The Potential protective effect of crocin against hyperhomocysteinemia induced oxidative stress in rats.

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A B S T R A C T

Crocin is the major yellow pigment of saffron and gardenia yellow, which are extracts of Crocus sativus stigmas and Gardenia jasminoides fruits, crocin can prevent chronic stress induced oxidative stress damage in the brain, liver and kidneys, the purpose of this study was to evaluate the protective and anti-inflammatory effect of crocin against L-methionine induced hyperhomocysteinemia and oxidative stress in rats. Thirty male albino rats were divided into three equal groups. Group I (normal group): rats administered distilled water. Group II (L-Methionine induced hyperhomocysteinemia (HHcy): rats received L-methionine (1.7 g/kg b.w) orally daily for continuous 8 weeks. Group III (HHcy + crocin treated group) rats received crocin for 4 weeks after induction of hyperhomocysteinemia inrta peritoneal once per day at a dose of (50 mg/kg body weight/day, I.P). The obtained results showed significant increase in serum homocysteine, lipid profile (cholesterol, triglyceride), liver enzymes (ALT, AST and ALP) activities, liver tissue L-MDA levels and inflammatory markers (TNF-α and IL-8) in hyperhomocysteinemic (HHcy) rats. However, activities of liver tissue antioxidant enzymes SOD and GSH concentration were markedly decreased. Administration of crocin to HHcy rats caused significant improvement of all previous parameters towards its normal ranges. These results suggested that, crocin treatment may have a protective effect against hyperhomocysteinemia induced oxidative stress in rats through free radical scavenging and anti-inflammatory activity as well as regenerating endogenous antioxidant defense system mechanisms.

Keywords: Hyperhomocysteinemia, crocin, oxidative stress

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1. INTRODUCTION

Hyperhomocysteinemia is the result of perturbed Hcy metabolism where regulating enzyme activities are disturbed, in condition such as dietary deficiencies in folic acid, vitamin B6, and/or vitamin B12 (Obeid et al., 2004). Increased Hcy levels are associated with several disorders, like cardio- and cerebrovascular diseases and neurodegenerative diseases, that affect the central nervous system (CNS), such as epilepsy, stroke, Alzheimer's disease, dementia, as well as with classical homocystinuria (Seshadri, 2012). Homocysteine (Hcy) is an intermediate sulfhydryl-containing amino acid derived from methionine. Hcy has two fates: remethylation to methionine (with the ease of methionine synthase enzyme) or transsulfuration to cysteine

(with cystathionine-β-synthase, enzyme (CBS) (Huang et al. 2007).

The increased production of ROS caused by Hcy may induce the subsequent oxidation of proteins, lipids and nucleic acids (Zou and Banerjee, 2005) and can lead to the endothelial dysfunction and damage to the vessel wall, followed by platelet activation and thrombus formation, accumulation of oxidized biomolecules alters the biological functions of many cellular pathways. Hcy acts as a potent oxidizing agent of -SH groups by reactive species production, such as superoxide anion (O2-) and hydrogen peroxide (H2O2), mainly during its auto-oxidation (Faraci and Lentz, 2004).

Crocin is one of these components, which is a natural carotenoid found in saffron (Crocus sativus L.) and gardenia (Gardenia jasminoides J. Ellis) flowers. It is a compound formed by a disaccharide called gentiobiose and a carboxylic acid called crocetin, which is soluble in water, and is in diester form with high thermal stability (Sánchez et al., 2011). The main active constituent of saffron is picrocrocin and its derivatives including safranal, flavonoid derivatives and crocin (Boskabady et al., 2008). Crocin pretreatment significantly prevented these increases. In agreement, it has been reported that crocin reduced the level of these enzymes after nicotine-induced hepatic injury (Jalili et al., 2015). Therefore, crocin, due to its antioxidant properties, could protect the liver cells from the damage caused by oxidative stress. The actions of crocin and crocetin in the manipulation of the inflammatory response have been scarcely studied by demonstrating the suppressive activities of crocin and crocetin on diverse pro inflammatory mediators including IL-8, TNF-α, and ROS (Yang et al., 2006). The powerful hypolipidemic activities can be directly linked with the presence of flavonoids in saffron as it is known that flavonoids have powerful hypolipidemic properties (Koshy and Vijayalakshmi, 2001). Moreover, in another study, authors demonstrated that crocin pretreatment protected the gastric mucosa against IR-induced insult via up regulating the mRNA expression and activity of antioxidant enzymes in rats So that the activity of all studied antioxidant enzymes (SOD) increased after crocin pretreatment (Mard et al., 2016). This study was to investigate the possible beneficial effect of spirulina against deleterious effect of hyperhomocysteinemia in adult male rats through investigation of Hcy, lipid profile, liver functions, inflammatory markers, oxidative stress biomarkers and enzymatic antioxidant status.

2. MATERIAL AND METHODS

2.1. Experimental animals:

Thirty white male albino rats of 10-12 weeks old and average body weight 150-200 g were used in this study. Rats were housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The rats were fed on constant ration and fresh, clean drinking water was supplied ad-libitum. All rats were acclimatized for minimum period of 15 days prior to the beginning of study.

2.2. Chemicals and antioxidant:

The antioxidant and chemicals used in the present study were:

A-L-methionine; 63-68-3; Methionine; H-Met-oh; (S)-2-Amino-4-(methylthio)

butanoic acid; L-(-)-Methionine was Purchased from El Gomhouria Company for Trading Chemicals and Medical Appliances, Egypt. Methionine was dissolved in in 1M HCL freshly prepared and orally administered at a dose (1.7 g/kg body weight/day) for 8 weeks (Sain et al., 2011)

B-Crocin was purchased from Sigma Company. It is used to treatment of hyperhomocysteinemia, dose level (50 mg/kg dissolved in saline I.P) (Hariri et al., 2011).

C- Other chemicals used in this study were of the highest purified grades available purchased from El Gomhouria Company for Trading Chemicals and Medical Appliances, Egypt.

2.3. Experimental design:

After acclimatization to the laboratory conditions, the animals were randomly divided into three groups (10 rats each) placed in individual cages and classified as follow:

Group I (normal control group): Rats received no drugs, served as control non- treated for all experimental groups.

Group II (L-Methionine induced hyperhomocysteinemia (HHcy): rats received L-methionine (1.7 gm/kg b.wt/ day) orally for continuous 8 weeks.

Group III (HHcy + crocin treated group) rats received crocin for 4 weeks after induction of hyperhomocysteinemia orally once per day at a dose of (50 mg/kg I.P).

2.4. Sampling:

2.4.1. Blood samples:

Blood samples were collected by ocular vein puncture from all animal groups after overnight fasting in dry, clean tubes and allowed to clot for 30 minutes and serum was separated by centrifugation at 3000 r.p.m for 15 minutes. The serum was taken by automatic pippte and received in dry sterile tubes, then kept in deep freeze at -20 °C until use for subsequent biochemical analysis. All sera were analyzed for determination of the following parameters: Hcy, Total cholesterol, Triacylglycerols, AST, ALT and ALP.

2.4.2. Tissue samples:

2.4.2.1. Liver tissue for biochemical analysis:

About 0.5 g of liver tissue specimen was taken from each group of rats after had been sacrificed. The specimens were immediately removed and washed several times with saline and blotted between two damp filter papers, weighed and stored at -20°C for subsequent biochemical analyses.

2.4.2.2. Preparation of liver tissue homogenate:

Briefly, liver tissues were cut, weighed and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH 7.4) to make 10 % homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4°C then the resultant supernatant was used for the determination of the following parameters: L-MDA and SOD.

About 0.2 g liver tissues were minced into small pieces homogenized with a glass homogenizer in 0.4 ml of 25% metaphosphoric acid (MPA) (ref. No.: 253-433-4, Sigma-Aldrich, Germany), then 1.4 mL of distilled water was added, mixed and incubated for 1 hour and centrifuged for 10 min at 3,000 r.p.m then the clear supernatant

was removed and used for determination of GSH concentration.

2.4.2.3.Liver tissue for molecular gene expression:

About 0.5 of liver tissue put in eppendorf tubes and were immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for determination of TNFα and IL-8 gene expression level.

2.5. 2.5. Biochemical analysis:

Serum Hcy was determined according to the method described by Rat homocysteine (Hcy) ELISA kit (My Bio Source, Cat# MBS703069), Total cholesterol, Triacylglycerols, ALT, AST and ALP were determined according to the method described by Ellefson and Caraway, (1976) and Stein, (1987), Schumann et al., (2002) and EL-Aaser and EL-Merzabani (1975) respectively. Liver tissue L-MDA, SOD and GSH were determined according to the method described by Mesbah et al., (2004), Kakkar et al., (1984), and Patterson and Lazarow, (1955), respectively. Moreover, the mRNA expression level of TNF-α and IL-8 was determined by real-time quantitative polymerase chain reaction (real- time qPCR) analysis in liver of rats. Target gene was normalized with β –actin by used the 2-ΔΔCt method (Livak and Schmittgen, 2001).

2.6. Statistical analysis:

The results were expressed as mean ± SE using SPSS (13.0 software, 2009) program. The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparison among the groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p<0.05$.

3. RESULTS

The data presented in table (1) showed a significant increase in serum homocysteine, total cholesterol and Triacylglycerol concentrations in L-methionine induced HHcy in rats when compared to normal control group. However, crocin treatment to HHcy male rats caused a significant decrease in elevated serum homocysteine, total cholesterol and Triacylglycerol concentrations when compared with L-methionine treated group.

The obtained results presented in table (2) revealed that, HHcy rats showed significant increase in serum ALT, AST and ALP activities when compared with normal control group. On the other hand, crocin treatment to HHcy male rats caused a significant decrease in elevated serum ALT, AST and ALP activities when compared with HHcy group.

The obtained data demonstrated in table (3) revealed that, HHcy rats showed significant increase in liver tissue L-MDA and significant up-regulation of TNFα and IL-8 all over the periods of the experiment when compared to normal control group. However, crocin treatment to HHcy rats caused a significant decrease in elevated liver tissue L-MDA and a significant down-regulation TNFα and IL-8 gene expression when compared with HHcy group.

The current results presented in table (4) exhibited significant decrease in liver SOD activity and GSH concentration in L-methionine treated rats when compared to normal control

group. Meanwhile, crocin treatment to HHcy male rats caused a significant increase in liver tissue SOD activity and marked increase in GSH level when compared with HHcy group.

Table (1): Effect of crocin administration on serum Hcy, total cholesterol and triacylglycerol concentration in HHcy induced in male rats.

Parameters Exp. groups	Hcy (nmol/ml)	TC(mg/dl)	TAG(mg/dl)
Group I: Normal control	10.24 ± 0.33 [e]	64.67 ± 4.63[b]	61.33 ± 2.60 [b]
Group II: (HHcy)	32.35± 0.72 [a]	106.0 ± 10.82 [a]	81.0 ± 3.06 [a]
Group III: HHcy + crocin	18.45±0.02 [c]	70.33 ± 3.28 [b]	61.33 ± 2.03 [b]

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (2): Effect of crocin administration on serum ALT, AST, and ALP activities in HHcy induced in male rats.

Parameters Exp. groups	ALT (U/L)	AST(U/L)	ALP(U/L)
Group I: Normal control	20.33 ± 1.45 [b]	22.67 ± 2.60 [b]	143.33 ± 11.14 [b]
Group II: (HHcy)	29.67 ± 2.19 [a]	38.67 ± 1.67	225.33 ± 17.74 [a]
Group III: HHcy + crocin	11.67 ± 3.28 [c]	12.0 ± 1.53 [c]	159.33 ± 8.67 [b]

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (3): Effect of crocin administration on liver tissue L-MDA, TNF-α and IL-8 in HHcy induced in male rats.

Parameters Exp. groups	L-MDA (mmol/g tissue)	Fold change in TNFα gene expression	Fold change in IL-8 gene expression
Group I: Normal control	5.34 ± 0.11 [b]	1.00 ± 0.02 [d]	1.00 ± 0.06 [d]
Group II: (HHcy)	7.71 ± 0.40 [a]	8.06± 0.31 [a]	9.99± 0.42 [a]
Group III: HHcy + crocin	5.43 ± 0.11[b]	2.50±0.11 [c]	2.22±0.1 [c]

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (4): Effect of crocin administration on liver tissue SOD activity and GSH concentration in HHcy induced in male rats.

Parameters Exp. groups	SOD (u/g.tissue)	GSH (ng/g.tissue)
Group I: Normal control	47.48 ± 2.05 [a]	5.06 ± 0.13 [b]
Group II: (HHcy)	21.75 ± 1.21 [b]	3.21 ± 0.21 [d]
Group III: HHcy + crocin	45.53 ± 4.31[a]	4.77 ± 0.07 [bc]

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

4. DISCUSSION

Hyperhomocysteinemia can be caused by genetic deficiencies in methionine and homocysteine metabolism, including cystathionine b-synthase, methionine synthase and methylene tetrahydrofolate reductase (MTHFR) deficiencies (Testai and Gorelick, 2010). The obtained results showed that, L-methionine induced HHcy rats showed significant increase in serum homocysteine, Total cholesterol and Triacylglycerol concentration all over the periods of the experiment when compared to normal control group. These results are nearly similar to those reported by (Prasanna and Ashok, 2011) recorded that, treatment of methionine (1g/kg, p.o.) for 30 days in pathogenic control group rat's elevated level of serum homocysteine, total cholesterol and triglycerides and atherosclerotic index values. Additionally, (Lan et al., 2011), who reported that, L-Methionine in earlier studies has also been demonstrated to induce endothelial dysfunction so that, given high levels of lipids as well as Hcy have been documented to enhance the production of free radicals with subsequent increase in oxidative stress. This increase of homocysteine level due to several disorders, like cardio- and cerebrovascular diseases and neurodegenerative diseases (Maron and Loscalzo, 2009) that affect the central nervous system (CNS), such as epilepsy (Herrmann and Obeid, 2011), stroke (Sawula et al., 2008), Alzheimer's disease (Piazza et al., 2012). So that, the development and progression of atherosclerosis is considered to be a form of chronic inflammation (Ross et al, 1999). Hcy enhances the production of several pro inflammatory cytokines indicates that moderate HHcy directly leads to endothelial dysfunction and premature atherogenesis showed significant increase in serum homocysteine, Higher levels of Hcy (Lan et al., 2011) and cholesterol (Yunoki et al., 2011) are suggested to be responsible for development of endothelial dysfunction. L-Methionine in earlier studies has also been demonstrated to induce endothelial dysfunction. Also, Woo et al., (2005) concluded that, hyperhomocysteinemia caused an activation of several transcription factors in the liver leading to increased HMG-CoA reductase and cholesterol biosynthesis. As a consequence, hepatic lipid accumulation and hypercholesterolemia occurred.

Treatment with crocin to hyperhomocysteinemic male rats caused a significant decrease in serum Hcy, total cholesterol and triacylglycerol concentration in all over the periods of the experiment. These results are nearly similar to those recorded by (Rinki et al., 2015) who reported that significant decrease in level of homocysteine in saffron treated HHcy rats. Also, (Asdaq SMB, Inamdar, 2010) showed that crocin treatment to hyperhomocysteinemic male rats resulted in a significant decrease in in total cholesterol and triacylglycerol concentration after four weeks. Moreover, crocin is the main constituents of saffron that has antioxidant activities therefore; saffron was more efficient than crocin probably due to synergistic action of many constituents such as crocin, dimethyl crocetin, safranal, and flavonoids that have antioxidant effects. This may have a role in protective effect of saffron on hyperlipidemic stress (Zheng et al., 2007).

Presented findings showed that, HHcy significant increase in serum ALT, AST and ALP activities all over the periods of the experiment when compared to normal control group. These results are nearly similar to those reported by (Ramesh et al., 2012) who recorded that the increases in ALT, AST, ALP levels are thought enzymes are considered to be the markers of organ dysfunction, indicator of cellular damage, cell leakage and the loss of cell membrane integrity in the liver. The increase of methionine might lead to liver oxidative stress increment and Hcy itself has the ability to generate potent reactive oxygen species (ROS) when oxidized by highly reactive sulfhydryl group (Yamada et al., 2012).

Crocin treatment to HHcy male rats caused a significant decrease in elevated serum ALT, AST and ALP activities when compared with HHcy group. These results are nearly similar to those reported by (Jalili et al., 2015). who reported that, crocin decreases the concentrations of these enzymes. These results showed that administration of crocin as a potent antioxidant for a week effectively enhanced the antioxidant capacity in liver tissue (Jalili et al., 2015). Moreover, the carotenoids in saffron extracts may protect tissues from oxidative damages due to their antioxidant effect (Zheng et al., 2007) researches show remarkably modulation in the levels of oxidative markers in the brain, liver caused by crocin. As it was mentioned previously, the brain tissue is highly vulnerable to oxidative stress (Metodiewa and Kośka, 2000).

The obtained results demonstrated that, HHcy significant increase in liver tissue L-MDA and significant up-regulation of TNFα and IL-8 in all over the periods of the experiment when compared to normal control group. Similarly, (da Cunha et al., 2012) who reported that, one of the effects of HHcy is an increased lipid peroxidation and protein oxidation. Therefore, we have investigated the effect of chronic HHcy on some parameters of lipid oxidation and oxidative damage of proteins. This increase may be due to homocysteine is a thiol containing amino acid derived from demethylation of dietary methionine, may generate partially reduced ROS that are able to stimulate the lipid peroxidation involved in the atherosclerotic process. (Toborek et al., 1995). Additionally, (Zhang et al., 2011) recorded that, HHcy induced atherosclerosis and atherosclerosis activates further release of cytokine-signaling molecules that recruit more inflammatory cells. The inflammatory cells most involved are monocytes and T-cells that can release MCP-1 .The more mature plaques contain dendritic cells, mast cells, B cells, and natural killer T-cells .Several of these cells are activated, and produce inflammatory cytokines like TNF-α. Furthermore, Jablonski et al., (2011) studied that, activation of NF-κB has been demonstrated to induce endothelial dysfunction in HHcy. Also, Poddar et al., (2001) reported that, homocysteine has also been shown to increase expression of IL-8. Moreover, HHcy stimulates the expression of MCP-1, in rats, leading to increased monocyte adhesion to the aortic endothelium. Such an effect may contribute significantly to the development of atherosclerosis by facilitating monocyte/macrophage infiltration into the arterial wall .

Crocin treatment to HHcy rats caused a significant decrease in elevated

liver tissue L-MDA and a significant down-regulation TNFα and IL-8 gene expression when compared with HHcy group. These results are nearly similar to the findings of, (Fernandez, 2004) who reported that, the powerful hypolipidemic activities of crocin and saffron can be directly linked with the presence of flavonoids as it is known that flavonoids have powerful hypolipidemic properties saffron and crocin prevented the elevation of MDA, GSH in serum resulting in potent antioxidant effect .This may have a role in protective effect of saffron on hyperlipidemic stress (Asdaq and Inamdar, 2010). Additionally, Nam et al., (2010) who reported that orally administration of crocin at dose of 150 mg/kg for 6 weeks daily documented the anti-inflammatory effect of crocin was significant when its effects on inflammation developed in the diabetic group. So that, it was determined that plasma TNF-α pancreas tissue down regulate cytokine release and suppressed NF-κB activation. This increased pro-inflammatory state was markedly ameliorated after the 8-week treatment with saffron extract and crocin. A significant inhibition of TNF-a level by saffron observed in the recent study contributed to beneficial effects in diabetic encephalopathy (Samarghandianet al., 2014). Moreover, (Mashmoul et al., 2014) demonstrating that antioxidant-rich saffron has the potential to modulate obesity and related metabolic disorders such as hyperglycemia, hyperlipidemia, insulinaemia and pro inflammatory derived complications.

The Presented data showed that, significant decrease in SOD and GSH activity was observed in HHcy rats all over the periods of the experiment when compared to normal control group. These results are nearly similar to those reported by (Sharma and Singh, 2011) showed that, L-Methionine treatment has induced a rise in superoxide anion in aortic strip as well as TBARS in serum and brain, along with reduction in brain GSH levels in this study, which is a reflection of oxidative stress and is probably one of the major contributing factors in L-methionine, induced endothelial dysfunction. Furthermore, we have previously suggested that hyperhomocysteinemia induces typical apoptotic changes, which are believed to be associated with increased oxidative stress, in hippocampus of rats (Baydas et al., 2005). This concurs with the present findings, wherein the levels of LPO were found to be significantly increased in the animals subjected to methionine treatment. Due to this increased lipid peroxidation, GSH levels are lowered (Flohe, 1989). Moreover, SOD was targeted for oxidation by free radicals that reduced their activities in hyperhomocysteinemia rats. Excessive production of ROS may induce protein damages resulting in a significant accumulation of oxidized and ubiquitinated proteins observed both in heart and aorta tissues (Miller et al., 2000).

Crocin treatment to HHcy male rats caused a significant increase in liver tissue SOD activity and a significant increase GSH activity all over the period of experiment when compared with HHcy group. These results came in accordance with the recorded data of (Mehri et al., 2015).The combined data suggests that the most important mechanism underlying the protective effects of crocin is its antioxidant activity reducing levels of the lipid peroxidation while elevating GSH in

affected organs. Additionally, the generation of ROS, free radicals and decreased GSH levels significantly increased oxidative stress; the overall effect of depletion of glutathione with concomitant increases in lipid peroxide level provides (Nagaraj et al., 2011). Also, (Pari and Latha, 2004) documented that, the aqueous extract of Crocus Sativus plant has got antioxidant activity which may resist against pathological alterations caused by the free radicals. Crocin is able to suppress the generation of ROS by various oxidative stresses.

The present study demonstrated that, administration of spirulina relieved actions and harmful effects caused by exposure to L-methionine induced HHcy. HHcy affected different organs mainly liver and these occurred through affected in several parameters. L-methionine induced HHcy caused significant increase in serum Hcy, TC,TG, AST, ALT, ALP and liver tissue L- MDA, TNFα and IL-8, however, a significant reduce in liver tissue SOD and GSH. Crocin treatment in HHcy rats relieved all previous parameters towards its normal range with best result after 4 weeks. So, these results confirm the strong antioxidant, anti-inflammatory effects of spirulina in HHcy.

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