Food Research 7 (4) : 337 - 343 (August 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Carotenoids composition, antioxidant and antimicrobial capacities of *Crocus sativus* L. stigma

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Article history:
Received: 22 August 2022
Received in revised form: 25 September 2022
Accepted: 28 September 2022
Available Online: 31 August 2023

Keywords:
Saffron,
Crocin,
Maceration extraction,
Antioxidant,
Antimicrobial

DOI:
https://doi.org/10.26656/fr.2017.7(4).390

Abstract

Carotenoid compounds are effective free-radical scavengers because they display a singlet oxygen-quenching property and can trap peroxyl radicals. Many extracts derived from natural sources consist of unique bioactive compounds. For example, hydrophilic carotenoids, such as crocin, are present in saffron and possess antioxidant, anticancer, anti -inflammatory and antimicrobial properties. Crocin is responsible for preventing and curing many health issues. This study investigated the antioxidant and antimicrobial properties of hydrophilic carotenoid (C_{50}) molecules extracted from *Crocus sativus* L. (saffron stigma) grown in different locations. These compounds were tested for their antioxidant properties through 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, while antimicrobial activity was determined using disc diffusion method. Four carotenoid compounds (i.e. crocin, crocetin, β-carotene and zeaxanthin) were detected from the three saffron samples collected from various localities. High-performance liquid chromatography (HPLC) analysis was carried out to determine the physical and chemical properties of the carotenoid samples. The HPLC results indicated the presence of a single crocin peak at 440 nm. The crocin concentration in Iranian saffron was higher (11414.67±516.34 µg/g DW with 99.9% purity) than those in Turkish and Kashmiri saffron. This finding suggested that location significantly affected crocin concentration as well as carotenoid composition and content in saffron. Crocin molecules (100 µg/mL) showed the highest antibacterial activity against *Staphylococcus epidermidis*, with a 6.0 mm inhibition zone. Hence, crocin molecules (500 µg/mL) were effective antioxidant compounds as they showed 68% inhibition, with an IC_{50} value of 283.918±3.934 µg/mL. In addition to being a biocolourant source, crocin may be used for its antioxidant and antibacterial properties.

1. Introduction

Carotenoids are primarily lipophilic or fat-soluble compounds. However, some plant species contain water-soluble carotenoids. Compounds with many conjugated chains show higher antioxidant activity. The high polarity of end groups (such as hydroxyl and carbonyl groups) due to the presence of β-ionone rings can improve the antioxidant activity of carotenoid compounds. *Crocus sativus* (or saffron) contain hydrophilic carotenoids, such as crocin ($C_{44}H_{64}O_{24}$, Figure 1). Crocin is a major pigment of the saffron plant; the yellowish-red colour of the plant is attributed to the presence of the glycosylated esters of a dicarboxylic acid, and its solubility is ascribed to the presence of a saccharide linkage with gentibiose, glucose and neapolitanose (Licón *et al.,* 2012). The central unit of a carotenoid compound contains seven conjugated double bonds, while its side chains contain four methyl groups. Furthermore, the end groups of carotenoids are esterified with one, two or three glucose units (Liakopoulou–Kyriakides *et al.,* 1998; Vickackaite *et al.,* 2004). This compound has a set of hydrophilic glycosyl esters of

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

crocetin (8,8′-diapo-ψ,ψ′-carotenedioic acid) and a molecular weight of 976.972 g/mol. The high volume of water molecules can improve the mobility of a carotenoid solution (Tsimidou and Biliaderis, 1997). Crocin molecules can be easily extracted from saffron plants by using aqueous, methanolic or ethanolic solvents (Sánchez *et al.,* 2008; Mohajeri *et al.,* 2010).

In this study, the environmental stability of crocin molecules collected from various locations was determined. A bioactive hydrophilic carotenoid molecule was extracted from the saffron stigma by maceration and tested for antioxidant, antibacterial and antifungal properties. Antioxidant assay was conducted using DPPH while antimicrobial assay was performed through the disc diffusion method.

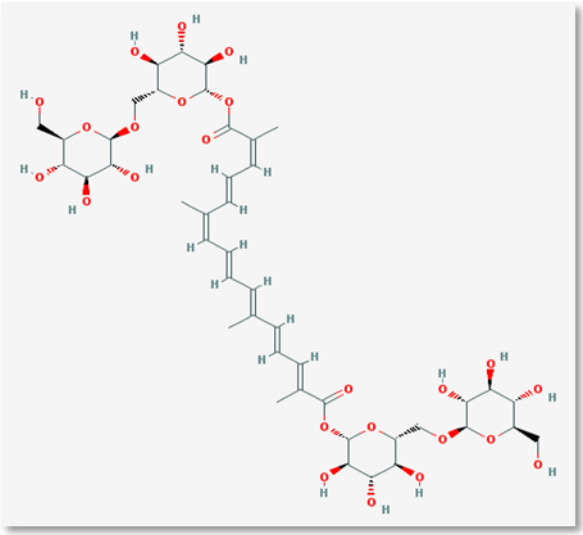

Figure 1. Chemical structure of crocin (National Center for Biotechnology Information (NCBI), 2022).

2. Materials and methods

2.1 Crocin extraction and quantification

2.1.1 Maceration extraction

The saffron stigma (1.0 g) was collected, weighed and placed in a volumetric flask containing distilled water (1 L) (Water Purification Systems, Millipore S.A.S). This mixture was agitated for 30 mins at room temperature on a platform shaker (Unimax 1010 DT, Heidolph, Germany) and heated at 60°C for 30 mins in an oven (UF55, Memmert). The mixture was placed in the dark at room temperature (i.e., 25°C) for 24 h. It was then filtered and transferred to another conical flask. This maceration technique was repeated many times until a colourless liquid was obtained. Crocin pigment was obtained by concentrating the sample on a rotary evaporator (Hei-VAP Precision, Heidolph, Germany) equipped with a water bath set at 45°C. The rotary evaporator was rotated at 75–100 rpm under a 45 mbar vacuum condition. The concentrated extract (10 mL) was freeze-dried (Alpha 1-4 LD Plus, Martin Christ) for 3 days. The freeze-dried sample was stored in a sealed, amber-coloured bottle at -20°C until further analysis (Mohd Hatta, 2019).

2.2 HPLC analysis

Crocin molecules were analysed using an HPLC instrument (Agilent 1200 series, USA) consisting of a quaternary pump in addition to an autosampler injector, micro degasser, thermostat-equipped column compartment and photodiode array detector. A ZORBAX Eclipse XDB-C_{18} end-capped reverse-phase column (5 mm × 4.6 × 150 mm) was used (Agilent Technologies, USA). The mobile phase consisted of solution A [acetonitrile: water (9:1 v/v)] and solvent B (ethyl acetate). The compounds were eluted using a gradient of Solution B, i.e. 0–40% Solvent B (0–20 mins), 40%–60% solvent B (20–25 mins), 60%–100% solvent B (25–25.1 mins), 100% solvent B (25.1–35 mins) and 100%-0 solvent B (35–35.1 mins) at a steady flow rate of 1.0 mL/min. After one run, the column was re-equilibrated using 100% solution A for 10 mins before injecting the next sample. The sample was run in the column at 20°C. About 10 µL of every sample was injected into the column. Crocin molecules were detected at the maximal absorption wavelength of 440 nm. The molecules were identified by co-chromatography using standard molecules and elucidated based on their spectral characteristics with the help of a photo-diode array detector (Nordiyanah *et al.,* 2018).

2.3 Antimicrobial activities

2.3.1 Media preparation

Before testing the antimicrobial properties of the elucidated compound, agar media were prepared for various types of microbes; in particular, Mueller Hinton Agar (MHA, Oxoid) media were used for different microbial species, and potato dextrose agar (PDA, Merck Millipore) was used for fungal species based on the compositions described earlier (Othman *et al.,* 2019). The media were added with distilled water, mixed in Schott bottles and autoclaved for 20 mins at 121°C (HVE-50, Hirayama). The molten media were poured into sterile Petri plates and allowed to solidify at room temperature in a biosafety cabinet to prevent contamination. The plates were sealed with parafilm strips and stored at 4°C for future use.

2.3.2 Inoculum preparation

Different bacterial species, namely *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, and *Pseudomonas aeruginosa*, as well as fungal species including *Aspergillus niger*, *Fusarium* sp., *Candida albicans*, *Microsporum gypseum*, and *Trichoderma* sp., were cultured in their respective liquid broths for a period of 2 to 4 hours at their appropriate temperatures (37°C for bacterial samples and 27°C for fungal species). The

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number of microbes that were added to the media plates was standardised for the disc diffusion test. The process was based on the McFarland standard and completed in two methods. In Technique 1, McFarland standard solution was prepared and used as the reference; in Technique 2, the optical density of the liquid inoculum was analysed to ensure that the final A_{625} value was 0.08. If the optical density was very high, then the inoculum was diluted with the sterile broth.

2.3.3 Antimicrobial activity test

After standardising the number of microbes in the inoculum broth, 100 µL of the inoculum was collected using a pipette and spread onto the labelled media plates for various microbial species. Sterile discs were also placed on five different spots on the freshly spread Petri dishes. Of the five discs, three were used as eluted compounds, while one each was used as a negative and a positive control compound. The test and control samples (10 µL) were placed onto sterile discs in their marked positions. The test samples included different crocin concentrations (10, 5 and 2.5 mg/mL), which were eluted from the HPLC column. The negative control included solvent A which was used for resuspending the eluted crocin molecules. The positive control sample included ampicillin (1 mg/mL), a known antibiotic compound that inhibited bacteria, while clotrimazole (1 mg/mL) was used against fungal species. All plates were sealed with parafilm and incubated at their respective temperatures (i.e. 37°C for bacterial species and 27°C for fungal species). The diameter of the inhibitory zone of the samples was measured.

2.4 Determination of antioxidant activities by DPPH scavenging assay

2.4.1 DPPH solution

DPPH solution (0.2 mM) was prepared by dissolving 0.0788 g of the compound in the solvent (1 mL). As this test needed 25 mL of DPPH solution, 2 g of DPPH was dissolved in 25 mL of methanol, yielding 0.2 mM of the DPPH solution.

2.4.2 Preparation of the ascorbic acid standard solution

MeOH (100 µL) solvent was pipetted using a multi pipettor device (six tips) into the holes labelled as B1–B6, C1–C6, until H1–H6. Thereafter, ascorbic acid (200 µL) was dissolved in MeOH at a final concentration of 1 mg/mL. The solution was pipetted into the holes labelled A1–A6. All the samples were serially diluted after pipetting ascorbic acid solution (100 µL) from A1–A6 to B1–B6. This step was repeated from B1–B6 to C1–C6, until H1–H6. Finally, 100 µL of the solution collected from the final holes was discarded. In the test solution, DPPH (0.2 mM, 100 µL) was pipetted into A4–A6 until F4–F6, while MeOH (100 µL) was pipetted in A1–A3 until H1–H3. The experimental setup is described in Figure 2. All the microplates were covered and kept in the dark for 40 mins at room temperature before recording absorbance A_{517}.

2.4.3 Preparation of crocin samples

MeOH (100 µL) was pipetted using a multi pipettor device (six tips) into the holes labelled B1–B6, C1–C6, until H1–H6. Thereafter, the crocin solution (200 µL of 1 mg/mL solution of crocin molecules in MeOH) was pipetted into the holes labelled A1–A6. All the samples were serially diluted after pipetting the crocin solution (100 µL) from A1–A6 to B1–B6. This step was repeated from B1–B6 to C1–C6, and so forth, until H1–H6. Finally, 100 µL of the solution collected from the final holes was discarded. In the test solution, the DPPH solution (0.2 mM, 100 µL) was pipetted into A4–A6 until F4–F6, while MeOH (100 µL) was pipetted in A1–A3 until H1–H3. The complete experimental setup is described in Figure 2.

2.4.4 Calculation of DPPH inhibition percentage

The DPPH inhibition was determined using the following formula (Gorinstein *et al.,* 2013; Othman *et al.,* 2017):

$$\text{Inhibition (\%)} = [1 - (Abs_{sample} / Abs_{DPPH})] \times 100$$

*Abs_{sample} = Absorbance (sample + DPPH) – Absorbance (sample + MeOH)

	Crocin + MeOH			Crocin + DPPH		
	1	2	3	4	5	6
A	500	500	500	500	500	500
B	250	250	250	250	250	250
C	125	125	125	125	125	125
D	62.5	62.5	62.5	62.5	62.5	62.5
E	31.3	31.3	31.3	31.3	31.3	31.3
F	15.6	15.6	15.6	15.6	15.6	15.6
G	7.8	7.8	7.8	7.8	7.8	7.8
H	3.9	3.9	3.9	3.9	3.9	3.9

Figure 2. Setup of the DPPH scavenging assay.

3. Results and discussion

3.1 Carotenoids composition of saffron derived from various locations

In this study, the saffron samples collected from Turkey, Iran and Kashmir were analysed for their carotenoid composition and content to elucidate environmental and geographical effects on carotenoid accumulation. The main carotenoids identified in all samples included crocin, crocetin, β-carotene and zeaxanthin. However, the concentration of the individual carotenoids differed in the samples depending on their

https://doi.org/10.26656/fr.2017.7(4).390

location. As shown in Table 1, Iranian saffron samples contained the highest crocin concentration (11.4±0.51 mg/g DW), followed by Turkish saffron samples (8.2±0.15 mg/g DW) and Kashmiri saffron (0.31±0.03 mg/g DW). The saffron collected from Kashmir showed the lowest carotenoid concentration. The saffron samples collected from Iran and Turkey showed a significantly higher carotenoid concentration, where four types of carotenoids were present (Figures 3 and 4). The Iranian saffron samples showed higher crocin and crocetin concentrations than the other samples. In an earlier study, saffron plants cultivated in a controlled environment in Malaysia showed a high crocin concentration (87.38 mg/g DW), followed by zeaxanthin (11.33 mg/g DW) and crocetin (7.52 mg/g DW) (Othman *et al.,* 2017; Nordiyanah *et al.,* 2018). Thus, location affected the individual carotenoid composition and concentration in saffron plants.

Crops cultivated using similar environmental, biological and agronomical practices show a similar biochemical composition, while crops exposed to varying environmental conditions would gradually adapt to their changing environments and possess different biochemical compositions (Othman *et al.,* 2017). Thus, the bioavailability of carotenoids is a complicated issue and is dependent on numerous factors, such as year, location, cultivar and interaction between all these factors (Kammona *et al.,* 2015). Previous studies investigated the biochemical composition (especially phenolic compounds) of saffron samples collected from various locations (Nordiyanah *et al.,* 2017).

3.2 Determination of the antimicrobial activities of crocin pigment

The antimicrobial activities of crocin molecules were tested using disc diffusion test. An inhibition zone was formed around the discs after the crocin-soaked discs were incubated with various microbial species (Tables 2 and 3). Crocin had no inhibition zones against different microbial species, except for *S. epidermidis.* The highest concentration of crocin molecules showed the presence

Table 1. Distribution of different concentrations and compositions of the saffron (*Crocus sativus*) plants which were extracted using maceration extraction methods.

Origin	Crocin (mg/g DW)	Crocetin (mg/g DW)	Zeaxanthin (mg/g DW)	β-carotene (mg/g DW)
Turkey	8.2±0.15	0.19±0.01	0.25±0.06	0.51±0.07
Iran	11.4±0.51	1.05±0.05	0.06±0.01	0.34±0.08
Kashmir	0.31±0.03	ND	ND	ND

Values are presented as mean±SD. DW: Dry Weight, ND: Not Detected

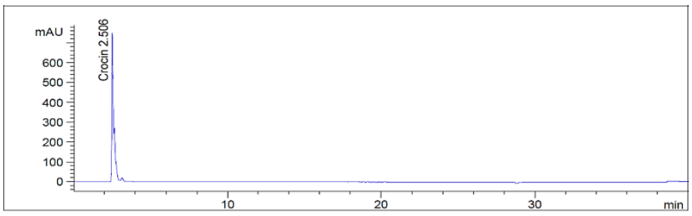


Figure 3. HPLC chromatogram of crocin detected in Iranian saffron sample.

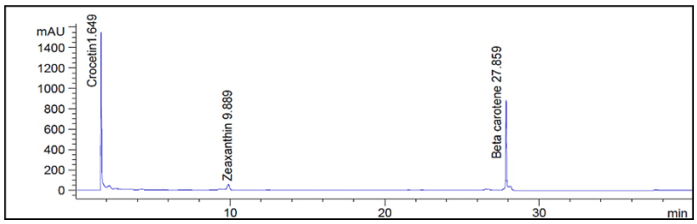


Figure 4. HPLC chromatogram of crocetin, zeaxanthin and β -carotene molecules present in saffron.

Table 2. Presence of a growth inhibition zone (mm) depicting antibacterial activity for different crocin concentrations.

Crocin	*Staphylococcus epidermidis*	*Staphylococcus aureus*	MRSA	*Escherichia coli*	*Pseudomonas aeruginosa*
25 µg/disc	-	-	-	-	-
50 µg/disc	-	-	-	-	-
100 µg/disc	+ +	-	-	-	-
Ampicillin	+ + +	+ + +	+ + +	+ + +	+ + +

- : No antimicrobial activity, inhibition zone (i.z.) of sample, < i.z. of methanol + 1 mm, + +: Clear antimicrobial activity, i.z. of sample 4–10 mm > i.z. of methanol, + + +: Strong antimicrobial activity, i.z. of sample > i.z. of methanol + 10 mm

Table 3. Measurement of growth inhibition zones (mm) depicting antifungal activities for various crocin concentrations.

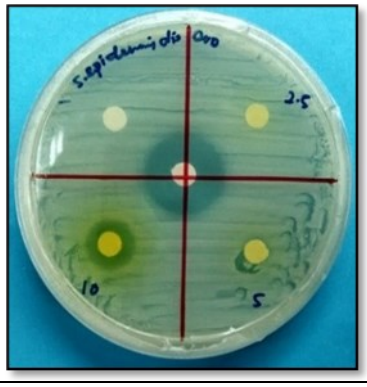
Crocin	*Aspergillus niger*	*Fusarium* sp.	*Candida albicans*	*Microsporum gypseum*	*Trichoderma* sp.
25 µg/disc	-	-	-	-	-
50 µg/disc	-	-	-	-	-
100 µg/disc	-	-	-	-	-
Clotrimazole	+ + +	+ +	+ +	+ + +	+ + +

- : No antimicrobial activity, inhibition zone (i.z.) of sample, < i.z. of methanol + 1 mm, + +: Clear antimicrobial activity, i.z. of sample 4–10 mm > i.z. of methanol, + + +: Strong antimicrobial activity, i.z. of sample > i.z. of methanol + 10 mm

https://doi.org/10.26656/fr.2017.7(4).390

of a 6 mm clear ring, indicating antibacterial activity (Table 4).

Table 4. Antibacterial activity and inhibitory zone for different crocin concentrations.

Bacterial Species	Result
Staphylococcus epidermidis	

Crocin molecules displayed a similar antimicrobial activity, as noted in an earlier study. The extracts did not show any antibacterial activity against the five test bacteria including common pathogens, such as *S. aureus* and *E. coli* (Soureshjan and Heidari, 2014). However, crocin could be used as an antimicrobial compound because it displayed excellent inhibition against *S. epidermidis*, a pathogenic organism (Vahidi *et al.,* 2002; Licón *et al.,* 2010; Jinous *et al.,* 2013; Okmen *et al.,* 2016). Furthermore, Jinous *et al.* (2013) reported the antibacterial activity of saffron crude extracts at high concentrations (1000–31.2 mg/mL).

Hence, antibacterial activity was affected by the technique used, purity, concentration and source. Antibacterial activity can be used to evaluate the potential of a compound as a preservative by inhibiting the growth of food-borne pathogens and preventing food spoilage. It also helps in enhancing the flavour of food and beverages (Licón *et al.,* 2010; Jinous *et al.,* 2013). Pathogenic organisms, such as *E. coli* and *S. aureus*, are associated with food poisoning and diarrhoea (Jinous *et al.,* 2013). They lead to many food-borne infections, such as bloody and watery diarrhoea, chronic renal disorders, meningitis and immunologic, respiratory and cardiovascular conditions (Mokhtarian *et al.,* 2004). Hence, many researchers have investigated the potential use of natural pigments as an antimicrobial compound to help prevent diseases caused by pathogens (Mokhtarian *et al.,* 2004; Soureshjan *et al.,* 2014).

3.3 Determination of the antioxidant activities of crocin pigment

DPPH is a stable free radical that can be used to scavenge free oxygen species (Suganya and Asheeba, 2015). Figure 5 presents the inhibition percentage for ascorbic acid and crocin standards. The crocin molecule showed a DPPH scavenging activity because it displayed 68% inhibition at a high concentration of 500 µg/mL. Furthermore, the IC_{50} value is the concentration of crocin that exhibits 50% inhibition. Crocin showed a high DPPH radical scavenging activity, with an IC_{50} value of 283.92±3.93 µg/mL (Table 5).

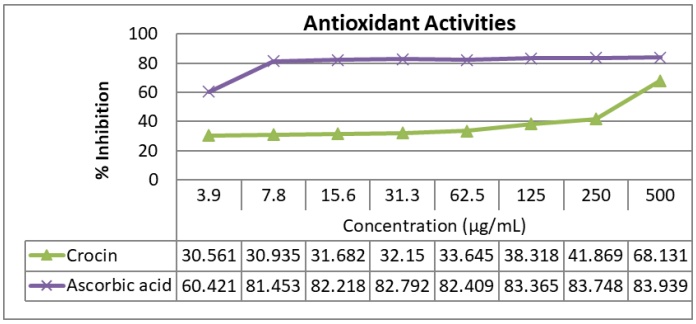


Figure 5. Antioxidant activities (inhibition percentage) of crocin and ascorbic acid standard.

Table 5. IC_{50} values (µg/ mL) and linear equation of crocin and ascorbic acid standards

Extracts	Linear equation	IC_{50} (µg/mL)
Ascorbic acid	$y = 15.759x - 1.024$, $R^2 = 0.999$	3.10±0.15
Crocin	$y = 0.072x + 29.473$, $R^2 = 0.963$	283.92±3.93

Crocin molecules show good antioxidant activity as they presented 68% inhibition. A similar result was noted by Okmen *et al.* (2016) when they carried out a DPPH scavenging activity assay. In the present study, high concentrations of the aqueous saffron extracts showed 72% inhibition. The crude saffron extracts showed high antioxidant activity due to the presence of different potential compounds that have a synergistic quenching effect on free radicals. Nevertheless, saffron showed a significant antioxidant activity. Few studies investigated the biological activity of saffron plant because of its many health benefits (Hosseinzadeh *et al.,* 2007; Urrutia *et al.,* 2007; Licón *et al.,* 2010; Okmen *et al.,* 2016). Hence, saffron has many applications in health drinks, functional food, pharmaceuticals and cosmetic products (Assimopoulou *et al.,* 2005; Rahaiee *et al.,* 2015). This plant can also prevent several diseases, such as cardiovascular and neurodegenerative diseases (Licón *et al.,* 2010). Furthermore, crocin derivatives extracted from saffron are pharmacologically active (Masi *et al.,* 2016).

Crocin is an effective antioxidant in addition to being a natural colourant. The presence of the conjugated double bonds in the molecular structure of crocin enables it to receive electrons from reactive species, thereby neutralising the free radicals (Rutz *et al.,* 2016). Carotenoids are not produced in human bodies, so they need to be consumed through the diet (Suganya *et al.,* 2015). Numerous researchers have attempted to investigate and explore natural sources of carotenoids to determine their antioxidant activity that could lead to radical scavenging activities, such as anti-ageing, anti-cancer and anti-inflammation (Licón *et al.,* 2010).

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4. Conclusion

This research determined the environmental stability of carotenoid molecules extracted from saffron stigma. Environmental and geographical factors significantly affected the composition and content of carotenoids in saffron. Carotenoids are effective free-radical scavengers due to their capacity to trap peroxyl radicals and quench singlet oxygen. Thus, the most hydrophilic carotenoid molecules (C_{50}) extracted from Iranian saffron plants were screened and analysed for their bioactive capabilities, such as antifungal, antibacterial and antioxidant properties. The chemical structure, number of conjugated double bonds, structural end-groups and oxygen-containing groups significantly affect the bioactivities of carotenoids. Therefore, hydrophilic crocin that makes up the majority of saffron is a superior biological compound that has great potential for use as a biocolourant in the food and beverage, pharmaceutical and cosmetic industries.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The research for this paper was funded by the Kursi Syeikh Abdullah Fahim Grant, the National University of Malaysia (RH-2020-006). We are also thankful to the Herbarium Unit Laboratory, International Islamic University Malaysia, for their support in completing this research work.

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