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The Effect of Crocin on Oxidative Stress Compared to Saffron Aqueous Extract, Glycerol and Myoinositol in Diabetic Rats

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is a prevalent and intricate disease globally. Despite various available treatments, there is no cure for it, and it is still associated with significant socioeconomic challenges. Small molecules with antioxidant activity and stabilizing effect on protein structure piqued our interest as potential antiglycating agents to decrease diabetes complications. Thus, this study aimed to evaluate and compare the antioxidant potential of Crocin with saffron aqueous extract (SAE) and two polyols (Glycerol and Myoinositol) in T2DM rats. **Method**: Neonatal male Wister rats were randomly divided into two groups. Diabetic groups at 3-5 days of birth received streptozotocin (STZ) (90 mg/kg) via intraperitoneal injection. After eight weeks, both groups were divided into subgroups and treated with SAE, Crocin, Glycerol, or Myoinositol. After five months, rats were sacrificed and their serum oxidative parameters and liver antioxidant enzymes were evaluated. **Result:** Crocin (100 mg/kg) significantly decreased advanced glycation end products (AGEs) and increased ferric reducing ability of plasma (FRAP) in serum of diabetic rats. Moreover, the reduction in liver catalase and superoxide dismutase specific activities, as well as glutathione (GSH) concentration in diabetic rats' liver significantly increased after treatment with the same amount of Crocin. There were no significant changes in advanced oxidation protein products (AOPP) and Nitric Oxide (NO). **Conclusion:** Among the mentioned treatments, Crocin at 100 mg/kg was more effective than SAE, Glycerol, and Myoinositol in decreasing AGEs, increasing serum antioxidant capacity, and increasing the activity of hepatic antioxidant enzymes and GSH.

Introduction

Type 2 diabetes mellitus (T2DM), or non-insulin dependent diabetes mellitus (NIDDM), is one of the most common and complex diseases accompanied by severe socioeconomic problems (Zheng *et al.*, 2018). The pathogenesis of T2DM is a complex phenomenon involving the progressive development of insulin resistance in the liver and peripheral tissues, resulting in pancreatic β-cell damage and defective insulin secretion. In addition, a long-term increase of glucose in biological fluids results in biomacromolecules (especially proteins) glycation and misfolding, which is one of the critical reasons for oxidative stress induction (Mahdavifard *et al.*, 2015).

Protein glycation initiates a chain of chemical reactions from Schiff base formation to early,

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intermediate, and advanced glycation end products (AGEs). AGEs are non-functional protein aggregates that might induce changes in the structure and function of other proteins (Bahmani *et al.*, 2012a). Pathologic effects of non-enzymatic glycation and AGEs are not limited to diabetes. These are also observed during aging, arthritis, and cancer (Ansari and Rasheed, 2010). One of the most harmful consequences of AGEs formation is the induction of reactive oxygen species (ROS) (Bahmani *et al.*, 2012a, Mahdavifard *et al.*, 2014).

It has been shown that ROS formation and oxidative stress play a central role in diabetic complications. This claim is supported by evidence indicating the increase in the production of free radicals by some biochemical pathways associated with hyperglycemia. These pathways are the mitochondrial electron transport chain, glucose autoxidation, and AGE production pathways (Assmann *et al.*, 2016, Mirmiranpour *et al.*, 2021). Prolonged hyperglycemia affects the overall body homeostasis and leads to chronic complications of diabetes, including nephropathy, angiopathy, retinopathy, and deficit of the antioxidant defense system (Mahdavifard *et al.*, 2015, Papatheodorou *et al.*, 2017).

Various agents, such as chemical chaperones, inhibited or delayed protein glycation and oxidative stress induction (Bahmani *et al.*, 2012a, Jafarnejad *et al.*, 2008a, Mahdavifard *et al.*, 2014). Chemical chaperones are low molecular weight compounds used to increase protein stability and inhibit non-enzymatic glycation and aggregation of proteins (Jafarnejad *et al.*, 2008a, Jafarnejad *et al.*, 2008b). Polyols are a class of chemical chaperones with antioxidant properties that stabilize protein structure (Okiyoneda *et al.*, 2013).

Although the exact molecular mechanism(s) involved in managing diabetes mellitus is still the subject of active discussion, various drugs are prescribed for diabetes. However, there is no cure for diabetes (Hossain and Pervin, 2018, J Meneses *et al.*, 2015). Thus, patients with diabetes use complementary strategies. Recently, there has been growing interest in using natural products to manage and treat various diabetic complications (Alam *et al.*, 2018).

According to our previous studies, the present study examined the protective effect of saffron aqueous extract (SAE) and Crocin as potential antioxidants in a rat model of T2DM. In addition, since Crocin has four glucose units with several OH groups, the study compared its antioxidant and antidiabetic effects with those of two members of polyol family of chemical chaperones, Glycerol and Myoinositol, in these rat models of T2DM.

Materials and Methods

Chemicals

Crocus sativus L. stigmas were collected from Ghaenat (Khorasan province, Northeast of Iran). Using the methods explained previously, SAE and Crocin were prepared. The ground saffron stigma was placed in distilled water in a shaker incubator for three days. Then, filtrated, and the aqueous extract was dried using a freeze dryer. For Crocin isolation, the ground stigma was washed with *n*-hexane, extracted with 50% ethanol, centrifuged, and the supernatant was loaded on a neutral aluminum 90-active column. After elution with 50% ethanol and then 50% ethanol: acetic acid, 4:1 v/v, the fractions containing Crocin were collected and dried using a Rotary Evaporator and then a Freeze Dryer (Bolhasani *et al.*, 2005, Shirali *et al.*, 2012). Streptozotocin (STZ) was purchased from Sigma-Aldrich Co. All other materials were of analytical grade.

Samples and study design

Neonatal male Wister rats aged 2-5 days old were housed in humidity- and temperature-controlled rooms with a 12-h light and 12-h dark photoperiod. Then, they were randomly divided into two groups: diabetic and healthy. Diabetic groups received an intraperitoneal injection of STZ (90 mg/kg body weight) (Shirali *et al.*, 2013). After that, only rats with blood glucose levels ≥ 270 mg/dl were included in experiments.

After eight weeks, the diabetic rats were divided into seven subgroups; four groups were treated with SAE and Crocin by intraperitoneal injection. Two other groups received Glycerol

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and Myoinositol in drinking water (0.5% w/v) ad libitum. The control group consisted of healthy rats with no more treatment or receiving higher doses of each treatment (**Table 1**). The doses were according to our previous studies or other references. After five months, animals were sacrificed under anesthesia, and their blood and tissues were collected and stored at -70 °C.

Table 1. The name and treatment of rat groups.

Groups name treatment		SAE (mg/kg body weight)		Crocin (mg/kg body weight)		Glycerol (0.5% w/v)	Myoinositol (0.5% w/v)
Diabetic	D	-----	-----	-----	-----	-----	-----
	D+S100	100	-----	-----	-----	-----	-----
	D+S150	-----	150	-----	-----	-----	-----
	D+C50	-----	-----	50	-----	-----	-----
	D+C100	-----	-----	-----	100	-----	-----
	D+G	-----	-----	-----	-----	√	-----
	D+I	-----	-----	-----	-----	-----	√
Healthy	H	-----	-----	-----	-----	-----	-----
	H+S	150		-----		-----	-----
	H+C	-----		100		-----	-----
	H+G	-----		-----		√	-----
	H+I	-----		-----		-----	√

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy.*

Biochemical analysis

Each animal's blood sample was taken by puncturing the retro-orbital plexus at the beginning (month 0), middle (month 3), and end of the study (month 5). Then, blood samples were clotted at room temperature. Finally, the serum was separated and stored at -70 °C until the desired experiments.

Several methods have been introduced to measure the total antioxidant capacity of biological samples. However, in the present research, serum antioxidant activity was evaluated by measuring the levels of AGEs, ferric reducing ability of plasma (FRAP), advanced oxidation protein products (AOPP), and nitric oxide (NO). AGE level was determined according to the previously explained method and a Shimadzu Spectrofluorometer, Model RF-5000 (Jafarnejad *et al.*, 2008b). FRAP was determined using Benzie's method (Benzie and Strain, 1996). AOPP was determined spectrophotometrically according to Kalousova's method (Kalousova *et al.*, 2002). NO release can be determined spectrophotometrically by measuring its stable end products, nitrite, and nitrate accumulation. Serum NO levels (nitrite+nitrate) were determined according to Kirkali *et al*. method using Griess reagent and enzymatic methods. Griess reagent (1% sulfonilamide in 1 N HCl, 15% N-1-naphtyletylenediamine dichloride) was added to the serum sample to measure the nitrite level and was evaluated by absorption at 550 nm. The serum nitrate was also converted to nitrite using nitrate reductase. Then, sum of nitrites was measured (Kirkali *et al.*, 2000).

Hepatic antioxidant capacity was evaluated by measuring activities of two oxidative enzymes, Catalase (CAT) and Superoxide dismutase (SOD), and the concentration of Glutathione (GSH). To this end, rats' liver tissues were frozen using liquid nitrogen and then powdered in a porcelain mortar. Phosphate buffer saline (PBS) was then added, homogenized, and centrifuged at 4 °C, 12,000 rpm, and for 15 min. The supernatant was separated and kept at -70 °C until use. As previously described, the CAT activity was determined by ultraviolet absorption of peroxide in liver homogenates prepared in phosphate buffer (Beers and Sizer, 1952). The level of SOD activity in liver homogenates was measured using SOD Assay Kit-WST according to technical manual of Dojindo Molecular Technologies, Inc. GSH concentration

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was measured using Ellman's reagent and based on Selak method (Bahmani *et al.*, 2012b).

Ethical considerations

The Animal Ethical Committee of Tarbiat Modares University approved the experimental protocol, following guidelines for the care and use of laboratory animals according to national and international laws and policies (National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication no. 85-23, 1985).

Data analysis

All data are presented as mean±SD in rat groups. Differences between mean values were analyzed for significance at a 95% confidence limit by one-way or two-way ANOVA statistics using SPSS 16.0 and GraphPad Prism version 9.0.

Results

Protein glycation

Table 2 shows changes in AGE formation in all groups during the study. It indicates a significant (P<0.001) and progressive increase of AGE formation in diabetic group, which was significantly (P<0.001) prevented due to the administration of each of the mentioned treatments. The observed changes were also significant between 3 and 5 months (P<0.0001) in all diabetic rats. The best treatment was 100 mg/kg Crocin. The order of effectiveness at the end of the experiment was as follows:

D+C100>D+C50>D+S150>D+S100>D+G> D+I

Treatments had no significant effect on AGE formation in healthy rats.

Table 2. Advanced glycation end products (AGEs) levels in the serum of rats in different groups and time course of the experiment.

Groups	0 month	3rd month	5th month
H	8.5 ± 1.3 [j]	10.0 ± 1.7 [j]	10.5 ± 1.8 [j]
H+C	8.4 ± 1.3	8.0 ± 1.3	5.5 ± 1.2
H+S	8.4 ± 1.1	7.5 ± 1.3	6.3 ± 1.6
H+G	8.4 ± 1.1	7.5 ± 1.3	7.0 ± 1.6
H+I	8.4 ± 1.1	7.5 ± 1.3	7.2 ± 1.6
D	74.0 ± 2.1 [j]	75.0 ± 1.6 [a,j]	81.0 ± 2.7 [a,j]
D+C50	72.0 ± 2.6	50.0 ± 1.1 [b,i]	35.0 ± 2.9 [b,i]
D+C100	72.0 ± 1.4	41.0 ± 1.4 [b,c,d,e,i]	30.0 ± 3.6 [b,c,d,g,i]
D+S100	71.0 ± 2.6	55.0 ± 2.8 [i]	40.0 ± 3.4 [i]
D+S150	72.0 ± 2.9	52.0 ± 2.7 [c,f,g,i]	37.0 ± 4 [c,f,g,i]
D+G	73.0 ± 2.7	60.0 ± 1.1 [d,f,I,i]	50.0 ± 2.9 [e,g,I,i]
D+I	73.0 ± 3.2	65.0 ± 3.2 [e,g,I,i]	58.0 ± 2.4 [e,g,I,i]

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; [a]: Significant differences between diabetic group and all other groups; [b]: Significant difference between diabetic groups treated with different Crocin concentrations (P<0.0001); [c]: Significant difference between high doses of Crocin and SAE (P<0.0001); [d]: Significant difference between diabetic group treated with high doses of Crocin and Glycerol (P<0.0001); [e]: Significant difference between diabetic group treated with high doses of Crocin and Myoinositol (P<0.0001); [f]: Significant difference between diabetic group treated with high doses of SAE and Glycerol (P<0.0001); [g]: Significant difference between diabetic group treated with high doses of SAE and Myoinositol (P<0.0001); [h]: Significant difference between diabetic group treated with Glycerol and Myoinositol (P<0.0001); [i]: Significant differences between 3-month period and 5-month period in each group (P<0.0001*); [j]: Significant difference between the Diabetic and Healthy groups (P<0.001).

Antioxidant activity

Table 3 shows results of FRAP measurement in different groups, indicating a significant decrease (P<0.001) in diabetic rats compared to the control group. However, it was significantly (P<0.001) increased due to the mentioned treatments in diabetic rats. The observed changes were also significant between 3 and 5 months (P<0.001) in all diabetic rats. However, treatments did not significantly affect FRAP in healthy rats during five months.

The AOPP level increased significantly (P<0.001) and in a time-dependent manner in diabetic rats compared to the control group (**Table**

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4). Although it was lower in the groups treated with two doses of Crocin, these changes were not significant compared to diabetic rats.

Table 3. Ferric reducing ability of plasma (FRAP) levels (µmol/l) in different groups and time course of the experiment.

Groups	0 Month	3rd Month	5th Month
H	1220.0 ± 60.8 [j]	1251.0 ± 63.6 [j]	1272.0 ± 61.4 [j]
H+C	1220.0 ± 65.0	1285.3 ± 78.0	1348.8 ± 87.0
H+S	1218.0 ± 64.4	1277.0 ± 83.0	1316.4 ± 82.0
H+G	1227.0 ± 60.0	1272.0 ± 58.9	1285.0 ± 69.0
H+I	1225.0 ± 65.5	1268.0 ± 59.1	1288.0 ± 62.2
D	373.0 ± 43.6 [j]	359.0 ± 37.2 [a,j]	325.0 ± 37.1 [a,j]
D+C50	370.0 ± 42.4	552.0 ± 40.5 [b,k]	683.0 ± 42.6 [b,k]
D+C100	374.0 ± 43.9	970.0 ± 51.7 [b,d,e,f,k]	1233.0 ± 54.2 [b,d,e,f,k]
D+S100	384.0 ± 44.1	545.0 ± 43.0 [c,k]	770.0 ± 37.5 [c,k]
D+S150	393.0 ± 48.3	697.0 ± 42.9 [c,d,g,h,k]	910.0 ± 40.0 [c,d,g,h,k]
D+G	374.0 ± 45.4	418.0 ± 42.2 [e,g]	500.0 ± 38.1 [e,g]
D+I	377.0 ± 41.7	434.0 ± 39.0 [f,h,k]	555.0 ± 37.2 [f,h,k]

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; [a]: Significant differences between Diabetic group and all other groups; [b]: Significant difference between diabetic groups treated with different Crocin concentrations (P<0.01); [c]: Significant difference between diabetic groups treated with different SAE concentrations (P<0.01); [d]: Significant difference between high doses of Crocin and SAE (P<0.01); [e]: Significant difference between diabetic group treated with high doses of Crocin and Glycerol (P<0.01); [f]: Significant difference between diabetic group treated with high doses of Crocin and Myoinositol (P<0.01); [g]: Significant difference between diabetic group treated with high doses of SAE and Glycerol (P<0.01); [h]: Significant difference between diabetic group treated with high doses of SAE and Myoinositol (P<0.01); [j]: Significant difference between diabetic and Healthy groups (P<0.01); [k]: Significant differences between 3-month period and 5-month period in each group (P<0.001).*

NO Level

Table 5 shows that NO production increased significantly (P<0.05) in diabetic groups. However, no significant differences existed between the healthy and diabetic groups with or without treatment. In addition, there were no significant differences between diabetic group without treatment, and diabetic groups received the mentioned treatments at the end of the experiment. These treatments did not significantly affect AOPP in healthy rats during five months.

Hepatic oxidative stress enzymes

CAT: The specific activity of catalase is shown in **Figure 1**. It significantly (P<0.001) decreased in diabetic rats compared to the control group. Although there were no significant changes in the healthy groups under treatment, it significantly (P<0.001) increased after administration of the mentioned treatments in diabetic rats. The order of effectiveness was as follows:

D+C100>D+C50>D+S150>D+S100> D+G≈D+I

SOD: **Figure 2** shows SOD specific activity in the liver of rats in different groups. Healthy rats had no changes in this parameter during the experiment. However, the results indicate a significant (P<0.001) decrease in SOD specific activity in diabetic rats compared to the control group. After administration of the mentioned treatments in diabetic rats, SOD significantly (P<0.001) increased. The order of effectiveness was as follows:

D+C100>D+C50>D+S150>D+S100>D+G≥ D+I

GSH: The mean GSH level in the liver tissues of healthy and diabetic rats is shown in **Figure 3**. No significant differences were observed between healthy groups before and after treatments. However, GSH levels in all diabetic groups were significantly less than in healthy groups (P<0.001). Although various treatments used in this study significantly increased the GSH level in diabetic rats, there were different degrees of increase in each group. The order of effectiveness was as follows:

D+C100>D+S150≥D+C50>D+S100>D+I≥ +G

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Table 4. The serum advanced oxidation protein products (AOPP) (μmol/l) levels in different groups and time course of the experiment.

Groups	0 month	3rd month	5th month
H	25.0 ± 5.6 [j]	29.0 ± 6.3 [a]	34.0 ± 6.1 [a]
H+C	25.0 ± 5.7	28.5 ± 6.1	33.5 ± 6.4
H+S	24.5 ± 5.6	29.0 ± 6.1	34.0 ± 6.5
H+G	24.5 ± 5.7	29.5 ± 6.3	34.5 ± 7.4
H+I	25.0 ± 6.8	29.5 ± 7.0	34.5 ± 8.3
D	50.0 ± 6.4 [a]	75.0 ± 8.4 [a]	95.0 ± 10.1 [a]
D+C50	50.0 ± 6.7	70.0 ± 8.4	87.0 ± 8.6
D+C100	50.0 ± 6.7	65.0 ± 8.4 [b]	82.0 ± 8.6 [b]
D+S100	50.0 ± 6.8	71.0 ± 8.1	88.0 ± 8.7
D+S150	50.0 ± 6.4	69.0 ± 6.1 [c]	86.0 ± 6.2 [c]
D+G	50.0 ± 6.4	73.0 ± 6.4	93.0 ± 6.2
D+I	50.0 ± 6.1	74.5 ± 6.6 [b,c]	94.0 ± 6.2 [b,c]

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy;* [a]: Significant difference between Diabetic and Healthy groups (P<0.001); [b]: Significant difference between Diabetic group treated with high doses of Crocin and Myoinositol (P<0.05 [c]: Significant difference between Diabetic group treated with high doses of SAE and Myoinositol (P<0.05).

Table 5. The serum Nitric Oxide (NO) levels (μmol/l) in different groups and time course of the experiment.

Groups	0 month	3rd month	5th month
H	27.0 ± 4.1 [a]	29.0 ± 4.1 [a]	30.0 ± 4.1 [a]
H+C	27.0 ± 4.1	27.5 ± 4.1	28.0 ± 4.1
H+S	26.0 ± 4.1	27.0 ± 4.1	28.0 ± 4.1
H+G	26.0 ± 4.1	27.5 ± 4.1	29.0 ± 7.1
H+I	27.0 ± 4.1	28.0 ± 4.1	29.0 ± 4.1
D	74.0 ± 6.2 [a]	82.0 ± 6.2 [a]	88.1 ± 6.3 [a]
D+C50	75.0 ± 4.2	75.0 ± 4.2	76.8 ± 4.2 [a]
D+C100	75.0 ± 4.2	74.0 ± 4.2	74.0 ± 4.2 [a]
D+S100	76.0 ± 8.1	77.0 ± 4.2	79.6 ± 4.2
D+S150	74.0 ± 4.2	75.0 ± 6.2	77.5 ± 4.3 [a]
D+G	75.0 ± 4.2	79.3 ± 4.2	82.0 ± 4.2
D+I	75.0 ± 4.1	78.1 ± 4.2	80.0 ± 5.4

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; [a]: Significant difference between Diabetic group and all other groups (P<0.01).*

Discussion

The present study evaluated the antioxidant activity of two doses of SAE and Crocin, a digentiobiosyl derivative of Crocetin containing several OH groups, in STZ-induced T2DM rats. Then, the obtained results were compared on liver antioxidant enzymes (SOD and CAT) and some oxidative stress markers (AGEs, AOPP, NO, and GSH) in serum and FRAP in plasma of T2DM rats treated with two chemical chaperones from polyol family, Glycerol, and Myoinositol. The results indicated various degrees of effectiveness of all treatments, but high dose of Crocin was more effective than the others.

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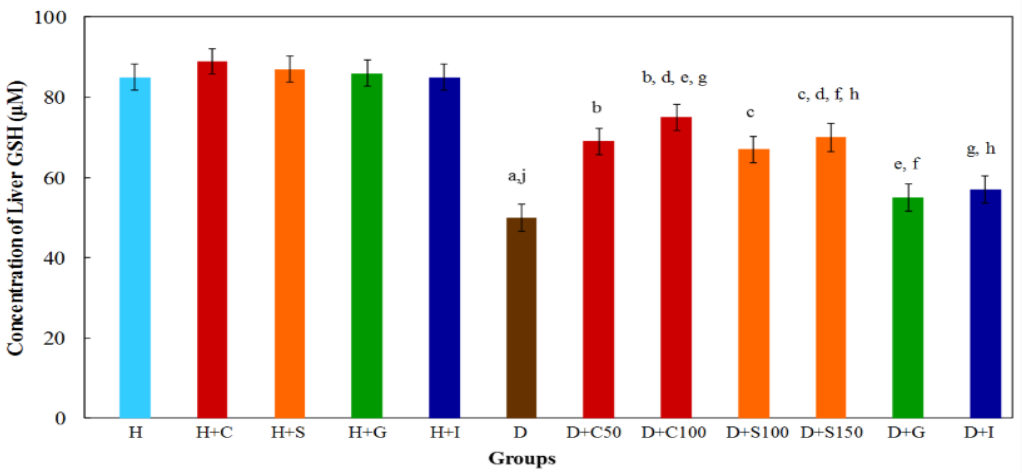

Figure 1. The levels of liver Glutathione (GSH). At the end of the experiment, GSH concentration in liver of all groups of rats. The statistical significance of the data is shown with letters over each column.

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; The letters show a significant difference between different groups as follows: a: Significant differences of the diabetic group with all other groups. b: Significant difference between diabetic groups treated with different Crocin concentrations (P<0.001); c: Significant difference between diabetic groups treated with different Saffron aqueous extract (SAE) concentrations (P<0.05;. d: Significant difference between high doses of Crocin and SAE (P<0.001;. e: Significant difference between diabetic group treated with Crocin and Glycerol (P<0.001); f: Significant difference between diabetic group treated with Crocin and Myoinositol (P<0.001); g: Significant difference between diabetic group treated with SAE and Glycerol (P<0.001); h: Significant difference between diabetic group treated with SAE and Myoinositol (P<0.001); i: Significant difference between diabetic group treated with Glycerol and Myoinositol (P<0.001); j: Significant difference between diabetic and Healthy groups (P<0.001).*

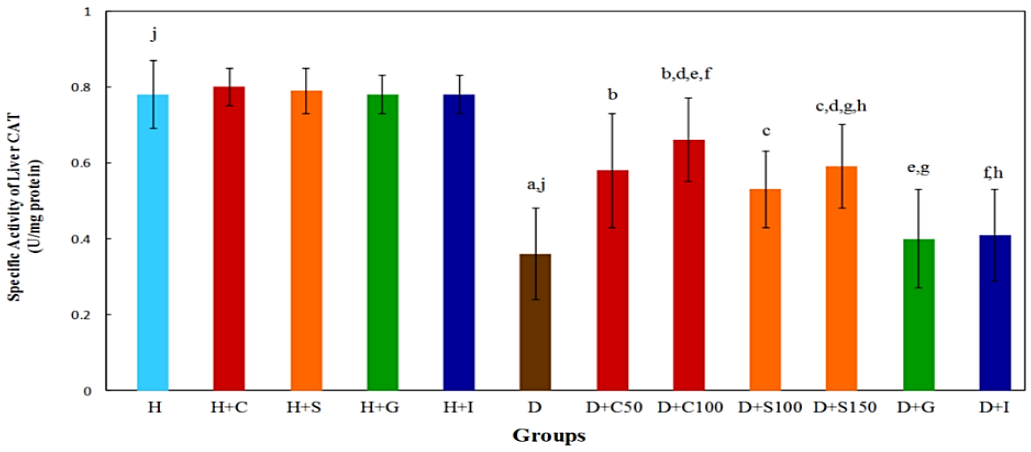

Figure 3. The Liver Catalase (CAT) activity. CAT specific activity in liver of all group rats at the end of the experiment. The statistical significance of the data is shown with letters over each column.

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; The letters show a significant difference between different groups as follows: a: Significant differences between diabetic group and all other groups; b: Significant difference between diabetic groups treated with different Crocin concentrations (P<0.001); c: Significant difference between diabetic groups treated with different Saffron aqueous extract (SAE) concentrations (P<0.001); d: Significant difference between high doses of Crocin and SAE (P<0.001); e: Significant difference between diabetic group treated with Crocin and Glycerol (P<0.001); f: Significant difference between diabetic group treated with Crocin and Myoinositol (P<0.001); g: Significant difference between diabetic group treated with SAE and Glycerol (P<0.001); h: Significant difference between diabetic group treated with SAE and Myoinositol (P<0.001); i: Significant difference between diabetic group treated with Glycerol and Myoinositol (P<0.001); j: Significant difference between giabetic and healthy groups (P<0.001).*

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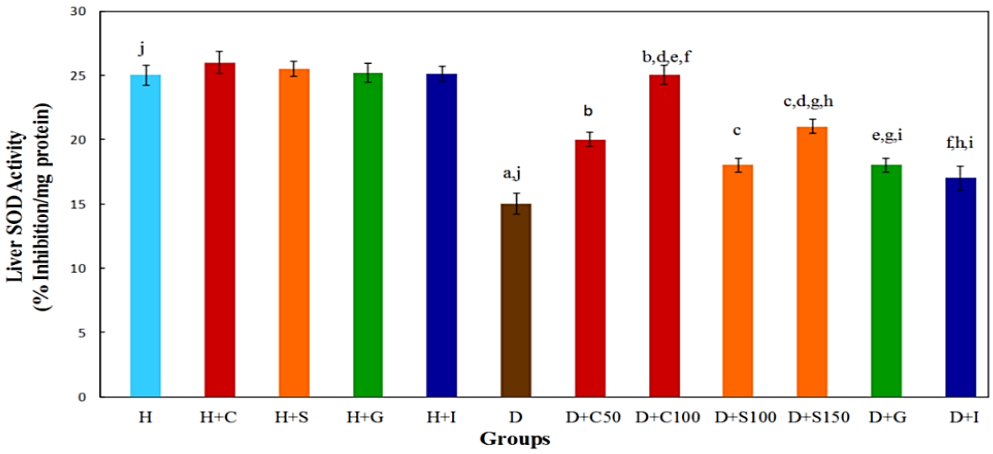

Figure 2. The Liver Superoxide dismutase (SOD) activity. The inhibition of SOD activity per mg protein in liver of all groups of rats at the end of the experiment. The statistical significance of data is shown with letters over each column.

***D**: diabetic; **S**: Saffron aqueous extract (SAE); **C**: Crocin; **G**: Glycerol; **I**: Myoinisitol; **H**: Healthy; The letters show a significant difference between different groups as follows: a: Significant differences of diabetic group and all other group;. b: Significant difference between diabetic groups treated between different Crocin concentrations (P<0.001); c: Significant difference between diabetic groups treated with different Saffron aqueous extract (SAE) concentrations (P<0.001); d: Significant difference between high doses of Crocin and SAE (P<0.001); e: Significant difference between diabetic group treated with Crocin and Glycerol (P<0.001); f: Significant difference between diabetic group treated with Crocin and Myoinositol (P<0.001); g: Significant difference between diabetic group treated with SAE and Glycerol (P<0.001); h: Significant difference between diabetic group treated with SAE and Myoinositol (P<0.001); i: Significant difference between diabetic group treated with Glycerol and Myoinositol (P<0.001); j: Significant difference between diabetic and healthy groups (P<0.001).*

Different pathways are involved in the excess free radical production in diabetes. Abnormally high levels of free radicals may damage cellular organelles and enzymes, increase lipid peroxidation, and develop insulin resistance. In addition, more damage occurs in cells and organelles because of the failure of antioxidant defense mechanisms (Unuofin and Lebelo, 2020). Determination of oxidative parameters in animals' liver, serum, and plasma confirmed these claims due to diabetes induction. The data indicated increased AGEs, AOPP, and NO levels and decreased FRAP, GSH, SOD, and CAT activities in diabetic rats. These observations are consistent with previous reports in diabetic patients (Kalousova *et al.*, 2002) and rats (Drikvandi *et al.*, 2020)

As in previous studies (Jafarnejad, 2007, Sato *et al.*, 1996), we administered Glycerol and Myoinositol in drinking water. The role of Glycerol (Jafarnejad, 2007) and Myoinositol (Shirali, 2011) in inhibiting protein glycation and misfolding was demonstrated. The proposed mechanism for protein stabilization by polyols was preferentially hydration and increased viscosity of target proteins, which caused editing of misfolded proteins (Arakawa *et al.*, 2006). Polyols change the environment of proteins and conserve their native structure (Arakawa *et al.*, 2006, Jafarnejad, 2007).

Pharmacokinetic studies have shown that oral administration of Crocin results in its intestinal excretion, and most of administered Crocin was detected in feces of rats (Xi *et al.*, 2007). In addition, intestinally absorbed Crocin is hydrolyzed in the blood and converted to Crocetin in rats and mice (Asai *et al.*, 2005, Xi *et al.*, 2007). Human studies have also indicated that Saffron administration results in Crocetin appearance in serum of participants (Chryssanthi *et al.*, 2011, Mohammadpour *et al.*, 2013). These findings are confirmed by the present study on the effectiveness of SAE and Crocin in preventing metabolic syndrome in Schizophrenia (Fadai *et al.*, 2014). Safety evaluation studies in animals and humans indicated high safety of SAE and Crocin (Mousavi *et al.*, 2015, Taheri *et al.*, 2014). We previously reported hypolipidemic and hypoglycemic effects of SAE and Crocin in STZ-induced T2DM in rats

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(Shirali *et al.*, 2012, Shirali *et al.*, 2013). Thus, these parameters are not studied/reported here.

The present study indicated different degrees of decrease in serum level of AGEs in all rat groups treated with the treatments. It means that SAE, Crocin, Myoinositol, and Glycerol prevented AGE formation and significantly inhibited the process of protein glycation. The results showed that these herbal preparations are more effective than the mentioned polyols. When comparing SAE and Crocin, their higher doses were more effective than lower doses. In addition, the high dose of Crocin (100 mg/kg) was more effective than the high dose of SAE (150 mg/kg). Crocin is a dominant component and main carotenoid of saffron stigma (Bathaie *et al.*, 2014). Thus, the current study used lower doses of Crocin (50 and 100 mg/kg) compared to SAE (100 and 150 mg/kg). However, Crocin was still more potent than SAE in inhibiting AGEs formation. Between polyols, Glycerol was more effective than Myoinositol in AGE formation inhibition.

Similar findings were observed in FRAP and AOPP levels, which means that the mentioned treatments decreased the serum oxidants. Although Crocin antioxidant activity has been reported in diabetes from different aspects (Bastani *et al.*, 2022, Behrouz *et al.*, 2020, Radmehr *et al.*, 2022, Ronsisvalle *et al.*, 2023, Samaha *et al.*, 2019, Yaribeygi *et al.*, 2021), its effectiveness was not compared with saffron extract and polyols.

This study demonstrated the increased serum level of NO in T2DM rats. There was controversy in serum NO level of T2DM in patients and animal models. Some studies indicate an increase in NO levels (Abou-Seif and Youssef, 2004, Zahedi Asl *et al.*, 2008), while others report a decrease (Ghosh *et al.*, 2011, Tessari *et al.*, 2010). However, an increased level of serum NO has been attributed to the activation of the inducible isoforms of NO synthase. Overproduction of ROS in diabetes mellitus stimulates the activation of both endothelial and inducible isoforms of NO, increasing NO levels (Abou-Seif and Youssef, 2004). Moreover, hyperglycemia-induced ROS production, in turn, activates several other damaging pathways, including the AGEs pathway (Brownlee, 2005) and NO synthase activation (Stirban *et al.*, 2014). However, the mentioned treatments here had no significant effect on this parameter, especially after five months.

The superoxide radical is transformed into H_2O_2 through the action of SOD, which is then converted to molecular oxygen and H_2O by CAT. Previous research has shown a decrease in antioxidant defense enzymes like SOD and CAT in diabetes (Diniz *et al.*, 2022). The present study confirmed decreased activities of hepatic enzymes and GSH (key antioxidant markers) similar to past findings (Drikvandi *et al.*, 2020). Reduced activity of SOD and CAT could be due to two mechanisms: inefficient ROS scavenging leading to oxidative enzyme inactivation (Brownlee, 2005), and enzymes glycation causing their inactivation. Glycation and inactivation of some proteins, including molecular chaperones, were also observed (Bathaie *et al.*, 2010). The present study also reported low CAT specific activity, consistent with previous observations in diabetic patients due to prolonged oxidative stress (Goth *et al.*, 2001). Reactivation effects of Crocin and SAE on these enzymes have been documented (Bahmani *et al.*, 2016).

The liver has been known as the major organ of GSH synthesis, a free radical scavenger that transfers it to other tissues. GSH detoxifies various xenobiotics in the liver (Seven *et al.*, 2004). A significant reduction was observed in GSH concentration in T2DM rats' livers, which increased to different degrees after treatments.

This study used a limited number of doses of each component. Although the choice of these doses was according to our previous studies, using a wider range of doses may have resulted in more convenient results. A key strength of the study lies in its *in vivo* evaluation and comparison of the antidiabetic properties of Crocin, SAE, and various polyols, particularly focusing on their antioxidant and antiglycation effects. The limitation of the study is a different method of Crocin administration compared to other polyols.

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Conclusion

Both polyols and Saffron components show beneficial effects on overcoming diabetes complications. There were no significant changes between healthy rats treated with these compounds with control rats. Thus, there were no adverse effects. Moreover, 100 mg/kg Crocin after five months of treatment was the best choice and more effectively improved oxidative and glycation markers in the serum (FRAP and AGEs) and antioxidant parameters in the liver (GSH, SOD, and CAT) of rats. This effect of Crocin is related to its higher potency as an antioxidant and prevention of protein glycation.

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Authors' contribution

SZ Bathaie designed the research; S Shirali conducted the research; M Mohyadini analyzed the data; S Shirali, M Mohyadini, and SZ Bathaie wrote the draft. M Mohyadini and SZ Bathaie completed and edited the paper. SZ Bathaie had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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