeks. In all the cases, ethanolic extract and tolbutamide were dissolved in 10 mL kg^{-1} body weight of ISS. Fasting blood glucose and the plasma insulin levels were estimated at the beginning and after 14 days of experiment.

Histopathological study: Histopathological studies of the pancreas were conducted in normal healthy and diabetic rats treated with ethanolic extract of *C. sativus* L. stigma at a dose of 40 mg kg^{-1} for 30 days. Animals of the different groups were sacricified by cervical dislocation and the pancreas was removed. To prepare pancreatic sections for light microscopy, a piece of splenic regions of the pancreas was fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin.

Immunohistochemical study: The removed pancreas from sacricified animals were fixed in Bouin, dehydrated in a graded series of ethanol and embedded in paraffin wax before sectioning. Sections were dewaxed and rehydrated. After the step of washing in Phosphate-Buffered Saline (PBS), sections were immersed in a solution of 3% H_2O_2 for 10 min. The sections were then pre-incubated with non-immune serum for 20 min. They were labelled with Streptavidin Biotin following incubation with primary monoclonal anti-insulin clone antibody (dilution 1/1000 μg mL^{-1}). The localization of the antigen was indicated by a yellow-brown color obtained with 3-Amino-9-Ethyl-Carbazole (AEC) as chromogenic substrate for peroxidase activity. Primary anti-insulin antibody (Sigma I-2018) and Histostain-Plus kit (Zymed Code 85-9943) were used for immunohistochemistry. Slides were counter stained with hematoxylin for microscopic observation. The specificity of the immunohistochemical staining was checked by omission of the primary antibody, or by using an inappropriate antibody (anti-gastrin). All these controls gave negative results. Control pancreas sections with (+) signals were used as a positive control.

In order to evaluate insulin immunoreactivity, the intensity of the corresponding signals from the tissue sections was measured. More than 10 islets in each rats group were randomly selected and transferred to a pathology image analyzing system (VNT, Beijing, China). Staining signals of the islets selected on the captured image were converted to gray density which can be automatically calculated as a staining intensity per unit area (mm^2). The insulin immunoreactivity was calibrated from 0-10.

LD_{50} experiments: Wistar rats, of both sex and weighing about 200-250 g were divided into six groups of 6 animals each (three females and three males). The test substance was administered in the dose of 5, 20, 40, 60, 80 and 100 mg kg^{-1} i.p. in a volume of 10 mL kg^{-1} to the animals of I, II, III, IV, V and VI groups, respectively. Then the rats were observed continuously for 1 h, intermittently for 6 h and at the end of 24 h for any gross behavioral changes and deaths. Food consumption, feces and urine were also examined at 2 h and then at 6 h intervals for 24 h.

Statistical analysis: All biochemical results were expressed as Mean±SD significant differences among the groups were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test or unpaired Student's t-test using the SPSS statistical analysis program. Statistical significance was considered at $p<0.05$.

RESULTS

Effect in normoglycemic rats using two routes of administration: Results of the effect, of graded doses of ethanolic extract of *C. sativus* stigma on blood glucose level of normal healthy rats in oral and intraperitoneal routes are presented in Table 1 and 2, respectively. Basal glycemia stays without significant variations in control groups (ISS). The extract in all the three doses (20, 40 and 80 mg kg^{-1}) produced significant hypoglycemic effect after 4 h of intraperitoneal administration ($p<0.05$). However, it was more marked in animals receiving ethanolic extract equivalent to 40 mg kg^{-1} b.wt. This dose produces a significant fall of 33.9% in BGL after 6 h of i.p. administration. A fall of 21.5 and 27.3% was observed in BGL at dose 20 and 80 mg kg^{-1}, respectively, after 6 h of i.p. administration. However, slight rise in BGL was observed after 8 h of extract administration. Although oral route of *C. sativus* shows hypoglycemic effect at 6 h, remarkable effect was observed using intraperitoneal route, with significant glucose reduction. However, when tolbutamide was administered, significant reduction of glycemia at 2 h were observed ($p<0.01$).

Therefore, subsequent studies were carried out after giving 40 mg kg^{-1} of ethanolic extract in MD and SD rats.

Acute effects produced by i.p. administration on the fasted blood glucose levels in mild and severely diabetic rats: Table 3 depicts hypoglycemic effect of i.p. administration of ethanolic extract of *C. sativus* stigma in MD and SD rats. Ethanolic extract of *C. sativus* stigma produced a significant reduction of glycemia in the MD rats, from 152.4±5.6-133.3±6.6 at 120 min and to 112.4±6.6 mg dL^{-1} at 240 min. In SD rats, ethanolic extract of *C. sativus* stigma produced a significant reduction of glycemia from 260.4±9.5 to 210.2±9.2 at 120 min and to 198.8±7.5 mg dL^{-1} at 240 min. Tolbutamide produced a significant attenuation in blood glucose only at 240 min when compared to the diabetic control groups.

Table 1: Hypoglycemic effect of graded doses of ethanolic extract of *Crocus sativus* L. stigma p.o. administered to healthy rats

		Blood glucose levels (mg dL^{-1})				
		Pretreatment (h)	Post treatment (h)			
Experimental groups	Dose (mg kg^{-1})	0	2	4	6	8
ISS (control)	-	85.4±2.4	87.7±3.2	85.9±3.6	84.5±2.6	83.4±5.2
Tolbutamide	200	88.6±5.2	46.3±4.1**	58.5±5.4*	64.9±3.7*	64.1±3.1*
Ethanolic extract	20	83.4±2.3	81.7±4.2	79.4±5.3	72.2±2.2*	74.3±4.6*
Ethanolic extract	40	86.2±4.1	83.8±2.2	79.7±2.8	68.9±3.4*	70.9±5.3*
Ethanolic extract	80	84.8±4.8	82.1±4.6	78.2±7.3	71.1±6.3*	72.3±4.0*

Mean±SD. Significantly different from the control: *$p<0.05$, **$p<0.01$

Table 2: Hypoglycemic effect of graded doses of ethanolic extract of *Crocus sativus* L. stigma i.p. administered to healthy rats

		Blood glucose levels (mg dL^{-1})				
		Pretreatment (h)	Post treatment (h)			
Experimental groups	Dose (mg kg^{-1})	0	2	4	6	8
ISS (control)	-	87.3±4.1	87.7±3.4	87.1±4.7	86.1±3.9	84.3±4.4
Tolbutamide	200	86.9±4.2	44.8±5.1**	57.3±7.2*	63.3±4.6*	61.7±3.1*
Ethanolic extract	20	81.9±4.7	80.7±4.4	72.7±4.8*	64.3±3.9*	71.1±4.3*
Ethanolic extract	40	88.1±3.4	79.8±4.6	64.6±4.4*	58.2±5.1*	62.7±3.7*
Ethanolic extract	80	86.2±4.2	80.1±3.9	69.7±4.1*	62.7±4.6*	66.6±4.2*

Mean±SD. Significantly different from the control: *$p<0.05$, **$p<0.01$

Table 3: Hypoglycemic effect of the ethanolic extract of *C. sativus* L. stigma i.p. administered to alloxan induced diabetic rats

Experimental groups	Dose (mg kg^{-1})	Blood glucose levels (mg dL^{-1}) 0 (min)	120 (min)	240 (min)
MD rats				
Control (vehicle treated)	-	158.4±7.5	155.4±6.3	154.2±7.2
Tolbutamide	200	152.3±5.2	149.2±6.4	123.2±7.1*
Ethanolic extract	40	152.4±5.6	133.3±6.6*	112.4±6.6**
SD rats				
Control (vehicle treated)	-	261.3±8.5	259.2±7.8	258.4±9.3
Tolbutamide	200	271.6±8.3	255.1±8.5	219.3±9.6*
Ethanolic extract	40	260.4±9.4	210.2±9.2*	198.8±7.5**

Mean±SD. Significantly different from the respective control: *p<0.05, *p<0.01

Table 4: Effect of the i.p. daily administration of ethanolic extract of *Crocus sativus* L. stigma at a dose of 40 mg kg^{-1} on blood glucose levels and plasma insulin concentrations in normal and diabetic rats

Experimental groups	Dose (mg kg^{-1})	Blood glucose levels (mg dL^{-1}) 0 (day)	14 (day)	Plasma insulin concentrations (μU mL^{-1}) 0 (day)	14 (day)
Normal rats					
Control (vehicle treated)		86.4±2.7	84.8±4.2	31.8±1.5	32.7±1.3
Ethanolic extract		87.7±5.1	60.9±2.3*	34.8±1.2	33.8±2.1
MD rats					
Control (vehicle treated)	-	156.2±5.8	152.2±6.4	18.9±1.2	18.1±1.1
Tolbutamide	200	152.5±6.7	122.3±6.1*	18.4±1.9	21.4±1.5*
Ethanolic extract	40	153.7±7.8	90.0±6.7**	19.2±1.4	25.6±1.6**
SD rats					
Control (vehicle treated)	-	263.0±6.1	260.5±6.2	15.2±0.8	14.4±0.6
Tolbutamide	200	261.0±5.3	221.0±5.2*	15.5±0.6	16.7±0.8
Ethanolic extract	40	260.7±6.5	180.6±4.5**	16.1±0.9	20.5±1.4**

Mean±SD. Significantly different from the respective control: *p<0.05, **p<0.001

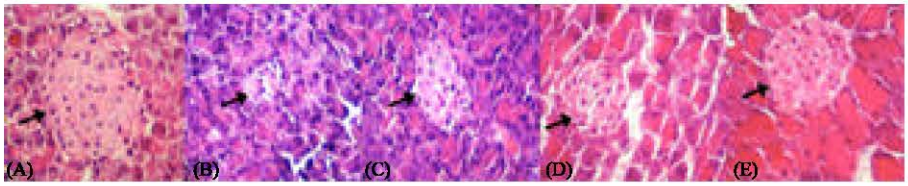

Fig. 1: Photomicrograph of the pancreas of normal healthy rat (A) showing normal structure of langerhans islet (arrow) (H and E, x400). Pancreas of SD rat (B) showing severe destruction of langerhans islet (arrow) and severe atrophy with an occasional cell (H and E, x400). Photomicrograph of the pancreas of MD rat (C) showing destruction of langerhans islet (arrow) and moderate atrophy with reduced number of islet cells. Islet cells show vacuolation (H and E, x400). Pancreas of extract (40 mg kg^{-1} b.wt.) treated SD rat (D) showing partially reversion of damaged pancreatic islet (arrow). Vacuolation of the islet cells is also not prominent (H and E, x400). Extract (40 mg kg^{-1} b.wt.) treated MD pancreas and (E) showing prominent recovery of islet (arrow). Many islet cells are absent showing atrophy of cells (H and E, x400)

Effect on FBG and basal plasma insulin concentrations of mild and severely diabetic rats: The stigma extract produced significant hypoglycemic effect in MD and SD diabetic rats after 2 weeks of administration. This dose produces a significant fall of 41.4% after 2 weeks in MD rats. In this manner, the FBG was decreased by 30.7% in SD rats (Table 4).

Significant changes in plasma insulin concentrations were observed in both MD and SD rats after daily treatment with the dose of 40 mg kg^{-1} for 14 days (MD 33.3%, SD 27.3%). Basal plasma insulin level in normal extract treated group had no significant changes.

Histopathological study: Histological studies of pancreas were carried out in healthy normal and alloxan-induced diabetic rats, which were sacrificed after 1 month of the experiment. Pancreas of the normal rats showed normal structures in histological examination. Histopathology of the pancreas in diabetic rats showed a spectrum of changes ranging from moderate atrophy with reduced number of islet cells to severe atrophy with an occasional cell, mild to severe destruction of the islets of langerhans by lymphocytic infiltration. Islet cells showed vacuolation. Exocrine cells were similar to those of the vehicle-injected rat pancreas (Fig. 1A-C). Recovery of

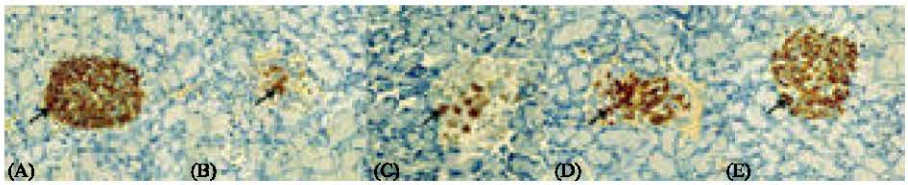

Fig. 2: Insulin immunoreactivity (arrow) in rat pancreatic islets. (Magnification: 320x): (A) Control rat showing normal structure and β-cells (arrow); (B and C) SD and MD rats showing the prominent decrease in insulin immunoreactivity and the number of immunoreactive β-cells in comparison with control group (arrows) and (D and E) SD and MD rats treated with the extract (40 mg kg^{-1} b.wt.) showing increase of insulin immunoreactivity and the number of immunoreactive β-cells in comparison with respective untreated diabetic rats (arrows)

pancreatic β-cells after treatment with *Crocus sativus* L. stigma in alloxanized mild diabetic rats was prominent as their pancreases were histologically near normal after 1 month of extract administration. Many islet cells were absent showing atrophy of cells. In the pancreas of the extract treated animals vacuolation of the islets cells was also not prominent. Partially reversion also occurred in the damaged pancreatic islets of severe diabetic rats after treatment with *Crocus sativus* L. stigma (Fig. 1D, E).

Immunohistochemical study: Figure 2A-E demonstrate the immunohistochemical results on pancreas in experimental rats. In the control (intact) and extract treated control animals, the islets showed the normal structure with a large central core formed by insulin-secreting β-cells. The control group given ethanolic saffron extract was no different from the other intact control group considering the insulin immunoreactivity in β-cells (Fig. 2A). In the pancreatic islets of SD and MD rats, decreases in insulin immunoreactivity and the number of immunoreactive β-cells were observed by comparison with the control group. This reduction was prominent in SD rats (Fig. 2B, C). On the other hand, in the pancreatic islets of SD and MD rats given extract (40 mg kg^{-1} b.wt.), marked increases in insulin immunoreactivity and the number of immunoreactive β-cells were observed as compared with respective untreated diabetic rats. However, it was still weaker than that of control rats.

Figure 3 shows the result of image analysis for insulin immunoreactivity of the β-cells by immunohistochemical intensity. It represented a remarkable decrease in the SD and MD diabetic rats when compared to control rats. After administration of extract, the insulin immunoreactivity was relatively more numerous than that of untreated diabetic rats.

LD_{50} experiments (behavioral effect and toxicity): The extract of the test substance (*Crocus sativus* L. stigma) was found to be safe for further biological studies as no

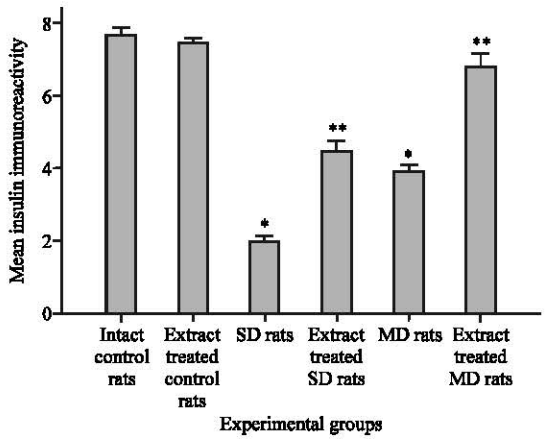

Fig. 3: Comparative evaluation of the expression of insulin immunoreactivity in the pancreatic islets. Intensity of the immunoreaction in each group was measured by the image analyzing system and recorded with the range from 0 to 10. Mean±SE levels of significance were determined by paired-samples t-test. *$p<0.01$ vs. healthy rats. **$p<0.01$ vs. respective diabetic rats

toxic effect and lethality was observed up to 100 mg kg^{-1} i.p. in rat. Only the consumption of food was increased by 20% in the dose of 80 and 100 mg kg^{-1} during 4 h but remaining normal afterwards.

DISCUSSION

The results showed important differences when the same doses of the ethanolic extract of *Crocus sativus* L. (Saffron) stigma were administered through different routes. Intraperitoneal injection produced the most important reductions of blood glucose levels in healthy rats. Consequently, this route of administration was selected for further investigation.

Ethanolic saffron extract showed a dose-dependent effect on FBG up to a dose of 40 mg kg^{-1}. The FBG

decreases by 26.6 and 33.9% after 4 and 6 h, respectively in normal rats treated with a single dose of 40 mg kg^{-1} of the extract whereas the dose of 80 mg kg^{-1} produces a fall of 19.1 and 27.3% in FBG of normal rats after 4 and 6 h of extract administration in i.p. route. Thus, higher dose of 80 mg kg^{-1} did not show any dose-dependent effect although, it caused a significant decrease in the FBG level. It is likely that the bigger doses could not produce the expected higher hypoglycemic effect by the presence of some other substances in the ethanolic extract, which interfere with the hypoglycemic effect. Such a phenomenon of less hypoglycemic response at higher dose is not uncommon with indigenous plants and has already been observed in *Aegle marmelos* (Gupta *et al*., 2005; Kesari *et al*., 2005; Sharma *et al*., 1996a), *Cinnamomum tamala* (Kesari *et al*., 2006; Sharma *et al*., 1996b). However, the dose of 40 mg kg^{-1} has almost same effect as of synthetic drug tolbutamide (200 mg kg^{-1}) after 6 h of administration.

The present investigation shows that in MD and SD alloxan-diabetic rats, ethanolic extract of *C. sativus* caused significant reductions of blood glucose levels after 2 h of extract administration. Tolbutamide (200 mg kg^{-1}) caused a lesser hypoglycemic effect than *C. sativus* ethanolic extract in diabetic rats after 4 h of drug administration. In addition, ethanolic extract of *Crocus sativus* L. (Saffron) stigma caused significant hypoglycemic effect in MD and SD rats after 14 days treatment, while tolbutamide exhibited a mild hypoglycemic activity in these animals.

Tolbutamide is a sulphonylurea that produces experimental and clinical hypoglycemia (in normal animals, in mild alloxan-diabetic animals and in type 2 diabetes) because it induces the release of insulin by the pancreatic β-cells. However, in severe alloxan-diabetic animals, such as in type 1 diabetes, these animals do not have pancreatic β-cells. Thus, tolbutamide does not produce hypoglycemic effect in these situations (Alarcon-Aguilar *et al*., 2002). Although, the percent fall in blood glucose was found to be more in extract treated MD rats, which have functioning pancreatic β-cells, significant fall in SD rats suggests that the active hypoglycemic compound present in the ethanolic extract of saffron does not necessarily require the presence of functioning β-cells and acts in the absence of insulin. Therefore, saffron extract may be classified as a direct hypoglycemic agent, by checking hyperglycemia due to alloxan-induced diabetes, in contrast to the tolbutamide as an indirect agent that act by stimulating the pancreatic β-cells to release more insulin.

The results demonstrated that ethanolic extract of saffron induces significant decrease of plasma glucose levels in diabetic rats and this effect was more potent after repeated i.p. administration as, a marked normalization of blood glucose levels in these animals was achieved after 2 weeks of treatment. Therefore, the effectiveness of the extract depends, probably on the accumulative effect of active principles. Therefore, it is possible for the test substance to exert its hypoglycemic activity by both the direct and indirect mechanism. On other hand, a significant change in plasma insulin concentrations was noted in diabetic rats after daily treatment with ethanolic saffron extract for 14 days, but no changes were observed in basal plasma insulin concentrations after treatment in normal rats indicating that the underlying mechanism of this pharmacological effect seems to be independent of insulin secretion in normal rats. In addition, the control group given ethanolic saffron extract was no different from the other intact control group considering the insulin immunoreactivity in β-cells. This result was consistent with the biochemical findings. Tolbutamide showed a mild increase in basal plasma insulin levels in MD rats and this effect was not significant in SD rats. Considering the histopathology and immunohistochemical findings in present study, the mechanism involved in this activity, appears to be both pancreatic, helping in regeneration of damaged pancreas and extra pancreatic. However, ethanolic extract of saffron may exert its hypoglycemic action by mechanisms such as stimulating of glucose uptake by peripheral tissues (Yang *et al*., 2003), inhibition of insulinase activity in both liver and kidney (Achrekar *et al*., 1991), inhibition of endogenous glucose production (Eddouks *et al*., 2002), inhibition of intestinal glucose absorption (Youn *et al*., 2004) inhibition of renal glucose reabsorption (Maghrani *et al*., 2005) or correction of insulin resistance (Hu *et al*., 2003). However, possibilities of other mechanisms to exert hypoglycemic effect cannot be ruled out.

The mechanism of alloxan diabetes has been the subject of many investigations and it is now generally accepted that free radicals are selectively involved in the initiation of the damage that ultimately leads to β-cell death (Minami *et al*., 1999; Vanco *et al*., 2004). Therefore, the pancreas is especially susceptible to the action of alloxan-induced free-radical damage. Many substances have been shown to ameliorate the diabetogenicity of alloxan in animals, which protect by reacting with free radicals formed from alloxan during its interaction with the β-cell, or prevent radical formation (Jörns *et al*., 1999). Recently, it was reported that the saffron extract, crocin and safranal exhibited significant radical scavenging activity and thus antioxidant activity (Assimopoulou *et al*., 2005) and the present finding indicates that administration of *Crocus sativus* L. stigma confirms the possibility that the major function of the

extract is on the protection of vital tissues including the pancreas, thereby reducing the causation of diabetes in these animals.

Therefore, protective effect of saffron extract on pancreas of alloxan-induced diabetic rats could be attributed directly to scavenging activity and for more extent to the regenerative properties of the extract. In conclusion, present study indicates that saffron ethanolic extract produced antihyperglycemic effects in experimental diabetes by providing a regenerative modification against damage caused by to endocrine cells of the pancreas. Protective effect of saffron extract is also evident from the immunohistochemical results obtained.

CONCLUSION

Taken in all, ethanolic extract of saffron may have value as a safe preventive or therapeutic agent against diabetes mellitus and the use of this plant in diabetes is then supported but more study is warranted to elucidate the precise active substance(s), site(s) and myriad mechanism(s) of this pharmacological effect.

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