www.sphinxsai.com

PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.1, No.4, pp 1317-1321, Oct-Dec 2009

THE FREE RADICAL SCAVENGING AND THE LIPID PEROXIDATION INHIBITION OF CROCIN ISOLATED FROM KASHMIRI SAFFRON *(CROCUS SATIVUS)* OCCURRING IN NORTHERN PART OF INDIA

Bakshi Hamid[1,2*],Smitha Sam[1] ,Tajamul Islam[2] ,Priyam Singh[2] and Manik Sharma [2]
[1]Jawaharlal Nehru Cancer Hospital and Research Center Bhopal, India-462001.
[2]Department of Cancer biology and Biotechnology, Bhoj College of science, Bhopal, India-462001.

***Corres.author: hamid_bakshi@rediffmail.com, Cell No. 09826979751.**

ABSTRACT: Background: Kashmiri saffron is considered as a panacea for several disease conditions. The cellular damage caused by reactive oxygen species (ROS) has been implicated in several diseases and hence antioxidants have significant importance in disease management. This study elucidates the free radical scavenging and lipid peroxidation inhibition of crocin from *Crocus sativus*.
Methods: Free radical scavenging and lipid peroxidation were evaluated by DPPH (1, 1- diphenyl 2- picryl hydrazyl) assay, FRAP (Ferric reducing antioxidant power) assay, Fe^{2+}ascorbate induced lipid peroxidation and hydroxyl radical scavenging.
Results: The total crocin isolated from the *Crocus sativus* showed potent DPPH radical scavenging capacity. 0.1% of the total crocin showed 29.36% DPPH radical scavenging activity and, 0.5% showed 44.52% where as 1.0% showed 50.07% DPPH radical scavenging activity equivalent to 0.019% of trolox . The crocin at a concentration of 0.1% showed ferric reducing ability equivalent to 0.058% of trolox, for 1.0% ferric reducing ability was equivalent to 0.356% of trolox. The IC_{50} value of crocin in inhibiting lipid peroxidation was 179 ± 6.7 where as the IC50 value of crocin for hydroxyl radical scavenging was 227 ± 3.8. It was considered statically significant ($P<0.05$).
Conclusion: Our preclinical studies demonstrate that crocin is effective in inhibiting free radical formation and also to scavenge the free radicals. Our data also suggest that the lipid peroxidation inhibiting effects of crocin induced by Fe^{2+} ascorbate system and shows potential for treatment/or prevention .
Keywords: *Crocus Sativus,* Crocin, Antioxidant, DPPH assay, FRAP assay

INTRODUCTION

Reactive oxygen species (ROS) are produced in the cells by cellular metabolism and other exogenous environmental agents. They are generated by a process known as red ox cycling and are catalyzed by transition metals, such as Fe2+ and Cu2+ [1.] In vivo, some of these ROS play positive roles such as energy production, phagocytosis, regulation of cell growth and intercellular signaling, or synthesis of biologically important compounds [2]. However, ROS may also be very damaging as they can induce oxidation of lipids, causing membrane damage, decreasing membrane fluidity, and leading to cancer via DNA mutation [3, 4]. A potent scavenger of these ROS may serves as a possible preventative against free radical mediated diseases [5] . Crocin, one of the active components of saffron, is a carotenoid pigment and has
the structure of crocetin di-gentiobiose ester [6] . Crocin exhibits a variety of pharmacological effects in mice including inhibition of skin tumour growth [7], improvement of learning behavior previously impaired by ethanol [8], and prevention of long-term potentiation inhibition caused by ethanol in rat [8], anti-hyperlipidemic effect [9], and treatment of colon adenocarcinoma in rat [10] anti-atherosclerotic effects [11].
The aim of the present study is to assess the free radical scavenge and lipid per oxidation inhibition of crocin from Kashmiri saffron. Our results suggested that the crocin isolated from *C.sativus* exhibited significant *in vitro* antioxidant activity.

MATERIALS AND METHODS

Animals

Male Swiss albino mice 6-8 weeks of age and weighing 25 ± 2 g, were selected from our mouse colony. They were maintained in environmentally controlled conditions with free access to standard food (Sai Durga Foods, Bangalore.) and water. Animal experiments were carried out with the approval and guidelines of Institutional Animal Ethical Committee.

Chemicals

Acetic acid, methanol, anhydrous sodium sulphate, sulphuric acid, ferrous sulphate ($FeSO_4$), hydrochloric acid, potassium chloride (KCl), ferrous ammonium sulphate ($FeSO_4(NH_2)_2SO_4$ $7H_20$), sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), pyridine, butanol, ethylene diamine tetra acetic acid (EDTA), hydrogen peroxide (H_20_2), potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), sodium chloride, acetic anhydride and formalin were purchased from Merck India Ltd, Mumbai. 1.1-diphenyl-2-picryl hydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8–tetramethyl chroman –2-carboxylic acid (Trolox), 2, 4, 6-tripyridyl-s-triazine (TPTZ) and carrageenan were purchased from Sigma Chemical Co. USA. Chloroform, anisaldehyde, ferric chloride ($FeCl_3$), tris-HCl, ascorbic acid and deoxyribose were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai.

Isolation of Crocin From saffron

Crocin was isolated from saffron by previously described method[12] with some modification. Briefly 500-mg saffron was washed thrice with 20-ml ethyl ether, and the residual ether was evaporated in air. It was then suspended in 15 ml of 30% methanol (v/v) in distilled water and stirred for 5 min at room temperature. The extract was filtered through a 0.45-µm Millipore filter. It was then diluted with 10 mmol/lphosphate-buffered saline (PBS, pH=7.4), and the concentration of crocin was adjusted to 25 µmol/l, using the coefficient e443=89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution [13] . Crocin was stored at -20 °C in the dark for a maximum of 2 months [14] .The crocin extracted from Kashmiri saffron was shown to possess similar spectral characteristics.

DETERMINATION OF *IN VITRO* ANTIOXIDANT ACTIVITY

DPPH radical scavenging assay

In this method, a commercially available and stable free radical ($DPPH^+$, 2, 2-diphenyl-1-picrylhydrazil), which was soluble in methanol, was used[15]. DPPH in its radical form has an absorption peak at 515 nm, which disappeared on reduction by an antioxidant compound. An aliquot (37.5 µl) of Crocin was added to 1.5ml of freshly prepared DPPH solution (0.25g/l in methanol). Absorbance was measured at 515 nm, 20min after the reaction. DPPH radical scavenged was calculated from the calibration curve of %DPPH scavenged versus concentration of the standard antioxidant (Trolox) [16].

Ferric reducing antioxidant power (FRAP) assay

The ferric- reducing ability was measured at low pH [17] . The stock solution of 10mM 2, 4, 6-tripyridyl-s-triazine (TPTZ), 40mM HCl, 20mM $FeCl_3.6H_2O$, and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent was mixed with 90µl water and 30µl test sample/methanol/distilled water/ standard antioxidant solution. The reaction mixture was then incubated at 37^0C for 30 min, and absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to the ferrous (Fe^{2+}) form and the absorption at 595nm was recorded. The calibration curve was plotted with absorbance at 595 nm versus concentration of $FeSO_4$ which inturn plotted against concentrations of standard antioxidant (Trolox) [16].

Inhibition of lipid peroxidation

The reaction mixture contained 0.1 ml of rat liver homogenate (25 %, w/v) in Tris- HCl buffer (20mM, pH 7); $FeSO_4$ $(NH_4)_2$ SO_4. $6H_2O$ (0.16mM); ascorbate (0.06 mM); and various concentrations of the crocin from *Crocus Sativus* in a final volume of 0.5 ml.[18].The reaction mixture was incubated for 1 hour at 37°C. After the incubation period, 0.4 ml was removed and treated with 0.2 ml SDS (8.1 %), 1.5 ml thiobarbituric acid (0.8%) and 1.5ml acetic acid (20%, pH 3.5). The total volume was made upto 4 ml with distilled water and then kept in a water bath at 95-100°C for 1 hour.

After cooling, 1 ml of distilled water and 5 ml of n-butanol and pyridine mixture (15:1) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 minutes. The n-butanol-pyridine layer was removed and its absorbance at 532 nm was read. Inhibition of lipid peroxidation was determined by comparing the optical density of the treatments with that of control[19].

Hydroxyl radical scavenging activity

The reaction mixture contained deoxyribose (2.8 mM); $FeCl_3$ (0.1 mM); KH_2PO_4-KOH buffer (20mM, pH 7.4); EDTA (0.1 mM); H_2O_2 (1mM); ascorbic acid (0.1mM) and various concentration of crocin of *C.Sativus* in a final volume of 1 ml. The reaction mixture was incubated at 37°C for 1 hour. The TBARS formed was estimated by thiobarbituric acid method of [18]. The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treatments [19].

Statistical analysis:

All data were expressed as mean ±SD of number of experiments (n=3). The statistical significance was evaluated by two-way analysis of software. A value of $p<0.05$ was considered significant.

RESULTS

DPPH radical scavenging assay.

The total crocin isolated from the *Crocus sativus* showed potent DPPH radical scavenging capacity. 0.1% of the total crocin showed 29.36% DPPH radical scavenging activity, equivalent to 0.011% of trolox, 0.5% showed 44.52% DPPH radical scavenging activity equivalent to 0.017% of trolox and 1.0% showed 50.07% DPPH radical scavenging activity equivalent to 0.019% of trolox. This indicated the significant DPPH radical scavenging activity of crocin isolated from this spice **(Fig. 1).**

Ferric reducing antioxidant power (FRAP) assay

The crocin isolated from the *Crocus Sativus* showed ferric reducing ability. The crocin at a concentration of 0.1% showed ferric reducing ability equivalent to 0.058% of trolox, for 0.5% ferric reducing ability was equivalent to 0.212% of trolox and for 1.0% ferric reducing ability was equivalent to 0.356% of trolox. This indicated the significant ferric reducing ability of the crocin isolated from saffron **(Fig.2).**

Inhibition of lipid per oxidation

The crocin isolated from the saffron were effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate. The IC_{50} value of crocin in inhibiting lipid peroxidation was 179 ± 6.7. This indicated the significant lipid peroxidation inhibiting activity of total crocin isolated **(Table: 1).**

Hydroxyl radical scavenging activity

The crocin isolated from the Kashmiri saffron showed significant scavenging activity of hydroxyl radical generated from Fe^{2+} ascorbate – EDTA – H_2O_2 system. The IC_{50} value of crocin for hydroxyl radical scavenging was 227 ± 3.8. **(Table: 1).** The hydroxyl radical scavenging activity was found to increase in a concentration dependent manner.

DISCUSSION

Saffron or *Crocus sativus* has been attributed many properties , is used in folk medicine as an anti-spasmodic, eupeptic, gingival, sedative, anti-catarrhal, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac and emmenagogue [20] . Chemical studies have shown that *C. sativus* contains constituents such as crocin, crocetin safranal and picrocrocin [21, 22]. All these constituents have been reported to possess significant biological activity. The present investigation reveals that the crocin isolated from this spice possesses profound free radical scavenging property. The DPPH assay and FRAP assay of crocin support this conclusion. The CS or *Crocus Sativus* crocin fraction also possess significant hydroxyl radical scavenging and lipid peroxidation inhibiting activities.

Reactive oxygen species such as superoxide anions and hydrogen peroxide are produced as byproducts in aerobic organisms and have been implicated in the pathophysiology of a vast number of human diseases including cancer. [23]. Therefore the elimination or inactivation of reactive oxygen species or their cellular function is considered as one practical approach to reduce the risk of cancer.

During this investigation antioxidant activity of the crocin is tested at various levels of antioxidant action. Non-enzymatic antioxidants react with pro-oxidants and inactivate them. In FRAP assay, an easily reducible oxidant Fe^{3+} is used in excess. Thus on reduction of Fe^{3+}-TPTZ complex by antioxidants, blue coloured Fe^{2+}-TPTZ is formed [17] and, this antioxidant power can be referred to as reducing ability.

The antioxidants scavenge free radicals to suppress chain initiation and/or break the chain propagation reactions. In the DPPH assay the ability of antioxidants to scavenge stable purple coloured primary radical DPPH is tested by its depolarization [15]. The present study shows that the crocin of Kashmiri saffron have strong ferric reducing and DPPH radical scavenging activities.

Membrane lipids are particularly susceptible to lipid peroxidation. Experimental and clinical evidence suggests that aldehyde products of lipid peroxidation can also act as bioactive molecule in physiological and pathological conditions. The initial products of peroxidation are conjugated dienes, to which is added oxygen to form lipid hydroperoxide that further breaks down to stable aldehyde and react with TBA to form thiobarbituric acid- malonaldehyde adduct [24]. The results of the present study indicate that crocins are capable of preventing lipid peroxidation that might lead to membrane damage.

Trace amount of metal ions, primarily ferrous ion, react with H_2O_2 in iron-catalyzed Fenton reaction to produce hydroxyl radical. Hydroxyl radicals cleave covalent bonds in protein and carbohydrates, cause lipid peroxidation and destroy cell membranes [25]. our results showed that crocin has significant hydroxyl radical scavenging activity. Since free radicals are involved in a variety of pathological events, their scavenging activity has significant role in normal biological functions.

In conclusion our results indicate the ability of Crocin to suppress formation of free radicals (FRAP assay) and also to scavenge the free radicals (DPPH) assay). Crude drugs or natural diet, which possess antioxidant or free radical scavenging activity, are of significant importance to prevent or ameliorate tissue injury and disease management. Since Saffron crocin possess profound antioxidant activity, a part of the medicinal properties of this spice can be attributed to its crocin components. The results of the present investigation support this conclusion. However before a conclusive statement on potential usefulness of crocin as adjuvant, there is a need for further studies including human trials.

.

Table-1: Hydroxyl radical scavenging and lipid peroxidation inhibiting activity of the crocins isolated from *Crocus sativus* (Inhibition %).

Activities	Crocins (IC_{50}) (µg/ml)	Catechin Standard (µg/ml)
Hydroxyl radical scavenging activity	227 ± 3.8*	850 ± 20.0
Lipid peroxidation Inhibiting activity	179 ± 6.7*	418 ± 28.6

***P<0.05 Values are mean ± S.D, n = 3.**

Fig: 1. DPPH radical scavenging activity of crocin isolated from *C.Sativus*.

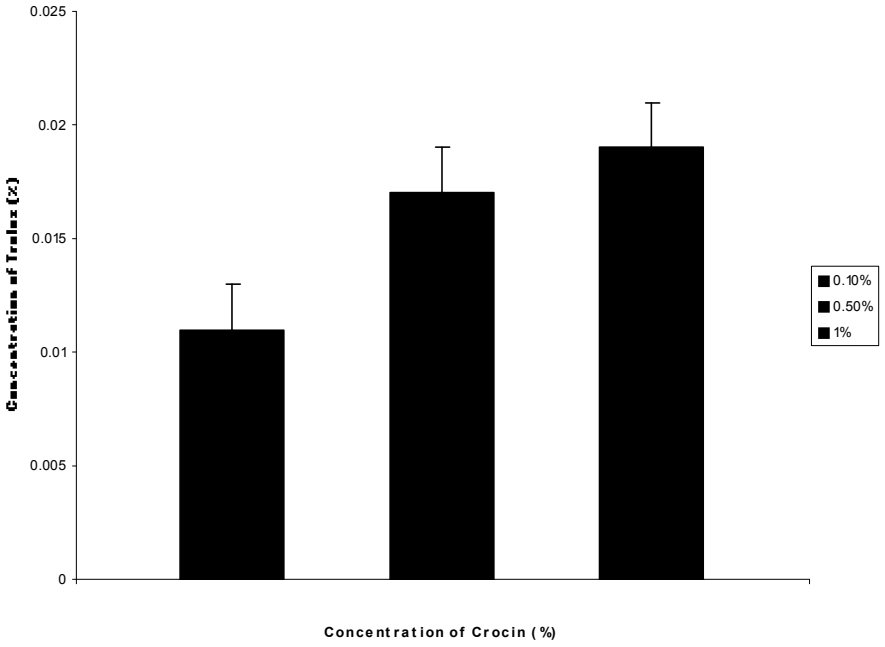
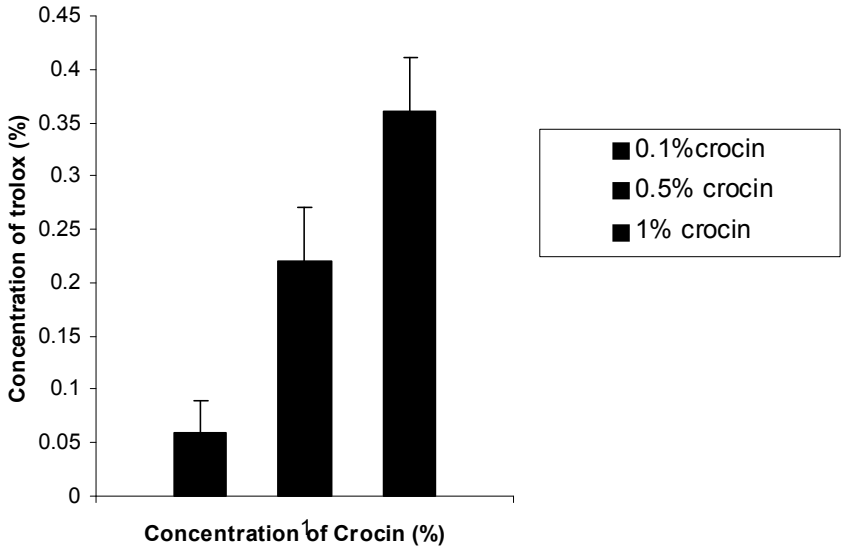


Fig: 2. Ferric reducing antioxidant capacity of Crocin isolated from *C.sativus*



.

Authors' contributions

Hamid bakshi and Smitha Sam, performed all major experiments and drafted the manuscript .Tajamul Islam and Priyam Sing reviewed the manuscript .Manik sharma was involved in the overall design of the study and writing the manuscript.

Acknowledgment

Authors are thankful to director of institution for providing facilities and humble support.

Conflict of interest

Authors have no competing conflict of interest.

REFERENCES

1) **Halliwell, B., & Gutteridge, J. M. C.** Free radicals in biology and medicine. Oxford:**1999;** Oxford University Press
2) **Halliwell, B.** Antioxidants and human diseases: a general introduction. *Nutrition Review*. **1997; 55**: S44–S52.
3) **Ames, B. N., Gold, L. S., & Willet, W. C.** The causes and prevention of cancer. *Proceedings of the National Academy of Sciences*. **1995; 92**:5258–5265

4) **Cerutti, P. A.** Oxy-radicals and cancer. *Lancet*. **1994; 344**:862–863.
5) **Pietta, P. G.** Flavonoids as antioxidants. *Journal of Natural Products*. **2000; 63:**1035–1042
6) **Ochiai, T., Soeda, S.H., Ohno, S., Tanaka, H., Shoyama, Y. and Shimeno, H.** Crocin prevents the death of PC-12 cells through sphingomyelinase-ceramide signaling by increasing glutathione synthesis. *Neurochem. Int.* **2004;** ***44****: 321-330*.
7) **Konoshima, T., Takasaki, M., Tokuda, H., Morimoto, S., Tanaka, H., Kawata, E., Xuan, L.J., Saito, H., Sugiura, M., Molnar, J. and Shoyama, Y.** Crocin and crocetin derivatives inhibit skin tumor promotion in mice. *Phytother. Res.* **1998;** ***12****: 400-404*.
8) **Abe, K. and Saito, H.** Effects of saffron extract and its constituent crocin on learning behavior and long-term potentiation. *Phytother. Res.* **2000; 14**: 149-152.
9) **Lee, I.A.H., Lee, J.H., Baek, N.I. and Kim, D.H.** Antihyperlipidemic effect of crocin isolated from the fractus of *Gardenia jasminoides* and its metabolite crocetin. *Biol. Pharm. Bull.* **2005;** ***28****: 2106- 2110*.
10) **Garcia-Olmo, D.C., Riese, H.H., Escribano, J., Ontanon, J., Fernandez, J.A., Atienzar, M. and Garcia-Olmo, D.** Effects of long-term treatment of colon adenocarcinoma with crocin, a carotenoid from saffron (*Crocus sativus L.*): an experimental study in the rat. *Nutr. Cancer.* **1999;** ***35****: 120- 126*.
11) **SH.Y. Qian, Z.Y., Tang, F.T., Wen, N., Xu, G.L. and Sheng, L.** Effects of crocin on experimental atherosclerosis in quails and its mechanisms. *Life. Sci.* **2005;** *77: 907-921*.
12) **Hamid bakshi, Smitha Sam, Manik Sharma and Tasduq Abdullah**. Assessment of cytotoxic and apoptogenic potential of *crocus sativus* against different human cancer cell lines. *Ind. J. applied Sci.* **2008;** ***4(1-2):****34-38*
13) **Lussignoli S, Fraccaroli M, Andrioli G, Brocco G, Bellavite P**. A micro plate based colorimetric assay of the total peroxyl radical trapping capability of human plasma. *Anal Biochem*. **1999; 269**:33–44.
14) **Tubaro F, Gheiselli A, Rapuzzi P, Maiorino M, Ursini F.** Analysis of plasma antioxidant capacity by competition kinetics. *Free Radic Biol Med*. **1998; 24:1228**– 34.
15) **Aquino R., Morelli S., Lauro M.R., Abdo S., Saija A., Tomaino A.** Phenolic constituents and antioxidant activity of an extract of *Anthurium versicolor* leaves. *J.Natl.Pro.* **2001; 64**, 1019-1023.
16) **Lakshmi B., Tilak J.C., Adhikari S., Devasagayam T.P.A and Janardhanan K.K** .Evaluation of antioxidant activity of selected Indian mushrooms. *Pharmaceutical Biology*. **2004; 42**, 179-185.
17) **Benzie I.F., Strain J.J.** The ferric reducing ability of plasma as a measure of "Antioxidant power". The FRAP assay. *Anal Bio. Chem*. **1996;239**, 70-76
18) **Ohkawa H., Ohishi W, Yagi K.** Assay for peroxidase in animal tissue by thiobarbituric acid reaction, *Biochemistry*. **1979; 95**, 351-358.
19) **Ajith T.A., Janardhanan K.K.** Antioxidant and antihepatotoxic activities of *Phellinus rimosus* (Berk) Pilat. *Journal of Ethanopharmacology*. **2002**; **81**, 387-391.
20) **Hosseinzadeh, H., Sadeghnia, H.R., Ziaee, T., Danaee, A.** Protective effect of aqueous saffron extract (*Crocus sativus L.)* and crocin, its active constituent, on renal ischemia-reperfusion induced oxidative damage in rats. *J. Pharm. Pharmaceut. Sci.* **2005;** ***8****: 387-393*.
21) **Tarantilis PA, Tsoupras G, Polissiou M)**. Determination of saffron (*Crocus sativus* L.) components in crude plant extracts using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. *J Chromatogr A*. **1995**; **699:** 107-18.
22) **Lozano P, Delgado D, Gomez D, Rubio M, Iborra JL .**A non-destructive method to determine the safranal content of saffron (*Crocus sativus* L.) by supercritical carbon dioxide extraction combined with high-performance liquid chromatography and gas chromatography. *JBiochem Biophys Method.* **2000; 43:** 367-8
23) **Pincemail, J.J.** Free radicals and antioxidatants in human diseases. In analysis of free radicals in biological systems. *Birkhauser Verlang, Berlin*. **1995;** 83-98.
24) **Girotti A.W.** Photodynamic lipid peroxidation in biological systems. *Photochemistry and Photobiology*. **1990; 51**, 497-509.
25) **Cuzzocrea S., Riley D.P., Caputi A.P., Salvemini D.** The American Society of Pharmacology and Experimental Therapeutics. *Pharmacol.Rev*. **2001; 53**,135-159.

*****