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Potential of *Crocus sativus* (saffron) and its Constituent, Crocin, as Hypolipidemic and Antioxidant in Rats

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Abstract The aim of the present study was to evaluate the hypolipidemic and antioxidant potential of saffron and its active constituent, crocin, in hyperlipidemic rats. The animals fed either with normal fat diet or high fat diet were administered orally saffron (25, 50, and 100 mg/kg) or crocin (4.84, 9.69, and 19.38 mg/kg) in their respective groups for five consecutive days. Biochemical estimations of triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), glutathione peroxidase enzyme activity (GSHPx), total glutathione (GSH), and oxidized glutathione (GSSG) in serum and superoxide dismutase (SOD), catalase (CAT), thiobarbituric acid reactive species (TBARS), ferric reducing/antioxidant power (FRAP), and total sulfhydryl (SH) groups in liver tissue homogenate were carried out. Both saffron and crocin were effective in decreasing the elevated levels of TG, TC, ALP, AST, ALT, MDA, GSHPx, GSH, and GSSG in serum and increasing SOD, CAT, FRAP, and SH values in liver tissue with reduction in TBARS. The saffron was found to be superior to crocin indicating the involvement of other potential constituents of saffron apart from crocin for its synergistic behavior of quenching the free radicals and ameliorating the damages of hyperlipidemia.

Keywords Antioxidant · Biochemical estimations · Crocin · Hypolipidemic · Saffron

Introduction

Elevated lipid levels are considered as major predisposing factor implicated in the development of cardiovascular diseases [1, 2]. Presently, search for new drugs capable of

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regulating and reducing serum cholesterol and triglyceride (TG) levels has been the focus of attraction with numerous reports showing remarkable activities of natural agents [3]. The products of plant are regarded as less toxic and free from side effects than synthetic agents. Also, it is well established that diet rich in vegetables and fruits can reduce cardiovascular diseases [4–6]. In view of the increasing use of herbal remedies by the general public and subsequent interest by the physicians, it is imperative to promote credible research for validation of their claimed activities based on modern scientific techniques.

Crocus sativus L. commonly known as saffron, is a perennial stemless herb of the Iridaceae family and principally grown in Spain and Iran as well as also cultivated on a lower scale in Greece, Turkey, India, Azerbaijan, France, Italy, India, and China [7]. Saffron is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anti-catarrhal, nerve sedative, carminative, diaphoteric, expectorant, stimulant, stomachic, and aphrodisiac [8]. The extracts of saffron and its active constituents have anticonvulsant [9], antidepressant [10], anti-inflammatory [11], and antitumor effects [12]. Saffron extract is also reported to be chemopreventive and showed protective effects on genotoxins-induced oxidative stress in animals [13, 14]. Moreover, free radical scavenging and antioxidant activity of saffron and crocin were also reported [15]. The constant decrease in lipoprotein oxidation susceptibility in healthy individuals after administration of 50 mg of saffron twice a day [16] is the evidence for hypolipidemic potential of saffron due to increased antioxidant potential.

The pharmacological activities of saffron are attributed to many of its active constituents such as volatile agents (e.g., safranal), bitter principles (e.g., picrocrocin), and dye materials (e.g., crocetin and its glycoside, crocin) [8]. The active constituent crocin and crocetin were found to be involved indirectly for lowering cholesterol level in the blood [17]. The crocins are unusual water-soluble carotenoids (cis and trans glucosyl esters of crocetin). However, there is no report on antioxidant status at times of hyperlipidemia after administration of saffron and its active constituent, crocin. Hence, we considered worthwhile to study the lipid profile, oxidative radicals, and antioxidant levels in presence and absence of high fat diet in animals treated with saffron or crocin.

Materials and Methods

Materials

Saffron was purchased from suppliers in Kashmir district (India). Crocin was procured from Sigma-Aldrich, Germany. 2,20-Dinitro-5,50-dithiodibenzoic acid (DTNB), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), 2-thiobarbituric acid (TBA), n-butanol, Tris, disodium ethylenediaminetetraacetic acid (EDTA), sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropane, ferric chloride ($FeCl_3.6H_2O$), ferrous sulfate, and hydrochloric acid were obtained from Merck. All other chemicals and estimation kits were purchased from standard companies.

Estimation of Crocin in Crocus Sativa and Selection of Dose of Crocin

The doses of saffron (25, 50, and 100 mg/kg p.o.) were selected based on earlier dose response study carried out in our lab [18]. The amount of crocin present in the saffron sample was estimated using high-performance liquid chromatography (HPLC). The doses

of the crocin for administration to animals were selected based on the amount of crocin present in 25, 50, and 100 mg of saffron sample.

Extraction of Crocin from Saffron

The crocin was extracted from saffron following method described by Li et al. [19]. Briefly, dried stigma powder was stirred with 80% aqueous methanol at ambient temperature in the dark for 2 h. The resultant mixture was filtered, and aliquots (20 µl) of the filtrate were analyzed by HPLC. The HPLC system consisted of a Hewlett-Packard HPLC 1100 chromatograph and a photo diode-array multiple-wavelength UV detector. The column configuration consisted of a Nova-Pak C_{18} reversed-phase column and an HP C_{18} guard column. Absorbance was measured spectrophotometrically at 442 nm.

Chromatographic Conditions

Gradient elution was employed using solvent systems A (methanol) and B (1% aqueous acetic acid *v*/*v*) at ambient temperature. The gradient program used was as follows: initial 0–1 min, A–B (40:60, *v*/*v*); 1–6 min, linear change to A–B (55:45, *v*/*v*); 6–23 min, linear change to A–B (75:25, *v*/*v*); linear change to A–B (90:10, *v*/*v*); this was maintained for 5 and 30 min, and there was return to initial conditions. The flow-rate was kept constant at 1.0 ml/min. Plotting peak area versus concentration made a standard calibration curve.

Experimental Animals

Albino Wistar rats of either sex weighing between 200 and 250 g were used. Institutional Animal Ethics Committee approved the experimental protocol. Animals were maintained under standard conditions in an animal house approved by the Committee for the Purpose of Control and Supervision on Experiment on Animals.

Experimental Protocol

The crocin content of the saffron sample was found to be 19.39%; hence, doses of 4.84, 9.69, and 19.38 mg/kg equivalent to 25, 50, and 100 mg, respectively, of saffron were selected for the study. The drug solutions were prepared as suspension using sodium carboxymethyl cellulose (0.5% *w*/*v*) in distilled water for oral administration. The animals were divided into 14 groups ($n = 8$) with the first seven groups fed with normal fat diet (NFD) and the second seven groups fed with high fat diet (HFD). The NFD animals were fed with standard rat chow (Amrut laboratory animal feed, Maharashtra, India) containing (percent *w*/*w*) protein 22.10, oil 4.13, fiber 3.15, ash 5.15, and sand (silica) 1.12, whereas, HFD animals were fed [20] with standard rat chow 68%, dalda (saturated fat) 30%, and cholesterol 2% for 2 weeks before and during treatment.

Treatment

For rats under group I up to group XIV, their diet and treatment are shown in Tables 1, 2, 3, and 4. Groups I to VII were fed with NFD; group I was treated with vehicle; groups II, III, and IV

Table 1 Effect of saffron and crocin on serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and LDL/HDL ratio (atherogenic index (AI)) in rats fed with normal fat diet (NFD) and high fat diet (HFD).

Treatment	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	AI
NFD control	73.06 ± 0.71	77.59 ± 1.51	36.19 ± 1.79	56.01 ± 1.25	1.43 ± 0.12
NFD + saffron 25	69.04 ± 0.86*	71.85 ± 1.36*	38.83 ± 0.71	46.82 ± 1.89**	1.19 ± 0.28*
NFD + saffron 50	64.80 ± 0.76***	63.10 ± 1.89***	40.53 ± 0.58*	35.53 ± 1.47***	0.87 ± 0.07***
NFD + saffron 100	52.82 ± 1.68***	53.64 ± 0.63***	45.38 ± 0.96***	19.20 ± 0.97***	0.42 ± 0.02***
NFD + crocin 4.84	71.07 ± 0.69	74.12 ± 1.01	34.14 ± 0.51	50.58 ± 1.61*	1.48 ± 0.30
NFD + crocin 9.69	64.49 ± 0.30***	66.99 ± 1.74***	38.49 ± 0.24	41.39 ± 1.91***	1.07 ± 0.21***
NFD + crocin 19.38	58.67 ± 0.20***	59.81 ± 0.49***	43.52 ± 0.41***	28.02 ± 1.26***	0.64 ± 0.06***
HFD control	126.50 ± 2.28****	127.53 ± 1.71****	36.67 ± 0.51	116.06 ± 1.82****	3.16 ± 0.33****
HFD + saffron 25	102.96 ± 2.90***	108.85 ± 1.77***	41.32 ± 0.93**	88.12 ± 1.65***	2.13 ± 0.43***
HFD + saffron 50	88.30 ± 2.01***	96.24 ± 0.44***	49.12 ± 0.65***	64.78 ± 1.17***	1.31 ± 0.21***
HFD + saffron 100	76.80 ± 0.89***	82.99 ± 0.66***	54.90 ± 1.79***	43.45 ± 1.45***	0.79 ± 0.05***
HFD + crocin 4.84	108.47 ± 0.98***	112.58 ± 1.34***	38.64 ± 0.36	95.54 ± 1.39***	2.64 ± 0.37***
HFD + crocin 9.69	97.16 ± 0.43***	103.80 ± 1.06***	42.34 ± 1.50**	80.89 ± 1.38***	1.89 ± 0.36***
HFD + crocin 19.38	88.62 ± 1.68 ***	94.28 ± 1.08***	47.79 ± 0.75***	64.21 ± 1.53***	1.35 ± 0.21***

Values are expressed as mean ± SEM of eight rats

Symbols represent statistical significance

Saffron 25, 50, and 100: saffron 25, 50, and 100 mg/kg p.o., respectively; crocin 4.84, 9.69, and 19.38 mg/kg p.o., respectively

*$P < 0.05$ (HFD fed vs HFD control)

**$P < 0.01$ (HFD fed vs HFD control)

***$P < 0.001$ (NFD fed vs NFD control)

****$P < 0.001$ (NFD control vs HFD control)

Table 2 Effect of saffron and crocin on serum alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), body weight change (percent), and daily diet intake in rats fed with normal fat diet (NFD) and high fat diet (HFD).

Treatment	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	Body weight (%)	Diet intake (g/day)
NFD control	204.7 ± 14.3	23.3 ± 2.5	16.9 ± 2.1	10.6 ± 1.7	17.2 ± 1.2
NFD + saffron 25	198.2 ± 12.7*	19.8 ± 2.0**	15.1 ± 2.2**	11.4 ± 1.6	16.3 ± 1.6
NFD + saffron 50	176.3 ± 10.5**	16.6 ± 2.4***	14.8 ± 1.7***	12.8 ± 1.7*	16.1 ± 1.9
NFD + saffron 100	157.8 ± 10.9***	14.2 ± 1.9***	12.4 ± 1.2***	15.2 ± 1.8***	15.6 ± 1.3*
NFD + crocin 4.84	201.4 ± 10.5	22.8 ± 1.6	15.8 ± 1.3	11.0 ± 1.6	17.1 ± 1.1
NFD + crocin 9.69	197.9 ± 9.2*	19.9 ± 1.9**	15.1 ± 1.2*	12.4 ± 1.3*	16.5 ± 1.7
NFD + crocin 19.38	174.5 ± 9.4***	17.0 ± 1.2***	14.2 ± 1.6***	14.7 ± 1.2***	15.9 ± 1.4*
HFD control	306.3 ± 22.3****	43.2 ± 1.4****	40.2 ± 1.2****	26.5 ± 1.8****	10.7 ± 1.7****
HFD + saffron 25	278.3 ± 10.2**	38.1 ± 1.6*	37.1 ± 2.4*	21.9 ± 1.9*	11.8 ± 1.7
HFD + saffron 50	249.1 ± 9.6***	31.7 ± 1.1***	31.3 ± 3.2***	16.3 ± 2.0***	13.4 ± 1.4*
HFD + saffron 100	214.9 ± 11.7***	29.4 ± 1.4***	24.7 ± 2.5***	11.8 ± 1.8***	15.9 ± 1.6***
HFD + crocin 4.84	287.6 ± 9.3*	40.5 ± 1.3*	38.6 ± 1.3*	24.4 ± 1.9	11.1 ± 1.3
HFD + crocin 9.69	265.3 ± 11.5**	37.8 ± 1.3**	34.8 ± 2.3**	20.1 ± 1.4*	12.8 ± 1.0*
HFD + crocin 19.38	237.7 ± 10.7***	34.2 ± 1.5***	30.3 ± 2.2***	14.2 ± 1.8***	14.2 ± 1.8***

Values are expressed as mean ± SEM of eight rats

Symbols represent statistical significance

Saffron 25, 50, and 100: saffron 25, 50, and 100 mg/kg p.o., respectively; crocin 4.84, 9.69, and 19.38 mg/kg p.o., respectively

*$P<0.05$ (HFD fed vs HFD control)

**$P<0.01$ (HFD fed vs HFD control)

***$P<0.001$ (NFD fed vs NFD control)

****$P<0.001$ (NFD control vs HFD control)

were given orally 25, 50, and 100 mg/kg saffron, respectively, whereas, groups V, VI, and VII were administered 4.84, 9.69, and 19.38 mg/kg of crocin orally. Groups VIII to XIV were fed with HFD; group VIII received vehicle; groups IX to XI were treated with 25, 50, and 100 mg/kg saffron orally, while groups XII, XIII, and XIV were given crocin 4.84, 9.69, and 19.38 mg/kg p.o., respectively. All treatments were done for five consecutive days. Animal weights were recorded both at the start and before sacrificing them, and percentage change in weight was calculated. Daily diet intake in grams per day for each animal was also recorded. At the end of the treatment period, the animals were fasted overnight and anesthetized using diethyl ether. Blood was collected by puncturing retro-orbital vein, centrifuged at 1,000×g for 10 min at +4°C, and upper plasma phase was drawn with pipette and transferred into polypropylene tubes and stored at −40°C.

Table 3 Effect of saffron and crocin on malondialdehyde (MDA), glutathione peroxidase enzyme activity (GSHPx), total glutathione (GSH), and oxidized glutathione (GSSG) in serum.

Treatment	MDA (nmol/ml)	GSHPx (U/mg protein)	GSH (nmol/ml)	GSSG (nmol/ml)
NFD control	0.49± 0.02	0.45± 0.05	4.59± 0.12	$4.45\times10^{-2}\pm0.6\times10^{-2}$
NFD + saffron 25	0.38± 0.07*	0.43± 0.01	4.21± 0.21*	$4.12\times10^{-2}\pm0.3\times10^{-2}$
NFD + saffron 50	0.35± 0.03**	0.37± 0.02***	3.98± 0.37***	$3.82\times10^{-2}\pm0.4\times10^{-2}$*
NFD + saffron 100	0.29± 0.02***	0.30± 0.04***	3.41± 0.22***	$2.83\times10^{-2}\pm0.5\times10^{-2}$***
NFD + crocin 4.84	0.45± 0.05	0.44± 0.01	4.48± 0.13	$4.25\times10^{-2}\pm0.3\times10^{-2}$
NFD + crocin 9.69	0.39± 0.02*	0.39± 0.02**	4.23± 0.24*	$3.90\times10^{-2}\pm0.4\times10^{-2}$*
NFD + crocin 19.38	0.34± 0.03**	0.32± 0.03***	3.45± 0.38***	$3.12\times10^{-2}\pm0.6\times10^{-2}$***
HFD control	0.78± 0.10****	0.67± 0.04****	6.21± 0.27****	$6.38\times10^{-2}\pm0.2\times10^{-2}$****
HFD + saffron 25	0.58± 0.08**	0.59± 0.08*	5.65± 0.41*	$5.75\times10^{-2}\pm0.1\times10^{-2}$
HFD + saffron 50	0.50± 0.05***	0.47± 0.07**	5.10± 0.28***	$4.92\times10^{-2}\pm0.6\times10^{-2}$**
HFD + saffron 100	0.43± 0.04***	0.38± 0.06***	4.70± 0.51***	$3.87\times10^{-2}\pm0.3\times10^{-2}$***
HFD + crocin 4.84	0.67± 0.03*	0.61± 0.04*	6.11± 0.31*	$6.14\times10^{-2}\pm0.4\times10^{-2}$
HFD + crocin 9.69	0.53± 0.05**	0.56± 0.06**	5.84± 0.41**	$5.89\times10^{-2}\pm0.3\times10^{-2}$
HFD + crocin 19.38	0.49± 0.10***	0.44± 0.05***	5.23± 0.26***	$4.92\times10^{-2}\pm0.2\times10^{-2}$***

Values are expressed as mean ± SEM of eight rats

Symbols represent statistical significance

Saffron 25, 50, and 100: saffron 25, 50, and 100 mg/kg p.o., respectively; crocin 4.84, 9.69, and 19.38 mg/kg p.o., respectively

*$P<0.05$ (HFD fed vs HFD control)

**$P<0.01$ (HFD fed vs HFD control)

***$P<0.001$ (NFD fed vs NFD control)

****$P<0.001$ (NFD control vs HFD control)

Biochemical Estimations in Serum

Determination of Protein Levels

Protein determinations in plasma were done according to modified micro method of Lowry et al. [21] using bovine serum albumin as standard.

Estimation of Glutathione Peroxidase Enzyme Activity

Glutathione peroxidase enzyme activity (GSHPx) measurements were conducted according to Lawrence and Burk [22]. Nine hundred microliter of 50 mM PBS solution (pH 7.4), including 5 mM EDTA, 2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 20 mM total glutathione (GSH), 10 mM NaN_3, and 23 mU of oxidized glutathione (GSSG) reductase were incubated at 37°C for 5 min. Fifty microliter of 0.25 mM H_2O_2 solution and 50 μL of samples were added to the assay mixture. The change in absorbance at 340 nm was monitored for 3 min. A blank with all ingredients except plasma sample was also monitored. Specific activity was calculated as unit per milligram protein for plasma samples.

Table 4 Effect of saffron and crocin on superoxide dismutase (SOD), catalase (CAT), thiobarbituric acid reactive species (TBARS), ferric reducing/antioxidant power (FRAP), and total sulfhydryl (SH) groups assay in liver tissue.

Treatment	SOD (unit/g tissue)	CAT (unit/g tissue)	TBARS (nmol/g tissue)	FRAP value (μmol/g tissue)	Total sulfhydryl (μmol/g tissue)
NFD control	80.7± 1.9	83.3± 2.5	30.93± 2.3	3.63± 0.7	0.62± 0.02
NFD + saffron 25	82.2± 2.7	85.8± 2.0	28.11± 2.4	3.79± 0.6	0.67± 0.06
NFD + saffron 50	86.3± 1.5	89.6± 2.4*	26.10± 1.7*	4.21± 0.7*	0.74± 0.09*
NFD + saffron 100	91.8± 1.9*	91.2± 1.9*	21.46± 2.2***	4.69± 0.8***	0.81± 0.03**
NFD + crocin 4.84	80.4± 1.5	83.8± 1.6	29.81± 1.3	3.69± 0.6	0.68± 0.01
NFD + crocin 9.69	87.9± 2.2	88.9± 1.9*	27.19± 2.2	3.98± 0.3*	0.76± 0.07*
NFD + crocin 19.38	90.5± 1.4*	90.0± 1.2*	24.21± 1.9**	4.41± 0.2***	0.89± 0.04**
HFD control	43.2± 2.3****	41.2± 1.4****	43.22± 1.4****	2.71± 0.8****	0.33± 0.07****
HFD + saffron 25	58.3± 1.2*	48.1± 1.6*	35.11± 2.1*	2.93± 0.9	0.38± 0.07
HFD + saffron 50	69.1± 2.6***	64.7± 1.1**	30.31± 3.0***	3.36± 0.8***	0.49± 0.04*
HFD + saffron 100	76.9± 1.7***	79.4± 1.4***	25.71± 2.6***	3.83± 0.5***	0.59± 0.06***
HFD + crocin 4.84	57.6± 2.3*	46.5± 1.2*	39.64± 2.3*	2.81± 0.9	0.41± 0.03
HFD + crocin 9.69	65.2± 1.5**	57.3± 1.4**	33.81± 2.7**	3.13± 0.4*	0.51± 0.04*
HFD + crocin 19.38	72.7± 1.7***	68.2± 1.3***	27.39± 2.4***	3.62± 0.8***	0.62± 0.08***

Values are expressed as mean ± SEM of eight rats

Symbols represent statistical significance

Saffron 25, 50, and 100: saffron 25, 50, and 100 mg/kg p.o., respectively; crocin 4.84, 9.69, and 19.38 mg/kg p.o., respectively

*$P<0.05$ (HFD fed vs HFD control)

**$P<0.01$ (HFD fed vs HFD control)

***$P<0.001$ (NFD fed vs NFD control)

****$P<0.001$ (NFD control vs HFD control)

Measurement of MDA Levels

Lipid peroxidation products were quantified by the TBA method [23]. Malondialdehyde (MDA) is formed as an end product of lipid peroxidation, which reacts with TBA reagent under acidic conditions to generate a pink-colored product. Plasma (0.5 ml) was made up to 1 ml with saline, and an equal volume of trichloroacetic acid (TCA) was added and incubated at 37°C for 20 min and centrifuged at 500 g. To 1 ml of TCA extract (the supernatant), 0.25 ml TBA was added and heated in a water bath at 95°C for 1 h till a faint pink color appeared. After cooling,

the color was extracted in 1 ml butanol, and the intensity was read at 532 nm. 1,1,3,3-Tetraethoxypropane (1–100 nmol/ml) was used as the standard.

Measurement of Glutathione Levels

Total Glutathione

Glutathione was assayed according to modified Owens and Belcher [24] method. Plasma was first treated with metaphosphoric acid and centrifuged at 4,000 g to obtain deproteinized samples. Then, supernatants were mixed with 5,5′-dithiobis-2-nitrobenzoat, $NADPH_2$, and oxidized glutathione reductase in a sodium potassium phosphate buffer. The samples were incubated in a water bath at 30°C for 15 min, and absorbance was read spectrophotometrically at 412 nm.

Oxidized Glutathione

At first, supernatants were treated with 2-vinylpyridine to prevent interference by any present reduced glutathione. Then, each sample was mixed with high concentrations of $NADPH_2$ and GSSG reductase in the sodium potassium phosphate buffer. The change in absorbance was observed spectrophotometrically at 340 nm. When all the GSSG was run out, a constant absorbance was recorded.

Measurement of Lipid Profile and Biomarkers

The TG, total cholesterol (TC), and high-density lipoprotein (HDL) cholesterol were estimated using autoanalyzer [25–27]. The low-density lipoprotein (LDL) cholesterol was calculated by using the formula: $[\text{LDL in mg/dl} = \text{Total cholesterol} - (\text{HDL} - \text{Cholesterol} - 1/5\text{Triglycerides})]$ [28].

The atherogenic index (AI) was also calculated: (AI=LDL/HDL) [29].

Aspartate transaminase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assayed using the corresponding commercial kits (Crest Biosystems Goa, India) [30, 31].

Biochemical Estimations in Liver Tissue Homogenate

The animals are sacrificed under deep anesthesia of diethyl ether; liver was immediately isolated and washed with normal saline and blotted with filter paper. Liver tissue homogenate was prepared in sucrose solution (0.25 M) and used for biochemical evaluation of superoxide dismutase (SOD), catalase (CAT), thiobarbituric acid reactive species (TBARS), ferric reducing/antioxidant power (FRAP), and total sulfhydryl (SH) groups assays.

Measurement of Thiobarbituric Acid Reactive Species (TBARS)

MDA levels, as an index of lipid peroxidation, were measured. MDA reacts with TBA as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex that has peak absorbance at 532 nm [32]. Three milliliter phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of muscle homogenate in a centrifuge tube, and the mixture

was heated for 45 min in a boiling water bath. After cooling, 4 ml of n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 20,000 rpm for 20 min. The organic layer was transferred to a fresh tube, and its absorbance was measured at 532 nm. The standard curve of MDA was constructed over the concentration range of 0–40 mM [33].

Ferric Reducing/Antioxidant Power (FRAP) Assay

The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from the colorless oxidized FeIII form by the action of electron donating antioxidants [34]. The FRAP reagent consist of 300 mM acetate buffer (3.1 g sodium acetate + 16 ml glacial acetic acid, made up to 1 l with distilled water; pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1. Briefly, 50 ml of muscle homogenate was added to 1.5 ml freshly prepared and pre-warmed (37°C) FRAP reagent in a test tube and incubated at 37°C for 10 min. The absorbance of the blue colored complex was read against reagent blank (1.5 ml FRAP reagent+50 ml distilled water) at 593 nm. Standard solutions of FeII in the range of 100 to 1,000 mM were prepared from ferrous sulfate ($FeSO_4 4.7H_2O$) in distilled water. The data was expressed as millimolar ferric ions reduced to ferrous form per liter (FRAP value) [35].

Total Sulfhydryl (SH) Groups Assay

Total SH groups were measured using DTNB as the reagent. This reagent reacts with the SH groups to produce a yellow colored complex which has a peak absorbance at 412 nm [36]. Briefly, 1 ml Tris-EDTA buffer (pH 8.6) was added to 50 ml muscle homogenate in 2 ml cuvettes, and sample absorbance was read at 412 nm against Tris-EDTA buffer alone (A1). Then, 20 ml DTNB reagent (10 mM in methanol) was added to the mixture, and after 15 min (stored in laboratory temperature) the sample absorbance was read again (A2). The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (millimolar) was calculated from the following equation:
$$\text{Total thiol concentration mM} = (\text{A2} - \text{A1} - \text{B}) \times (1.07/0.05) \times 13.6$$

Assay of Super Oxide Dismutase (SOD)

SOD activity was determined by the method of [37]. The assay mixture contained 0.1 ml of sample, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulphate (186 μm), 0.3 ml of nitro blue tetrazolium (300 μm), and 0.2 ml of NADH (750 μm). Reaction was started by addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4 ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged, and butanol layer was separated. The color intensity of the chromogen in butanol layer was measured at 560 nm against n-butanol, and concentration of SOD was expressed as units per gram of liver tissue. Absorbance values were compared with a standard curve generated from known SOD.

Assay of Catalase (CAT)

CAT was assayed according to the method of [38]. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction

mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2, and the enzyme extract. The specific activity of CAT was expressed in terms of units per gram of liver tissue. Absorbance values were compared with a standard curve generated from known CAT.

Statistical Analysis

Results are expressed as mean ± SEM. Significance of differences among the groups was assessed by one-way analysis of variance followed by Tukey's multiple comparison tests.

Results

Effect of Saffron and Crocin on Serum TG, TC, HDL, LDL, and LDL/HDL Ratio (AI) in Rats Fed with Normal Fat Diet (NFD)

Administration of saffron (100, 50, and 25 mg/kg p.o.) resulted in significant fall in TG, TC, and LDL levels in serum of animals fed with NFD. The AI was also found to be significantly low in serum of animals treated with saffron. The best activity was found with high and moderate doses of saffron (100 and 50 mg/kg p.o., respectively). However, HDL level was significantly elevated with saffron 100 mg/kg when compared to NFD control. Similarly, treatment of animals with low and moderate doses of crocin (9.69 and 19.38 mg/kg p.o., respectively) showed significant reduction in TG, TC, and LDL levels as well as in AI compared to NFD control. High dose of crocin (19.38 mg/kg p.o.) was also found to increase HDL level in serum compared to NFD control (Table 1).

Effect of Saffron and Crocin on TG, TC, HDL, LDL, and AI in Rats Fed with High Fat Diet (HFD)

Change of diet from NFD to HFD caused significant rise in TG, TC, LDL levels, and AI. However, there was no remarkable change in HDL level in serum upon shifting of diet. Administration of saffron (100, 50, and 25 mg/kg p.o.) as well as crocin (4.84, 9.69, and 19.38 mg/kg p.o.) for five consecutive days resulted in significant fall in TG, TC, and LDL levels as well as in AI compared to HFD control. The high dose of saffron was found to show the maximum fall in lipid levels than high dose of crocin, whereas, moderate dose of saffron was better effective in ameliorating the elevated lipid levels than moderate dose of crocin. The high and moderate doses of saffron and crocin were also shown to increase significantly the HDL level in serum compared to HFD control (Table 1).

Effect of Saffron and Crocin on Serum ALP, AST, ALT, Body Weight Change (Percent), and Daily Diet Intake in Rats Fed with Normal Fat Diet (NFD)

There was significant decrease in AST, ALT, and ALP levels in serum of animals treated with saffron (100, 50, and 25 mg/kg p.o.) and crocin (9.69 and 19.38 mg/kg p.o.) when compared to NFD control. There was significant increase in body weight with reduction in daily diet intake in animals treated with high doses of saffron (100 mg/kg p.o.) and crocin (19.38 mg/kg p.o.) compared to NFD control. Among all treated groups, saffron 100 mg/kg was found be most effective for keeping the integrity of cell membrane (Table 2).

Effect of Saffron and Crocin on Serum ALP, AST, ALT, Body Weight Change (Percent) and Daily Diet Intake in Rats Fed with High Fat Diet (HFD)

Shifting of diet from NFD to HFD showed remarkable incline in AST, ALT, ALP, and body weight with decline in daily diet intake. Upon treatment of animals with saffron (100, 50, and 25 mg/kg p.o.) and crocin (4.84, 9.69, and 19.38 mg/kg p.o.) for 5 days, there was significant fall in AST, ALT, ALP, and body weight with rise in daily diet intake compared to HFD control. The high dose of saffron was found to be more effective than high dose of crocin when compared to HFD control (Table 2).

Effect of Saffron and Crocin on MDA, GSHPx, GSH, and GSSG in Serum of Animals

Upon administration of saffron (100, 50, and 25 mg/kg p.o.) and crocin (9.69 and 19.38 mg/kg p.o.), there was significant fall in serum MDA, GSHPx, GSH, and GSSG levels compared to NFD control. In the animals fed with HFD, significant rise in MDA, GSHPx, GSH, and GSSG levels were seen compared to NFD control. However, treatment of HFD fed rats with saffron and crocin for five consecutive days resulted in significant fall in elevated levels of MDA, GSHPx, and GSH in serum compared to HFD control. The serum GSSG level was significantly lowered by moderate and high doses of saffron (100 and 50 mg/kg p.o.) and high dose of crocin (19.38 mg/kg p.o.) compared to HFD control (Table 3).

Effect of Saffron and Crocin on SOD, CAT, TBARS, FRAP, and Total SH in Liver Tissue

The high dose of saffron and crocin was shown to augment the SOD and CAT activities in liver tissue compared to NFD control. Also, FRAP and total SH values were significantly high in liver tissue of animals treated with moderate and high doses of saffron (100 and 50 mg/kg p.o.) as well as high dose of crocin (19.38 mg/kg p.o.). On the contrary, the moderate and high doses of saffron (100 and 50 mg/kg p.o.) and high dose of crocin (19.38 mg/kg p.o.) found to decline TBARS level in liver tissue compared to NFD control. Feeding of animals with HFD resulted in significant depletion in SOD, CAT, FRAP, and total SH values in liver tissue compared to NFD control. However, upon treatment of animals with saffron and crocin, there were significant incline from the depleted values of SOD, CAT, FRAP, and total SH in liver tissues. Similarly, HFD diet significantly causes rise in TBARS level in liver tissue that was reverted back to normalcy by administration of saffron and crocin in animals (Table 4).

Discussion

The present study was carried out with the objective to determine the hypolipidemic potential and antioxidant status of saffron and its active constituent, crocin, in experimental models of animals. The results obtained in the present investigation suggest that the saffron and crocin have an overall protective effect against hyperlipidemic manifestation in rat. The antihyperlipidemic and antioxidant actions of saffron (100, 50, and 25 mg/kg p.o.) were found to be better than that observed with crocin (9.69 and 19.38 mg/kg p.o.). High dose of saffron was superior in ameliorating the damages caused by high fat diet than the high dose of crocin, suggesting the possible role of other constituents apart from crocin for potent antioxidant nature of saffron.

Hyperlipidemia is an important risk factor in initiation and progression of atherosclerotic lesions and subsequent cardiovascular complications. The beneficial effect of lowering elevated serum cholesterol level in prevention of coronary heart disease is well established. Main causative factor for atherosclerotic diseases is the disturbance in lipid metabolism. There are several methods available for induction of hyperlipidemia. In the present study, high fat diet consisted of dalda (30%), which is saturated fat, and cholesterol (2%) in diet was used to induce hyperlipidemia. Administration of this diet for 2 weeks increased TG, LDL, and TC level without altering HDL level [21]. Saffron and crocin treatment decreased the serum TG levels. This was an important finding as TG is independently related to development of coronary heart disease. This might be related to increase in endothelium bound lipoprotein lipase, which hydrolyses TG into fatty acids [39]. High level of TC and, more importantly, LDL cholesterol are major coronary risk factors [40]. LDL carries cholesterol from liver to peripheral cells and smooth muscle cells of arteries, and a rise in LDL may cause deposition of cholesterol in the arteries and aorta. Hence, it is bad for health and a direct risk factor for coronary heart diseases [41]. Treatment of animals with moderate and high doses of saffron (100 and 50 mg/kg p.o.) and high dose of crocin (19.38 mg/kg p.o.) substantially depletes both TC and LDL levels in NFD and HFD rats indicating the cardioprotective nature of saffron and crocin during cholesterol deposition. Enhancement of cardioprotective lipoprotein HDL after administration of higher doses of saffron and crocin in HFD group might be due to increase in activity of lecithin-cholesterol acyl transferase (LCAT), which might contribute to regulation of blood lipids. LCAT plays a key role in incorporating free cholesterol into HDL and transferring back to very-low-density lipoprotein and intermediate-density lipoprotein, which is taken back by liver cells [42]. Several studies show that an increase in HDL is associated with decrease in coronary risk [43], and most of the drugs that decrease TC also decrease HDL. However, in the present study, both saffron and crocin decreased the TC and LDL but enhanced HDL, remarkably showing its advantage over other hypolipidemics. AI is a measure of atherogenicity. Elevated values are indications of hyperlipidemia, whereas, low values demonstrate low level of lipid. High AI ratio in HFD could be due to induction of hyperlipidemia that was not done in NFD. The reduction in AI values in saffron and crocin-treated groups could be due to increased antioxidant potential. The powerful hypolipidemic activities can be directly linked with the presence of flavonoids in saffron [44] as it is known that flavonoids have powerful hypolipidemic properties [45, 46]. Weggemans and Trautwein [47] demonstrated that flavonoids intake decreased LDL cholesterol and increased HDL cholesterol in hypercholesterolemic individuals that may hasten removal of cholesterol from peripheral tissue to liver for catabolism and excretion.

Induction of hyperlipidemia leads to damage to the membrane of hepatocytes resulting in leakage of endogenous enzymes into circulation [48, 49]. Therefore, the extent of protection offered by saffron and crocin to liver cell was evaluated by measuring the serum AST, ALT, and ALP levels. Both saffron and crocin remarkably brings back the normalcy in serum enzymes levels of AST, ALT, and ALP, demonstrating their protective behavior.

It is well established that hyperlipidemic stress leads to the release of oxidative free radicals. Millimole levels of hydrogen peroxide lead to the inactivation of CAT and glutathione peroxidase despite normal mRNA expression [50, 51]. In our study, we found increased MDA and GSHPx activity levels in plasma of HFD fed animals. CAT and glutathione peroxidase catalyze the decomposition of hydrogen peroxide. Cohen and Hochstein [52], using the H_2O_2 diffusion technique, offered evidence that CAT does not play an important role in protecting blood cells against endogenous H_2O_2. Rather, they

concluded glutathione peroxidase is the major route for disposing of H_2O_2. We know that concentration of H_2O_2 is high in areas of damage, and the diffusion of H_2O_2 into plasma will be relatively more limited and significantly high. GSHPx activities will be able to lead to the detoxification of H_2O_2 easily in the plasma. One of the sources of plasma antioxidant enzymes is lysis of erythrocytes. Increased plasma GSHPx activities may be the result of lysis of erythrocytes due to decreased GSHPx activity and increased reactive oxygen species levels in erythrocytes. The consequence of increased free radicals via H_2O_2 generation and imbalances in oxidant/antioxidant balance is oxidative stress, which leads to oxidative damage, resulting in increased MDA levels, which is the end product of lipid peroxidation. Our studies suggest that both saffron and crocin prevented the elevation of MDA, GSHPx, GSH, and GSSG in serum resulting in potent antioxidant effect.

Under pathologic conditions such as hyperlipidemia, the balance between oxidant and antioxidant system is known to get disturbed [53, 54]. Hence, we assessed the antioxidant/reducing potential of liver tissue muscle using FRAP assay. As anticipated, a remarkable fall in antioxidant power, as indicated by FRAP value, was observed in HFD control group. The treatment of animals with saffron and crocin rose antioxidant power of liver tissue homogenate samples. SH groups are highly reactive constituents of protein molecules, and they participate in important biochemical and metabolic process such as cell division, blood coagulation, maintenance of protein systems, and enzymatic activation including antioxidant enzymes (CAT, SOD, etc.) [55]. They are also important scavengers of oxygen-derived free radicals [56]. SH groups are known to be sensitive to oxidative damage and are depleted following hyperlipidemic stress; therefore, we studied the effect of these agents on total thiol concentration. We found the dramatic fall in SH groups in HFD control. Saffron and crocin exhibited higher SH contents in liver tissues than their HFD control, indicating their help in replenishing the total thiol pool. Higher concentrations of saffron were more effective than their crocin counterpart in showing high levels of total SH group as well as FRAP values. The treatment of saffron and crocin was also found to bring back the normal activities of SOD and CAT in liver tissues of animals subjected to hyperlipidemic stress.

As discussed earlier, crocin and crocetin are main constituents that have antioxidant activity. The crocins are unusual water-soluble carotenoids (cis and trans glucosyl esters of crocetin). It does not mean that saffron may not contain other active constituents with antioxidant potential. In our study, saffron was more potent than crocin. This may be attributed to synergistic action of many constituents such as crocins, dimethyl crocetin, safranal, and flavonoids that quench free radicals and have antioxidant effects and may have role in protective effect of saffron on hyperlipidemic stress.

Conclusion

The saffron and crocin was effective in decreasing the elevated lipid levels and oxidative radicals during hyperlipidemic stress with augmentation of antioxidant activities in liver tissue. The high dose of saffron was found to be superior in counteracting the manifestation of hyperlipidemia than high dose of crocin. These suggest that apart from crocin of saffron, there are other constituents responsible for synergistic antihyperlipidemic and antioxidant potential of saffron.

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