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Antioxidant potential of crocins and ethanol extracts of *Gardenia jasminoides* ELLIS and *Crocus sativus* L.: A relationship investigation between antioxidant activity and crocin contents

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Abstract

Crocins are water-soluble carotenoids responsible for the colour of saffron and gardenia. In this study, we isolated and identified three major crocins from gardenia, and then evaluated their antioxidant potential using four *in vitro* antioxidant tests in comparison with saffron ethanol extract (SE), gardenia ethanol extract (GE) and gardenia resin fraction (GRF). The relationship between total crocin contents and antioxidant activity of ethanol extracts of two herbs was investigated and the antioxidant potentials of three different polar crocins were compared. The crocins appeared to possess antioxidant activity when tested by four *in vitro* antioxidant models. However, in anti-hemolysis, DPPH radical-scavenging and lipid peroxidation assays, GRF exhibited significantly stronger antioxidant activity than crocins and no correlation between total crocin contents and antioxidative function was revealed, which implied that ingredients other than crocins in gardenia gave markedly strong antioxidant activity. In the phosphomolybdenum assay, antioxidant capacities of fractions and extracts correlated with total crocin contents ($R = 0.93$). Moreover, comparison of results indicated that sugars attached to the crocetin moiety seemed to be beneficial for the antioxidant activity of these water-soluble pigments.

Keywords: Gardenia; Saffron; Crocin; Antioxidant

1. Introduction

The constituents of gardenia fruits, *Gardenia jasminoides* ELLIS, are known as herb medicines and natural dyes in China. The yellow pigments of this herb have been used as a natural food colourant for a long time in Japan, mainly in coloured juice, jelly, candy and noodles, because of their water solubility (Watanabe & Terabe, 2000). Saffron, another herb containing the same yellow pigments of gardenia, is typically used as a spice with colouring properties in a wide range of culinary, bakery and confectionery preparations, as well as in alcoholic and non-alcoholic beverages (Selim, Tsimidou, & Biliaderis, 2000). Phytochemical studies of saffron and gardenia have shown that the main chemicals responsible for their colour are crocins, which are a series of mono and di-glucosyl esters of crocetin, a polyene dicarboxylic acid (8,8-diapocarotene-8, 8-dioic acid) (Pfister, Meyer, Steck, & Pfander, 1996; Van Calsteren et al., 1997). In contrast to most families of carotenoids, these compounds are known for their colouring properties owing to their unique water-soluble behaviour, which is the reason for their great application as a food colourants (Van Calsteren et al., 1997).

Numerous studies have shown crocins to be capable of a variety of pharmacological effects, such as protection against cardiovascular diseases (He et al., 2005; Shen & Qian, 2006; Xiang et al., 2006), inhibition of tumor cell proliferation (Magesh, Singh, Selvendiran, Ekambaram, & Sakthisekaran, 2006), neuroprotection (Ahmad et al.,

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2005; Ochiai et al., 2004) and protection of hepatocytes (Tseng, Chu, Huang, Shiow, & Wang, 1995). Among the mechanisms underlying their various protective actions, the antioxidant activity was hypothesised to be responsible for various pharmacological effects of crocins. The following evidence supports the notion that one of the important mechanisms by which crocetin or crocins exert their biological effects is their ability to modulate redox status of organisms. Growing evidence indicates that chronic or acute overproduction of reactive oxygen species (ROS) plays an important causal or contributing role in the development of the various above mentioned diseases (Cerutti, 1994; Ferrari et al., 1998; Smythies, 1999; Wattanapitayakul & Bauer, 2001) and the protective effects of crocin or crocetin against these diseases have been repeatedly demonstrated in various studies (Ahmad et al., 2005; He et al., 2005; Magesh et al., 2006; Ochiai et al., 2004; Shen & Qian, 2006; Tseng et al., 1995; Xiang et al., 2006), which may be attributed to antioxidant capacities of crocetin or crocins.

In the course of our previous study aimed to screen for antioxidants from gardenia fruits, we observed significant antioxidant capacity of saffron ethanol extract (SE), gardenia ethanol extract (GE) and gardenia resin fraction (GRF), tested in several *in vitro* antioxidant models. In order to study whether the antioxidant activity of the fraction and extracts was consistent with the contents of crocins present in the fractions and ethanol extracts of two spices from gardenia, we isolated, characterised and identified the major crocins (named crocin-1, crocin-2 and crocin-3) on the basis of NMR, ESI-MS, UV–visible and TLC data, and then quantified these three most abundant crocins in the fraction and extracts using RP-HPLC and UV–visible methods. In addition, antioxidant activity and radical-scavenging ability of crocins, in comparison with fractions and extracts of these two herbs, were assayed using anti-hemolysis, DPPH radical-scavenging, lipid peroxidation and phosphomolybdenum assays, and correlations between total crocin contents and antioxidative function of fractions and extracts were investigated. The present study might give insight into potencies of crocins and two herbs as biological antioxidants, and show if crocins are major contributors to the antioxidant properties of these two traditional medicines.

2. Materials and methods

2.1. Plant material

The dried gardenia (*Gardenia jasminoides* ELLIS) fruits and saffron (*Crocus sativus* L.) stigmas were purchased from Chengdu, Sichuan Province, in August 2004, and identified by Hao Zhang at West China School of Pharmacy, Sichuan University, China. The voucher specimens are deposited in West China School of Pharmacy, Sichuan University, China.

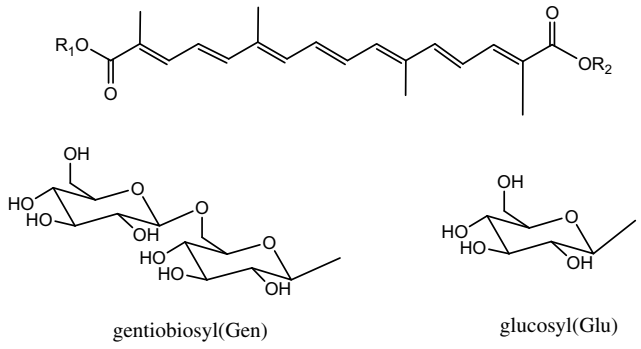
2.2. Chemicals and reagents

Methanol (Sigma, USA) was of chromatographic purity and water was double distilled for HPLC. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical (Sigma, USA) was used for testing the radical-scavenging activity of crocins and extracts. 2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) was used for anti-hemolysis assay and purchased from Wako (Japan). α-Tocopherol (Sigma, USA) and ascorbic acid (Sigma, USA) were used as references. Assay kits for malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute, China. All other regents and solvents were analytically pure and purchased from local firms.

2.3. Extraction and isolation

The dried gardenia fruits (40 kg) were ground to a coarse powder and extracted with ethanol–water (40%) by cold percolation (4 × 40 l). The alcohol extract was concentrated, suspended in water, and then partitioned with ethyl acetate. The ethyl acetate layer extract was subjected to CC (column chromatography) on silica gel (2000 g) eluting with CH_2Cl_2 containing increasing amounts (3%, 5%, 7%, 10%) of methanol. Upon concentration of the fraction eluted with MeOH–CH_2Cl_2 (3%), crocetin (**4**, 40 mg) (Compound No. **4** in Fig. 1 yield) crystallized. The water layer, further diluted with water, was subjected to HPD-100 macroporous resin (15 kg, Cangzhou bon, Hebei, China), and eluted with water containing increasing amounts (0%, 25%, 60%) of ethanol.

The ethanol–water (60%) fractions (gardenia resin fraction, GRF) were combined and evaporated to dryness and separated by CC on silica gel, eluting with ethyl acetate containing increasing amounts (5%, 10%, 15%, 20%) of methanol–water (16:13); the methanol–water, (16:13)–ethyl acetate (5%) was further purified by a preparative ODS column to yield crocin-3 (**3**, 3 g). Similar treatment of the



crocin-1: R_1=R_2=Gen (1)
crocin-2: R_1=Gen, R_2=Glu (2)
crocin-3: R_1=Gen, R_2=H (3)
crocetin: R_1=R_2=H (4)

Fig. 1. Structures of crocins and crocetin.

fractions eluted with 10%, and 20% (methanol–water, 16:13)–ethyl acetate yielded crocin-2 (**2**, 1.5 g) and crocin-1 (**1**, 3 g), respectively. The purity of each isolated compound was confirmed by TLC, HPLC, UV and NMR analysis. TLC analyses were carried out on precoated silica gel plates, using a solvent system of ethyl acetate–methanol–water (100:16.5:13.5) and CH_2Cl_2–methanol (4:1) and spots were detected with 15% H_2SO_4 in MeOH reagent. UV–visible spectra were recorded on the Cintra 10_e UV/Visible spectrometer (GBC, Australia).

Dried gardenia (100 mg) and saffron (100 mg) were extracted with 20 ml of 40% alcohol. Gardenia ethanol extract (GE) and saffron ethanol extract (SE) were obtained by evaporating and then lyophylizing alcohol extracts of gardenia and saffron.

2.4. Elucidation of chemical structures

ESI-MS, recorded in the positive and negative ion modes with a spray voltage of 4.5 kV on a Finnigan LCQ^{DECA} spectrometer, was used to determine the molecular weight. ^1H and ^{13}C NMR experiments were measured in DMSO-d_6, with TMS as internal standard, on a Varian Unity INOVA 400/54 NMR spectrometer.

2.5. HPLC analysis of crocins

High performance liquid chromatography (HPLC) was performed according to the method described by Li, Lin, Kwan, and Min (1999). HPLC was performed on a Shimadzu HPLC system equipped with two LC-10AT VP pumps, CTO-10AS VP column oven, UV–vis SPD-10A VP detector and fitted with a ODS column (150 × 4 mm; Shimadzu, Japan). Purified crocin-1 (0.26, 5.20, 10.4, 26.0 and 52.0 μg/ml), crocin-2 (0.22, 2.20, 4.40, 8.80 and 22.0 μg/ml) and crocin-3 (0.30, 3.00, 6.00, 12.0 and 30.0 μg/ml) were used to establish calibration curves under standard HPLC conditions.

2.6. Determination of total crocin

The visible absorbances of saffron and gardenia ethanol extracts in 50% ethanol, which showed absorption peaks at 440 nm, were proportional to total crocin. GRF (23 μg/ml), SE (18 μg/ml) and GE (92 μg/ml) in 50% ethanol were prepared, respectively. Visible spectra were recorded on the UV–visible spectrometer. The major pigment, crocin-1 (3.59, 6.28, 8.80, 11.7 and 14.4 μg/ml) was selected as a reference and used to establish calibration curve.

2.7. In vitro study of anti-hemolysis activity

The anti-hemolysis activity was assayed according to the method described by Zhang et al. (1997). Blood was collected from male SD rats (250 g) from the abdominal aorta. The RBC was separated from plasma by centrifugation at 1500*g* for 20 min. The crude RBC was then washed five times with 5 volumes of phosphate-buffered saline (PBS, pH 7.4). The RBC was suspended in 4 volumes of PBS solution for hemolysis assay. Two ml of RBC suspension were mixed with 2 ml of PBS solution containing varying amounts of crocins and gardenia or saffron ethanol extract; 2 ml of 200 mM AAPH in PBS solution were then added to the mixture. The incubation mixture was shaken gently in a water bath at 37 °C for 3 h. After incubation, 8 ml of PBS solution were added to the reaction mixture, followed by centrifugation at 1000*g* for 10 min. The absorbance of the supernatant at 540 nm was recorded in a spectrophotometer. Percentage inhibition was calculated by the following equation:

$$\%\ \text{Inhibition} = (A_{\text{AAPH}} - A_{\text{Sample}})/A_{\text{AAPH}}$$

A_{AAPH} refers to the absorbance of the control in the absence of samples and A_{Sample} is the absorbance of the sample in the presence of crocins, gardenia or saffron ethanol extracts. The anti-hemolysis activity of each sample, which was calculated from the graph, was expressed as mg of ascorbic acid (AA) equivalents per gramme dry weight. Ascorbic acid was selected as the anti-hemolysis capacity on the basis of a reference (Barros, Baptista, Estevinho, & Ferreira, 2007). The experiment was carried out in triplicate and the results are mean values.

2.8. DPPH free radical-scavenging assay

The DPPH free radical-scavenging activities of crocins and ethanol extracts of two plants were determined according to the method described by Leong and Shui (2002) with modification. Briefly, a 0.06 mM solution of DPPH$^{\cdot}$ in ethanol was prepared. The initial absorbance of the DPPH$^{\cdot}$ in ethanol was measured at 517 nm and did not change throughout the period of assay. A 0.5 ml solution of the samples at different concentrations was added to 3.5 ml of ethanolic DPPH$^{\cdot}$ solution. The change in absorbance at 517 nm was measured at 30 min and converted into the percentage of antioxidative activity (AA) using the following formula:

$$[A_0 - (A_1 - A_s)]/A_0 \times 100,$$

where A_0 is the absorbance of the control solution containing only DPPH$^{\cdot}$; A_1 is the absorbance of the DPPH$^{\cdot}$ solution containing samples, and A_s is the absorbance of the sample solution without DPPH$^{\cdot}$. The absorbance change at 517 nm was used to calculate the amount of DPPH$^{\cdot}$ reduced; the percentages of DPPH$^{\cdot}$ reduced were plotted against the concentrations of samples. The DPPH radical-scavenging capacity of each sample, which was calculated from the graph, was expressed as mg of α-tocopherol equivalents per gramme dry weight α-tocopherol was selected as the DPPH radical-scavenging capacity according to a reference (Loo, Jain, & Darah, 2007) and our preliminary experiment. The experiment was carried out in triplicate and the results are mean values.

2.9. In vitro lipid peroxidation

The antioxidant activities of crocins and ethanol extracts of two herbs were evaluated according to the method described by Mitra, Venkataranganna, Sundaram, and Gopumadhavan (1999). The drugs were dissolved in dimethyl sulphoxide (DMSO). The liver of the SD rat, after ether anaesthesia, was perfused with ice-cold 0.9% sodium chloride and the tissue was homogenized at a concentration of 10% w/v in 1.15% of potassium chloride using a glass homogenizer. The homogenate was centrifuged at 800*g* and the supernatant was used for the study. Various concentrations of samples were taken with 0.5 ml of the homogenate and the lipid peroxidation was induced using ferric chloride (100 μl of 2 mM solution). The mixture was incubated at 37 °C for 20 min and then subjected to the measurement of MDA. Absorbance of the supernatant was measured spectrophotometrically at 532 nm. The percentage inhibition of lipid peroxidation was calculated using the following equation:

$$\%\ \text{Inhibition} = (A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}$$

A_{blank} refers to the absorbance of the control in the absence of samples and A_{sample} is the absorbance of the sample in the presence of crocins, gardenia or saffron ethanol extracts. The lipid peroxidation inhibition effect of each sample, which was calculated from the graph, was expressed as mg of α-tocopherol equivalents per gramme of dry weight. α-Tocopherol was selected as the lipid peroxidation inhibition capacity on the basis of a reference (Mitra et al., 1999). The experiment was carried out in triplicate and the results are mean values.

2.10. Evaluation of antioxidant activity

The antioxidant activities of crocins and ethanol extracts of two herbs were evaluated according to the phosphomolybdenum method of Prieto, Pineda, and Aguilar (1999). An aliquot of 0.2 ml of sample solution (50 μg/ml in dimethyl sulfoxide) was combined in a 4 ml vial with 2 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed as mg of α-tocopherol equivalents per gramme dry weight. α-Tocopherol was selected as the antioxidative capacity according to a reference (Loo et al., 2007) and our preliminary experiment. The experiment was carried out in triplicate and the results are mean values.

2.11. Statistical analysis

All tests and analyses were run in triplicate. The experimental data were subjected to an analysis of variance for a completely random design to determine the least significant difference at the level of 0.05.

3. Results and discussion

3.1. Identification of crocins and crocetin

Crocins are a series of mono- and di-glucosyl esters of crocetin which exhibit similar NMR spectra. ^{1}H and ^{13}C NMR data of pigments 1 and 2 were very similar and featured a chiral structure on a polyene moiety in which only 7 polyene carbons signals were observed in the ^{13}C NMR spectrum (data not shown). However, pigment 3 was characteristic of chemical shifts of conjugated polyene moiety carbons and exhibited 14 polyene carbons signals in ^{13}C NMR (data not shown), which implied that the aglycone was not symmetric, due to a nonesterified carboxylic acid function on one side of the molecule. The visible absorption spectra of crocetin derivatives showed hypsochromic shifts when amounts of saccharide attached to the crocetin moiety decreased. Pigments 1, 2, 3 and 4 showed major peaks at 439, 439, 432 and 419 nm, respectively. The ESI-MS spectrum of pigment 1 displayed an ion at m/z 999.8 $[M+Na]^+$ and an additional signal was observed at *m/z* 675.4 [M+Na+H−Gen] which, as well as the NMR data, confirmed 1 as trans crocetin di-(-D-gentibiosyl) ester. Trans crocetin (-D-glucosyl)-(-D-gentibiosyl) ester (2) was determined on the basis of NMR data and ESI-MS spectrum: *m/z* 837.7 $[M+Na]^+$ and 675.4 [M+Na+H−Glu]. The asymmetric structure of pigment 3 was assigned to trans crocetin (-D-gentibiosyl) ester on the basis of ESI-MS spectrum data: *m/z* 675.4 $[M+Na]^+$. In addition, pigment 4 was assigned to crocetin by its NMR spectrum in which no saccharide signal was observed, and its structure was further supported by ESI-MS (negative) at *m/z* 327.2 $[M-H]^-$. These assignments were additionally supported by TLC analysis in which more polar pigments exhibited lower R_f values. All spectroscopic results were in agreement with data reported in the literature (Choi et al., 2001; Pfister et al., 1996; Van Calsteren et al., 1997).

3.2. Antioxidant capacities of crocins, extracts and fraction

3.2.1. In vitro study of anti-hemolysis activity

The oxidative hemolysis in erythrocytes induced by AAPH has been extensively studied as a model for peroxidative damage in biomembranes (Zhang et al., 1997). The *in vitro* experiments with AAPH, if not clinically relevant, may help to understand the specific role played by antioxidants (Zou, Agar, & Jones, 2001). The present study showed that anti-hemolysis activity of all samples was proportional to their used concentrations (Fig. 2). As shown in Table 1, GRF showed the most potent activity (1037 mg AA/g). Anti-hemolysis effects of SE and GE, 259 and 280 mg AA/g, were significantly weaker than those

of GRF. When the *in vitro* activities of crocin-1 and crocin-3 against AAPH-induced hemolysis were compared with that of GRF, crocins showed markedly weaker effects than did the extract but still remained comparable with SE and GE. However, crocin-2 showed an antioxidant effect of 452 mg AA/g which is stronger than SE and GE but weaker than GRF.

3.2.2. DPPH·-scavenging assay

The scavenging effects of crocins, fractions and extracts under investigation on DPPH radicals are shown in Fig. 3 and Table 1. All samples exhibited appreciable scavenging properties against DPPH radicals, and the inhibition percentage was proportional to the concentrations of each sample. It can be seen that GRF was proven once again to be the most powerful antioxidant, its inhibition being significantly different from that of any other extract. Among all samples examined, GRF exhibited 1229 mg α-tocopherol/g DPPH radical-scavenging activity. Although less potential was observed when comparing GE (421 mg α-tocopherol/g) with GRF, GE still exhibited significantly stronger efficiency than SE (107 mg α-tocopherol/g). However, crocins showed the weakest activity and DPPH radical-scavenging activities of 98.3, 90.8 and 33.1 mg α-tocopherol/g, respectively, for crocin-1, crocin-2 and crocin-3.

3.2.3. Lipid peroxidation assay

Lipid peroxidation is known to result in the formation of malonaldehyde and other structurally similar compounds, which react with thiobarbituric acid (thiobarbituric acid-reactive substances, TBARS) to produce a chromophore that absorbs at 532 nm. The inhibition of TBARS formation provides sound evidence concerning the potency of a compound for protecting against lipid peroxidation. In this study, the antioxidant activities of crocins, fraction and extracts were examined using an *in vitro* lipid peroxidation model. The current study showed that the inhibition percentage was proportional to the concentration of each sample (Fig. 4). As shown in Table 1, GRF exhibited significantly more potential (55.4 mg α-tocopherol/g) than SE (18.1 mg α-tocopherol/g) or GE (22.4 mg α-tocopherol/g). Among all pigments tested, crocetin, the aglycone of crocins, exhibited the most powerful potential (63.0 mg α-tocopherol/g) and its efficiency was even stronger than that of GRF. However, data again revealed that crocins were the weakest antioxidant agents in lipid peroxidation, showing inhibition activities of

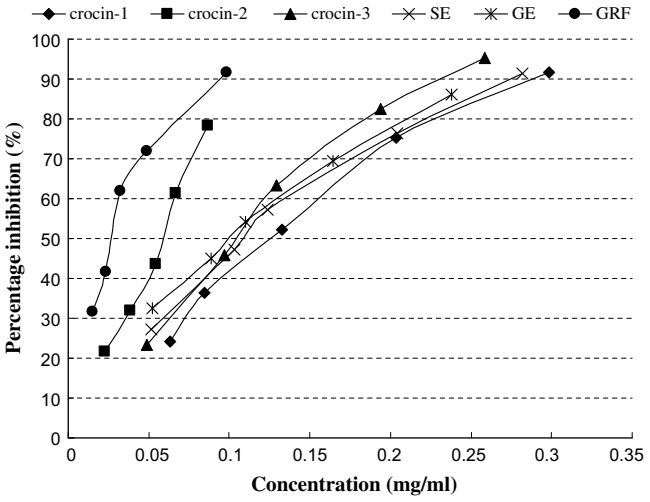


Fig. 2. Anti-hemolysis activity of different concentrations of crocins, fraction and extracts of gardenia and saffron. Values are mean ± SD, $n = 3$, $p < 0.05$, significantly different with Student's *t*-test.

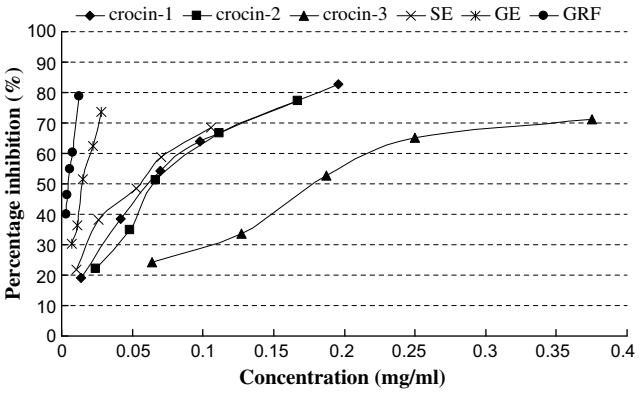


Fig. 3. Scavenging activity of different concentrations of crocins, fractions and extracts of gardenia and saffron on DPPH radical. Values are means ± SD, $n = 3$, $p < 0.05$, significantly different with Student's *t*-test.

Table 1
Antioxidant activity of crocins and ethanol extracts of gardenia and saffron

Samples	Anti-hemolysis (equivalent ascorbic acid) mg/g	DPPH (equivalent α-tocopherol) mg/g	Lipid peroxidation (equivalent α-tocopherol) mg/g	Antioxidant activity (equivalent α-tocopherol) mg/g
Crocin-1	221	98.3	10.6	84.7
Crocin-2	452	90.8	9.65	78.2
Crocin-3	267	33.1	13.5	77.2
Crocetin	–	–	63.0	110
SE	259	107	18.1	116
GE	280	421	22.4	29.9
GRF	1037	1229	55.4	82.3

The experiment was carried out in triplicate and the results are mean values. The relative error for average values (three independent determinations) was less than 5%.

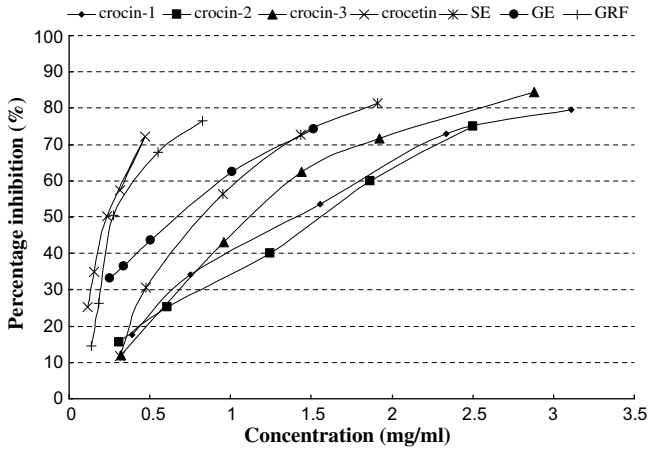


Fig. 4. Lipid peroxidation inhibition of different concentrations of crocins, fractions and extracts of gardenia and saffron. Values are means ± SD, $n = 3$, $p < 0.05$, significantly different with Student's t-test.

10.6, 9.65 and 13.5 mg α-tocopherol/g, respectively, for crocin-1, crocin-2 and crocin-3.

3.2.4. Antioxidant activity assay

The antioxidant activities of crocins, fractions and extracts, as measured by the phosphomolybdenum method, are presented in Table 1. The most powerful antioxidant efficiency was observed in SE (equivalent to 116 mg α-tocopherol/g) and the value was significantly higher than that of GE (equivalent to 29.9 mg α-tocopherol/g) and GRF (equivalent to 82.3 mg α-tocopherol/g) which, however, seemed very different from results obtained from three other models. Similar values were observed in crocins, as shown, 84.6, 78.2 and 77.2 mg α-tocopherol equivalent/g, respectively, for crocin-1, crocin-2 and crocin-3. Crocetin showed a stronger activity (equivalent to 111 mg α-tocopherol/g) than all crocins and the potential is comparable to SE. Among all samples assayed, GE exhibited the weakest efficiency.

3.3. Correlations between total crocin content and antioxidative function

Crocins, the major constituents isolated from saffron and gardenia, are recognized as natural antioxidants. In order to study whether the antioxidant activities of the fractions and extracts were consistent with the contents of crocins, the relationships between antioxidant capacities and total crocin contents were investigated. The crocin contents of fractions and extracts were determined using UV–visible and HPLC analysis, and the results are shown in Table 2. Quantitative determination by two methods indicated that macroporous resin treatment significantly increased pigment contents in gardenia extract, as evidenced by the increase of total crocin content from 9.2% (GE) to 45.9% (GRF) in HPLC analysis. Saffron extract showed markedly higher crocin content than the corresponding gardenia extract, which may be attributed to

Table 2
UV–visible and HPLC analysis of crocins in three extracts

Samples	UV–visible[a] total crocin[b]	HPLC[a]			
		Crocin-1	Crocin-2	Crocin-3	Total crocins
SE	39.9	26.9	15.9	4.7	47.4
GE	8.9	6.8	1.3	1.1	9.2
GRF	44.9	38.3	6.2	1.4	45.9

[a] Mean of triplicate determinations expressed as % dry extracts basis.
[b] Equivalent crocin-1.

the higher crocin content in saffron crude drug (34.2%) than in gardenia crude drug (1.7%). However, macroporous resin fractionation resulted in GRF in which total crocin content increased to a considerable high level, comparable to that of SE (47.4%). On the other hand, as shown in Figs. 5 and 6 and Table 2, the HPLC analysis showed that the three main peaks in the SE chromatogram corresponded to crocin-1, crocin-2 and crocin-3, with concentrations of 26.9%, 15.9%, and 4.7%, from which crocin-1 content in total crocin was calculated as 56.5%. However, a higher crocin-1 content in total crocin was calculated in two gardenia samples (73.7 and 83.2%, respectively for GE and GRF). In present study, no correlation was observed between antioxidant capacities and total crocin contents in anti-hemolysis, DPPH radical-scavenging or lipid peroxidation assays, which suggested that crocins probably did not play a main role in antioxidant capacities of fractions and extracts in these models. This hypothesis was further supported by the result that GRF exhibited 4.7-, 12.5- and 5.2-fold higher effects, in anti-hemolysis, DPPH radical-scavenging and lipid peroxidation assays, respectively, than crocin-1, the major pigment in the fruits of gardenia. However, the linear regression and a relationship between antioxidant capacities and total crocin contents was only observed in the phosphomolybdenum assay ($R = 0.93$).

Despite numerous biological investigations aimed to elucidate various pharmacological effects of crocetin or crocins, to our knowledge, there are few literature studies reporting *in vitro* antioxidant properties of crocins and extracts of gardenia and saffron. Pham, Cormier, Farnworth, Tong, and Van Calsteren (2000) reported that antioxidant capacity of crocin-1 was stronger than that of gardenia acetone extract, evaluated by the thiocyanate method (Pham et al., 2000). In our work, ethanol extract of gardenia exhibited stronger capacities than did crocin-1 tested by all models except in the phosphomolybdenum assay in which all crocins showed significantly stronger effects than ethanol extract of gardenia. The different results are probably due to various *in vitro* models and/or different solvents used in the preparation of crude extracts.

Phytochemical studies have revealed that the most investigated ingredients in gardenia fruits include terpenoids, iridoid glycosides and crocins (Machida, Onodera, Furuta, & Kikuchi, 1998; Machida et al., 2000; Van Calsteren et al., 1997; Wang, Tseng, Huang, & Tsai, 2004). Our work has

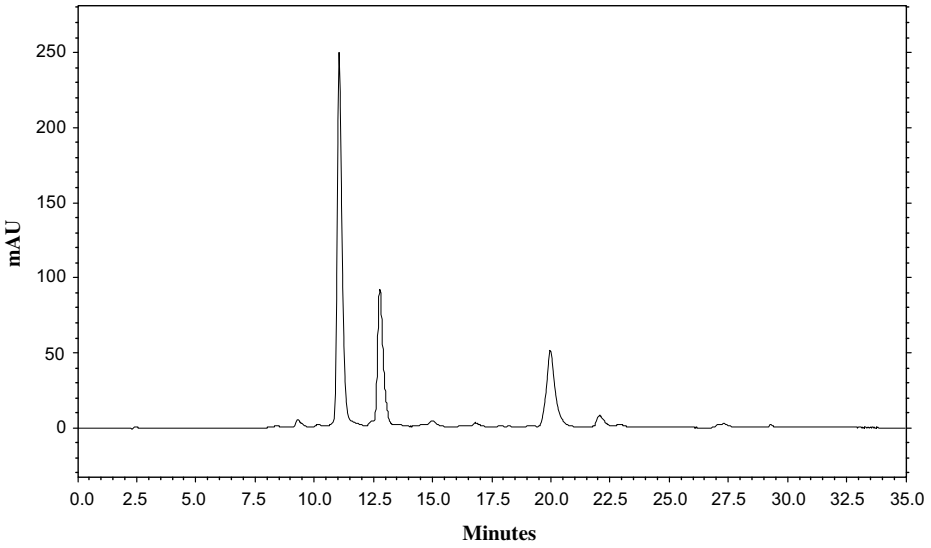


Fig. 5. HPLC chromatogram (440 nm) of crocins in saffron ethanol extract (SE).

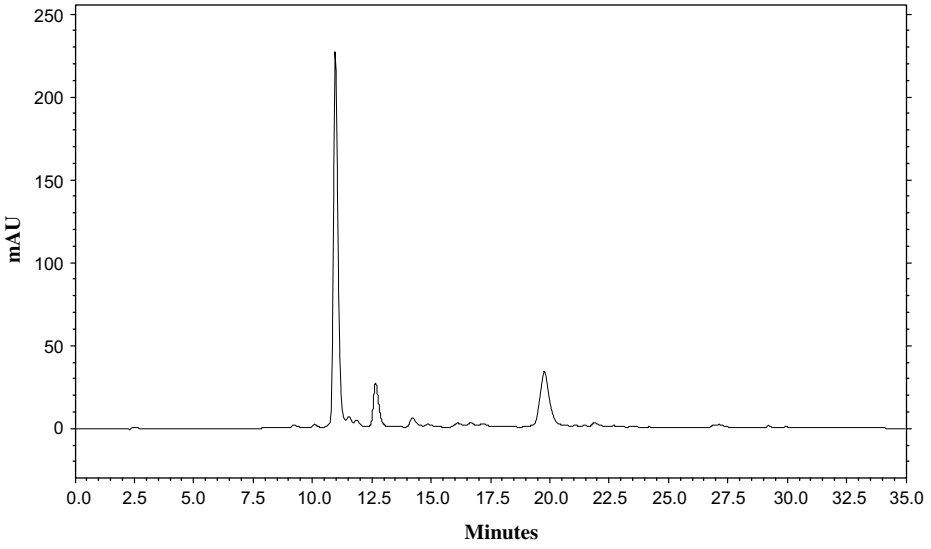


Fig. 6. HPLC chromatogram (440 nm) of crocins in gardenia ethanol extract (GE).

indicated that geniposide, the major iridoid glycoside in the fruits of gardenia, exhibits very weak capacities when tested in four *in vitro* models (data not listed). On the basis of the similarity to the chemical structures of iridoid glycosides, we suggest that iridoid glycosides probably do not contribute to the antioxidant activity of gardenia fruits. The current study implies that constituents other than crocins in gardenia possess marked antioxidant effects. Thus, more research work is required, and further study, aimed to isolate antioxidants other than crocins from gardenia fruits, is in progress in our laboratory.

Furthermore, it must be borne in mind that *in vitro* activities can be considered only potentially relevant in biological systems and that *in vivo* activities depend also on bioavailability and biotransformation. Thus, the protective effects of crocetin and crocins, as well as extracts of gardenia and saffron, require further *in vivo* comparative examination.

3.4. Structure–activity relationships of crocins

Structure–activity relationships of crocins were investigated in several studies which suggested that sugars attached to the crocetin moiety probably play a key role in biological effects of the crocins (Abe, Sugiura, Shoyama, & Saito, 1998; Escribano, Alonso, Coca-Prados, & Fernandez, 1996; Papandreou et al., 2006; Sugiura, Shoyama, Saito, & Abe, 1994). These reports revealed that the action of crocins was enhanced by the presence of the sugars. To our knowledge, however, little work has been done to investigate

the structure–antioxidant relationships of crocins. Purified crocins were subjected to some representative *in vitro* antioxidant tests in this work, to obtain clues about their potencies as biological antioxidants, and to compare their properties. The present study evidences that crocins have potent antioxidant effects and these properties are influenced by the sugars attached to the crocetin moiety. When comparing antioxidants with molar concentrations of active components, crocin-1 exhibited the strongest activity in all *in vitro* studies except the anti-hemolysis activity assay, in which crocin-2 showed the strongest effect among all crocins. Comparative study among the three analogues in the DPPH$^{\cdot}$ and phosphomolybdenum method assays clearly suggested that the more sugars the derivatives contain, the stronger the antioxidant activity exhibited by the pigments. Moreover, antioxidant activity of crocetin, the aglycone of crocins, was studied in two assays and results were very different (63.0 mg α-tocopherol equivalent/g) against lipid peroxidation in comparison with crocins (10.6, 9.65 and 13.5 mg α-tocopherol equivalent/g). In contrast, the aglycone exhibited the weakest activity (36.2 mg α-tocopherol equivalent/ g) in phosphomolybdenum assay among all compounds tested. Generally, our work indicated that sugars seemed to be beneficial for the antioxidant activity of the water-soluble carotenoids, which is in agreement with the above mentioned reports. To the best of our knowledge, the mechanism by which sugars attached to the crocetin moiety influence the antioxidant effects of crocins, however, remains uninvestigated. Further research concerning this issue, is therefore required.

4. Conclusions

The present study shows that crocins possess antioxidant capacities assayed in four models, which confirm their roles as antioxidant agents. However, these pigments, the main chemicals considered responsible for various pharmacological effects of gardenia fruit, seemed not to be major contributors to the antioxidant activity of gardenia in anti-hemolysis, DPPH radical-scavenging and lipid peroxidation assays. The antioxidant effects of gardenia extract require further investigation concerning isolation, identification and quantification of other components responsible for the antioxidant activity of the herb.

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