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 intraperitoneal (i.p.) route for decreasing Blood Glucose Levels (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 (Alarecon-Aguilera 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
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-Aguilera 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 hypolipidemic, 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; Rios 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 hepatoxic lesions
and has a modulatory effect on aflatoxin B1, cytotoxicity.
It also has a protective effect on the bladder toxicity,
induced by cyclophosphamide (Giacco, 2004). Crocus sativus is a promising compound for reducing
cisplatin-toxic side effects including nephrotoxicity, but
the exact mechanism by which the saffron extract exerts
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 monochrhydrate
(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
glucose 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 Betamatic 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 rats 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 sacrificed 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 sacrificed 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$_2$O$_2$ 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 L-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.
**I.D$_{40}$ 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 hr, intermittently for 6 hr and at the end of 24 hr for any gross behavioral changes and deaths. Food consumption, feces and urine were also examined at 2 hr and then at 6 hr intervals for 24 hr.

**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 hr 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 hr 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 hr of i.p. administration. However, slight rise in BGL was observed after 8 hr of extract administration. Although oral route of *C. sativus* shows hypoglycemic effect at 6 hr, remarkable effect was observed using intraperitoneal route, with significant glucose reduction. However, when tolbutamide was administered, significant reduction of glycemia at 2 hr 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>
<thead>
<tr>
<th>Experimental groups</th>
<th>Dose (mg kg$^{-1}$)</th>
<th>Blood glucose levels (mg dL$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pretreatment (h)</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>ISS (control)</td>
<td>-</td>
<td>85.4±2.4</td>
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<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>88.6±5.2</td>
</tr>
<tr>
<td>Ethenolic extract</td>
<td>20</td>
<td>83.4±2.3</td>
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<td>Ethenolic extract</td>
<td>40</td>
<td>86.2±4.1</td>
</tr>
<tr>
<td>Ethenolic extract</td>
<td>80</td>
<td>84.8±4.8</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
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<td>Pretreatment (h)</td>
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<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>ISS (control)</td>
<td>-</td>
<td>87.3±4.1</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>86.9±4.2</td>
</tr>
<tr>
<td>Ethenolic extract</td>
<td>20</td>
<td>81.9±4.7</td>
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<tr>
<td>Ethenolic extract</td>
<td>40</td>
<td>88.1±3.4</td>
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<tr>
<td>Ethenolic extract</td>
<td>80</td>
<td>86.2±4.2</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Dose (mg kg⁻¹)</th>
<th>Blood glucose levels (mg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>-</td>
<td>158.4±7.5</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>152.3±5.2</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>40</td>
<td>152.4±5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SD rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>-</td>
<td>259.2±7.8</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>255.1±8.5</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>40</td>
<td>210.2±9.2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Dose (mg kg⁻¹)</th>
<th>Blood glucose levels (mg dl⁻¹)</th>
<th>Plasma insulin concentrations (µU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>-</td>
<td>86.4±2.7</td>
<td>31.8±1.4</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>87.4±5.1</td>
<td>60.9±2.3</td>
<td>34.8±1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MD rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>-</td>
<td>156.2±5.8</td>
<td>18.9±1.2</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>152.5±6.7</td>
<td>21.4±1.5</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>40</td>
<td>155.7±7.8</td>
<td>25.6±1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SD rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle treated)</td>
<td>-</td>
<td>263.0±6.1</td>
<td>15.2±0.8</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>200</td>
<td>261.0±5.3</td>
<td>16.7±0.8</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>40</td>
<td>260.7±6.5</td>
<td>20.5±1.4</td>
</tr>
</tbody>
</table>

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

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 vacuolization (H and E, x400). Pancreas of extract (40 mg kg⁻¹ b.wt.) treated SD rat (D) showing partial recovery of damaged pancreatic islet (arrow). Vacuolization of the islet cells is also 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 lymphoeytic infiltration. Islet cells showed vacuolization. Exocrine cells were similar to those of the vehicle-injected rat pancreas (Fig. 1A-C). Recovery of
pancreatic β-cells after treatment with *Crocus sativus* L. stigma in alloxanized mild diabetic rats was prominent as their pancreases were histologically 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. Partial 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.

**LD₉₀ 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 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 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 insulase 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 (Jorns et al., 1999). Recently, it was reported that the saffron extract, crocin and safranal exhibited significant radical scavenging activity and thus antioxidant activity (Assimopoulos 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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