The Journal of Medicinal Plants and By-Products
Original Article

Wild *Crocus haussknechtii* BOISS Stigmas as a Rich Source for Crocin Extraction

Zahra Tahmasebi[1*], Hassan Feizi[2,3], Noosheen Fallahi[4] and Soheila Mohammadi[1]

[1]*Agronomy and Plant Breeding Department, Faculty of Agriculture, Ilam University, Ilam, Iran*
[2]*Department of plant production, University of Torbat Heydarieh, Torbat Heydarieh, Iran*
[3]*Saffron institute, University of Torbat Heydarieh, Torbat Heydarieh, Iran*
[4]*Plant Breeding (Molecular genetics and genetic engineering), Agronomy and Plant Breeding Department, Faculty of Agriculture, Ilam University, Ilam, Iran*

*Corresponding author: Email z.tahmasebi@ilam.ac.ir

Article History: Received: 14 Auguste 2024/Accepted in revised form: 04 November 2024

ABSTRACT

Crocus haussknechtii Boiss, commonly known as wild saffron, is the closest wild relative of cultivated saffron (*C. sativus* L.). Due to limited information on the presence of expensive chemical compounds responsible for color and aroma in its stigma, this study aimed to compare and measure crocin, picrocrocin, and safranal, (which are responsible for color, taste, and aroma, respectively) in both cultivated and wild saffron using HPLC. Additionally, the volatile metabolites present in the stigmas of both species were identified and quantified using gas chromatography-mass spectrometry (GC-MS). *C. sativus* corms were purchased from saffron cultivation fields in Torbat Heydariyeh, Iran. *C. haussknechtii* corms were collected from Zagros forests, Ilam, Iran, and cultivated in the field. The results revealed significantly higher levels of all compounds, particularly crocin, in wild saffron than cultivated saffron. The crocin content was 35.49 and 478.99 mg/g dry weight in cultivated saffron wild saffron, respectively. GC-MS analysis of *C. sati vus* stigma identified 5 major and 26 minor compounds. In contrast, 6 major compounds and 17 minor compounds were identified in *C. haussknechtii* stigma. These findings showed that the surprising amount of crocin in this unknown wild species suggested the value of further studies on this species.

Keywords: GC-MS, HPLC, Picrocrocin, Safranal, Metabolites

INTRODUCTION

Cultivated saffron (*Crocus sativus* L.) is a triploid (2n = 3x = 24) perennial plant with a genome size of 1C = 3.45 Gbp [1]. It is one of the most important medicinal herbs in the Iridaceae family, with a cultivation history dating back to 2500-1500 BC. Saffron is believed to have originated in Iran and Greece and has since spread to India, China, the Mediterranean, and Eastern Europe [2]. In Iran, the primary source of saffron was the Alvand and Zagros mountain ranges in the ancient land of Media, encompassing Hamadan, Borujerd, Nehavand, Kermanshah, and the regions around Isfahan and Qom. Its cultivation later spread to other regions [3].

C.haussknechtii BOISS, locally known as "Pēēshūkk," is a wild saffron species native to the Zagros Mountains of Iran, northern Iraq, and southern Jordan. This edible geophyte is harvested in spring in the western provinces of Iran (Kermanshah, Ilam, Lorestan, and Hamedan) [4]. Genetic diversity assessment of Iranian saffron species (Crocus spp.: *C. sativus*, *C. haussknechtii*, *C. cancellatus*, *C. speciosus*, *and C. caspius*) using SSR markers revealed that *C. haussknechtii* is the closest wild relative of cultivated saffron [5].

Saffron's value stems from its unique composition of primary secondary metabolites and their derivatives. The three key components of saffron stigmas are crocin, safranal, and picrocrocin, responsible for their color, aroma, and flavor, respectively [6]. Crocin pigments accumulate abundantly in saffron flower stigmas, imparting their distinctive deep red color [7]. Due to the labor-intensive harvesting and processing of the collected stigmas, these metabolites command high market prices [8].

Some of the wild Crocus species hold promise as alternative sources of saffron's primary metabolites [10, 9]. However, research on the primary compounds (crocin, picrocrocin, and safranal) in wild Crocus species remains limited [11].

A comprehensive chemical analysis of cultivated saffron stigmas has revealed over 694 distinct metabolites. Saffron is characterized by a diverse array of chemical compounds, including carbohydrates, minerals, mucilages, vitamins (particularly riboflavin and thiamine), pigments (crocin, anthocyanin, carotenoids, lycopene, and zeaxanthin), a fragrant terpenic essential oil called safranal, and flavoring compounds (picrocrocin) [12]. However, no chemical analysis of wild saffron stigmas has been conducted to date.

Given the limited information available on wild saffron, this study aimed to compare and measure the color and flavor compounds (crocin, picrocrocin, and safranal) using HPLC and volatile compounds using GC-MS in cultivated saffron and wild barley stigmas.

MATERIAL AND METHODS

Plant Materials

Cultivated saffron corms (*C.sativus* L.) were purchased from saffron cultivation fields in Torbat Heydariyeh, Khorasan Razavi, Iran. Wild saffron corms (*C. haussknechtii*) were collected from the Zagros forests, in Ilam, Iran. Corms of both species were cultivated in the educational-research farm of Ilam University. Ilam City is located at 33° 38' N and 46° 25' E and at an altitude of 1440 meters above sea level. Ilam has a temperate mountainous climate with an average annual rainfall of 619.5 mm and an average absolute temperature ranging from -13.6 to 41.2 °C. Each sample was planted in six 1 m × 1 m plots in September 2021. Flowers of both species were collected in November 2022. Stigmas of the collected flowers of each species were separated and then dried in the shade for 8 days.

HPLC Analysis

100 mg of finely ground saffron stigmas were weighed and transferred to a 10 mL test tube. 5 mL of 80% ethanol (v/v) in water was added, and the mixture was vortexed vigorously for 1 minute. The mixture was then centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected and transferred to a lahh25 mL test tube. This extraction procedure was repeated two more times using 5 mL of 80% ethanol each time [13].

Prior to HPLC analysis, 500 µL of nitroaniline internal standard (0.5 mg/mL in ethanol–water (80% V/V)) was added to 500 µL of the sample and mixed thoroughly. Standards of safranal (88% purity), picrocrocin, and crocin were purchased from Sigma-Aldrich (St. Louis, MO). Safranal, picrocrocin and crocin content were calculated from standard curves generated using 0-500 µg/mL, 0-500 µg/mL, and 0-25 mg/mL dilutions series, respectively (Figure 1).

The HPLC system used was a Philips system equipped with a Pu 41110 UV-visible detector and a Sunfire C18 column (250 mm × 4.6 mm, 5 µm particle size) (Waters Corporation, Milford, MA, USA). Injections were performed using a Hamilton syringe. All solvents used were HPLC grade and were filtered through 0.45 µm cellulose acetate filters before use and degassed. HPLC analysis was carried out on a dual-solvent delivery system equipped with a Waters 600E pump (Waters Corporation, Milford, MA, USA).

A C18 column and a 50/50 gradient of methanol and water (15% acetonitrile) were used as the mobile phase at a flow rate of 1 mL/min for 25 min at room temperature. The injection volume was 10 µL. The analyses were repeated three times for each sample. Picrocrocin was detected at 250 nm, crocin at 440 nm, safranal at 310 nm, and the internal standard above all three wavelengths. The amount of each compound was determined using the area under the curve and calculated based on the standard curve obtained from the injection of standard compounds. The standard curve was also obtained by injecting different concentrations of standard compounds [14].

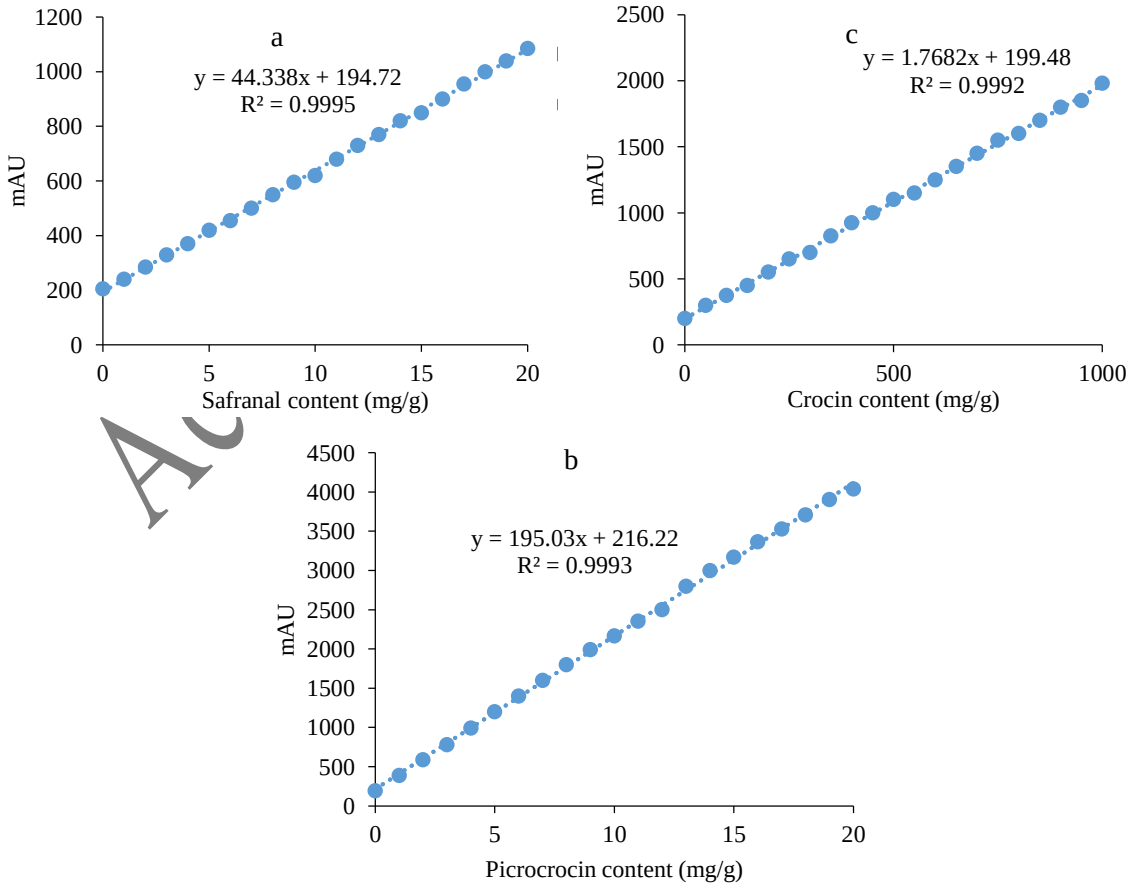

Fig. 1 standard curves for Safranal (a), picrocrocin (b) and crocin (c) content calculation

GC-MS Analysis

Saffron samples were initially ground into a powder using a porcelain mortar and pestle. Then, 500 mg of each saffron sample was weighed using a digital balance and placed in a dark-colored, sealed container.

The prepared saffron samples were extracted with diethyl ether in two steps. The extraction was carried out using an ultrasonic bath at a constant frequency of 35 kHz and a temperature of 25 °C. In each step, 5 mL of diethyl ether was added to each saffron sample, and the samples were then placed in the ultrasonic bath at a constant temperature of 25 °C for 15 minutes. After this time, the extracted saffron extract was transferred to another container, and the same procedure was repeated with 5 mL of diethyl ether and extraction by ultrasonic bath. After the ultrasonic treatment, the extracted organic phase was combined with the extract from the first extraction, resulting in a final extract volume of approximately 10 mL. A small amount of sodium sulfate anhydride was added to the extract, and the samples were then filtered and purified using filter paper before injection into the GC-MS instrument [15].

A gas chromatograph (GC) model 7890B-HP (USA Technologies) (Agilent) equipped with a mass selective detector (MSD) model HP-5977A (Agilent Technologies) was used for the analysis of saffron extract. The ionization energy was set to 74 eV and a capillary column HP-5MS (5% phenyl dimethyl) (silxan) was used. The column dimensions were 30 m × 0.25 mm × 0.25 µm (30 m long, 0.25 mm in diameter, and 0.25 µm in thickness). Helium was used as the carrier gas with a purity of 99.99% and a flow rate of 1 mL/min. The temperature program was as follows: the column temperature was initially held at 50 °C for 3 min, then increased at a rate of 3 °C/min to 180 °C, and finally increased at a rate of 15 °C/min to 250 °C and held for 5 min. The injector and detector temperatures were set to 220 °C and 290 °C, respectively. A 6 µL sample of saffron extract was injected manually using a Hamilton microsyringe into the injection port of the GC in Splitless mode. The experiment was repeated at least twice for each sample. If the data did not match, the experiment was repeated until the results were reproducible.

RESULTS

HPLC Analysis

The metabolites crocin, picrocrocin, and safranal were measured in both saffron species (Figure 2), and the results are presented in Table 1.

The amount of all three compounds differed significantly between the stigmas of the two saffron species. The mean crocin content was 35.49 mg/g dry weight for the cultivated species and, interestingly, 899.47 mg/g dry weight for the wild species. For *C. sativus* saffron, picrocrocin was 4.18 mg/g and for *C. haussknechtii* saffron, it was 8.14 mg/g. Safranal was less than 5 mg/g in cultivated saffron and 9 mg/g in wild saffron (Table 1).

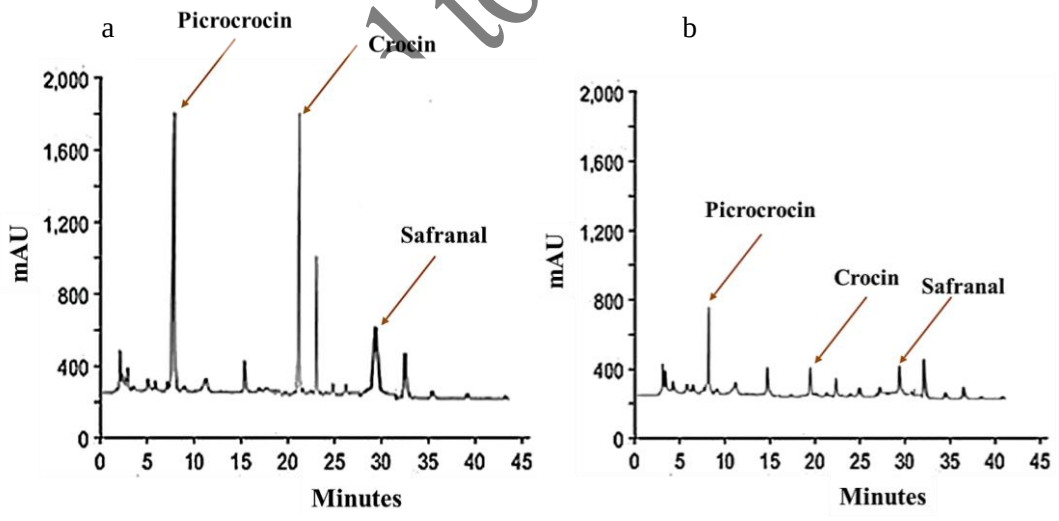

Fig. 2 HPLC chromatograms of *C.haussknechtii* (a) and *C.sativus* (b)

A wide range of values has been reported for the main metabolites of saffron (Crocus sativus L.) stigmas, with significant variation from country to country. Reported values for crocin range from 29 mg/g [16] to 67.3 mg/g for Indian saffron [17] and 45.99 mg/g for Iranian saffron [18]. Safranal levels reported by some researchers are around 0.88 mg/g [17], while other reported values for safranal range from a minimum of 0.06 mg/g to a maximum of 0.29 mg/g [19]. The amount of picrocrocin in Spanish saffron is between 0.79 and 12.94%, 1.07 and 2.16% in Indian saffron, and 2.18 to 6.15% in Iranian saffron [20]. A review paper, citing other studies, reported crocin ranges for saffron from different countries between 6.29 (China) and

41.21 (India), picrocrocin ranges from 0.53 (China) to 8.14 (Spain), and safranal ranges from 0.22 (China) to 8.14 (Spain) (all compounds extracted using HPLC) [11].

In a study, the amount of crocin in the stigmas of three saffron species, cultivated saffron (Crocus sativus L.) and two wild species (*C. caspius* and C. speciosus), was determined. The results showed that the crocin content was significantly higher in the two wild species, *C. caspius* (35.83 mg/g dry weight) and C. speciosus (35.40 mg/g dry weight), compared to the cultivated saffron (15.27 mg/g dry weight) [21].

Table 1 HPLC Analysis Results of Saffron Stigma Extracts (*C. sativus* and *C. haussknechtii*)

Samples	Crocin content (mg/g)	Safranal content (mg/g)	Picrocrocin content (mg/g)	LOQ
C.sativus	35.49 ± 0.02	<5 ± 0.01	4.18 ± 0.02	5
C.haussknechtii	899.47 ± 0.01	9 ± 0.03	8.14 ± 0.02	5

In a study, crocin was extracted from the stigmas of two species, *C. sativus*, and *C. haussknechtii*, and analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The relatively high concentration of pigments in the stigmas of *C. haussknechtii* plants and the similarity of the carotenoid composition of this species with that of *C. sativus* indicated that some wild Crocus species could be used as potential sources of saffron compounds [10]. To date, there has been no report on the amount of safranal and picrocrocin in *C. haussknechtii*, and this is the first report.

GC-MS Analysis

The results of the GC-MS analysis of *C. sativus* stigma extract are presented in Table 2. A total of 5 major compounds and 26 minor compounds were identified in the stigma.

The most abundant compound was 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester, with the formula $C_{24}H_{38}O_4$ and an abundance of 43.006%. This compound is also known as Diethylhexyl phthalate (DEHP). DEHP is an industrial colorless and oily organic carcinogen with a slight odor. In industry, bis(2-ethylhexyl) phthalate is mainly used as a plasticizer to make flexible materials for many household products. Inhalation, ingestion, and skin contact with this compound have been linked to an increased incidence of liver cancer in animals, and it is considered a probable human carcinogen [22].

Table 2 Constituents identified in the stigma of *C. sativus* and *C.haussknechtii* extracts by GC-MS.

Species	Number	Metabolite name	Relative peak area (%)	Retention time (min)
	1	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl	4.93	15.012
C. sativus	2	Benzeneacetonitrile, 4-chloro	0.042	18.339
	3	Phenol, 2-(1,1-dimethylethyl)	0.093	20.174
	4	2,6-DI-T-BUTYL-4-METHYLENE-2,5-CYCLOHEXADIENE-1-ONE	0.42	21.440
	5	Dodecanenitrile	9.088	21.652
	6	Heptadecane	0.242	21.710
	7	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.992	22.039
	8	Hexadecane	0.591	23.741
	9	2 2-Bromo dodecane	0.473	25.839
	10	Benzoic acid, 2-ethylhexyl ester	0.457	25.902
	11	Octadecane	0.844	27.409
	12	Hexadecane, 2,6,10,14-tetramethyl	0.607	27.579
	13	Nonadecane	2.808	29.115
	14	Hexadecanoic acid, methyl ester	0.753	29.547
	15	Palmitic acid	1.150	30.151
	16	Eicosane	3.44	30.744
	17	Heneicosane	3.98	32.298
	18	Docosene	3.513	33.231
	19	Tetratriacontane	0.559	34.309
	20	Tricosane	1.757	35.207
	21	Tetracosane	3.773	36.789
	22	Normal-docosane	0.918	38.733
	23	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	43.006	40.179
	24	Diisooctyl phthalate	10.251	40.463
	25	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	0.920	40.711
	26	Monopentyl Phthalate	1.304	40.763
	27	Dihydro-.beta.-ionol	0.494	42.08
	28	Squalene	0.535	44.473
	29	1-Hexacosene	0.631	44.941
	30	14B-PREGNANE	0.391	46.751

	31	E-11-Tetradecen-1-ol trifluoroacetate	0.635	46.832
C.haussknechtii	1	bis(2-ethylhexyl) phthalate	6.063	3.156
	2	Isothiocyanic acid	0.991	3.699
	3	4,5-Dimethyl-2-pentadecyl-1,3-dioxolane	0.549	3.879
	4	5-Methyl-2 (3H)-Furanone-	0.790	17.558
	5	trans-2-oxa-6-decalone	1.413	19.187
	6	Nookatone	0.496	21.433
	7	Dodecane	4.017	21.692
	8	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.655	22.033
	9	Hexadecane	1.253	25.835
	10	Pentacosane	0.882	29.539
	11	Hexadecanoic acid	0.998	30.067
	12	Eicosane	13.994	32.860
	13	Vitamin E	1.845	34.066
	14	Phthalic acid	11.867	39.906
	15	Octadecane	0.707	42.695
	16	Tetrapentacontane, 1,54-dibromo	8.446	43.612
	17	1,3-Cyclohexadiene-1-18carboxaldehyde, 2,6,6-trimethyl	6.931	43.914
	18	Tricosane	1.027	44.028
	19	Stigmasta-7,16-dien-3-ol, (3.beta.,5.alpha.)	13.55	44.497
	20	Nonadecane	1.796	44.903
	21	Cetyl palmitate	15.25	44.998
	22	Docosane	2.730	45.152
	23	Pentacosane	4.555	45.215

However, DEHP and similar compounds have been identified in some plant species and even microorganisms, and they have been shown to have antibacterial, antimicrobial, and anticancer properties. For example, these compounds have been found in *Calotropis gigantea* [23, 24] and *Aspergillus Awamori* [25]. GC-MS analysis of saffron samples from major saffron-growing regions in Turkey also showed the presence of this compound [26]. Analysis of 26 samples of saffron stigma from 9 countries with GC×GC-ToF-MS also identified the compound 1, 2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, but it was not reported as a major compound [27].

The next major compound identified in the extract was Diisooctyl phthalate (DIOP), accounting for 10.251% of the total composition. With the chemical formula $C_{24}H_{38}O_4$, DIOP exhibits low toxicity in plants and possesses promising antimicrobial properties. Additionally, DIOP has demonstrated potent anti-cancer activity by inhibiting melanogenesis, the process of melanin production in skin cells [28]. GC-MS analysis of *Citrullus colocynthis* (L.) seeds revealed DIOP as the major constituent, accounting for approximately 58% of the identified compounds [29]. Similar findings have reported the presence of DIOP in *Plantago major* [24] and *Haliclona caerulea* [30], highlighting its widespread occurrence in various plant species.

Another major compound found in cultivated saffron is Dodecanenitrile (9.088%), a nitrile with the chemical formula $C_{12}H_{23}N$. Nitriles (RC≡N or organic cyanides) are a family of molecules containing one or more cyano groups consisting of a carbon atom triple-bonded to a nitrogen atom. Today, over 400 natural nitrile compounds from various plant, animal, and microbial sources have been discovered worldwide in both terrestrial and marine environments. These molecules play important roles in carbon and nitrogen metabolism, wound response, and intraspecies communication, acting as a key interface for microorganism-plant-animal interactions [31].

Another major compound of saffron is safranal (2, 6, 6-trimethyl-1, 3-cyclohexadiene-1-carboxaldehyde) with the chemical formula $C_{10}H_{14}O$ and an abundance of 4.93%. It is an important metabolite of saffron and has been identified in other similar studies on saffron [27, 26]. According to various studies, safranal is the main chemical responsible for the aroma of *C. sativus* and exhibits pharmacological activities including anticonvulsant, hypnotic, and other effects, justifying its importance as a potential drug in the future. Safranal can be introduced as an anticonvulsant/antianxiety/hypnotic drug [32]. Safranal exerts its antioxidant effect by stabilizing membranes in various biological systems, reducing peroxidation of unsaturated fatty acids in membranes, and restoring the reduced activity of antioxidant enzymes present in the body [33].

Six major and 17 minor compounds were identified in *Crocus haussknechtii* species (Table 2).

Cetyl palmitate, with the formula $C_{32}H_{64}O_2$, is the most abundant compound in wild saffron, with a relative abundance of 15.25%. Cetyl palmitate is one of the most important waxes with wide applications in the cosmetic and pharmaceutical industries. It can be used as an emulsifier and thickener in creams. This compound is naturally found in the head cavities of sperm whales (*Physeter macrocephalus*), but extraction from this source is not feasible [34]. The most common synthesis method for cetyl palmitate is enzyme-catalyzed esterification. However, enzymes are expensive. Cetyl palmitate is the ester

of palmitic acid (a fatty acid found in plants and animals) and cetyl alcohol. Acids or enzymes [35, 36] can catalyze esterification of fatty acids. The compound Isopropyl palmitate has been previously reported in cultivated saffron [37].

One of the predominant compounds in the wild saffron stigma extract is Icosane, with the molecular formula $C_{20}H_{42}$ and an abundance of 13.994%. Icosane is a natural hydrocarbon compound found in several plants, including Drosera indica L. [38] and *Barringtonia asiatica* L. [39]. Previous studies have also identified it as a major component of the stigma of cultivated saffron [40]. Icosane exhibits remarkable anti-inflammatory and antimicrobial properties. For instance, in a diabetic mouse wound model, the administration of Icosane and octadecane accelerated wound healing [12]. Eicosane increases multiple metabolites, including L-arginine and L-carnitine, in the retina. Consequently, in a glaucoma mouse model, it protected retinal cells from N-methyl-D-aspartate-induced damage [38].

The next most abundant compound in wild saffron is Stigmasta-7, 16-dien-3-ol, (3β, 5α), with a relative abundance of 13.55% and a molecular formula of $C_{29}H_{48}O$.

Stigmasterol is a naturally occurring steroidal derivative found in many plants, including cabbage, *Gypsophila oldhamiana*, Arabidopsis, *Aralia cordata*, eucalyptus, and *Physcomitrella patens* [41, 42]. Literature review suggests that stigmasterol can act as a precursor to corticosteroids-1, progesterone, androgens, estrogens, and vitamin D3 and can readily cross the blood-brain barrier [43, 44]. Previous studies have proposed stigmasterol as a potential cancer treatment candidate due to its ability to inhibit tumor growth [45]. One study investigated the effect of crocin and stigmasterol from cultivated saffron on the in vitro growth of promastigotes and amastigotes of Leishmania major, confirming their efficacy in reducing parasite growth [46].

Another abundant compound present in wild saffron is Phthalic acid ($C_6H_4(CO_2H)_2$). This compound was identified in this plant with an abundance of 11.867%. It is the simplest member of phthalate esters. Phthalates are ubiquitous compounds and have been used as plasticizers in polymers for several decades. Phthalates have been isolated from a wide range of plants, algae, bacteria, and fungi [47, 48]. In some studies, phthalates have been classified as plant secondary metabolites, and there are reports of biological activity of phthalates isolated from living organisms [49, 23, 29]. However, further studies are needed to elucidate the mechanisms involved and the ecological consequences of these compounds [50].

Another prevalent compound in *C.haussknechtii* is Tetrapentacontane, 1, 54-dibromo, with an abundance of 8.446% and a molecular formula of $C_{54}H_{108}Br_2$. This compound is an essential oil. Similarly, this compound has been identified in the extract of the *Opuntiaficus-indica* plant [51].

Saffronal was also observed in this species similar to the cultivated species with a frequency of 6.931%.

CONCLUSION

The findings of this study revealed that the concentration of crocin in wild saffron is several times higher than that in cultivated saffron. Considering the high price of saffron carotenoid compounds, this species can be utilized for extracting higher quantities of these valuable compounds. Additionally, due to the genetic closeness of the wild saffron (*C.haussknechtii*) to the cultivated one, there is a possibility of transferring its fragrance and flavor genes to the cultivated species.

GC-MS analysis of the stigmas of wild saffron and cultivated saffron revealed the presence of several valuable medicinal compounds in both species. The antimicrobial, anticancer, and antioxidant properties of these compounds have been demonstrated in studies. These findings further enhance the potential of the wild species for future research.

Funding

This research has received financial support from the Saffron Institute at the University of Torbat Heydarieh. The grant number associated with this funding is 128852.

Authorship Contribution Statement

Zahra Tahmasebi: Writing – original draft, Validation, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Hasan Feyzi:** Writing – review & editing, Project partners. **Noushin Fallahi:** Project partners. **Soheila Mohammadi:** Project partners.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Aboobucker S.I., Suza W.P. Why do plants convert sitosterol to stigmasterol?. Front. Plant Sci. 2019; 10:354. https://doi.org/10.3389/fpls.2019.00354.
2. Addai Z.R., Abood M.S., Hlail S.H. GC-MS profiling, antioxidants and antimicrobial activity of prickly pear (Opuntiaficus-indica) pulp extract. Pharmacogn J.2022; 14(2):262-267.

3. Ahrazem O., Rubio-Moraga A., Nebauer S.G., Molina R.V., Gomez-Gomez L. Saffron: its phytochemistry, developmental processes, and biotechnological prospects. J. Agric. Food Chem. 2015; 63(40):8751-64. 10.1021/acs.jafc.5b03194.
4. Anastasaki E., Kanakis C., Pappas C., Maggi L., Del Campo C.P., Carmona M., Alonso G.L., Polissiou M.G. Geographical differentiation of saffron by GC–MS/FID and chemometrics. Eur. Food Res. Technol. 2009;229:899-905.
5. Asil H. GC-MS analysis of volatile components of Safranbolu and Kirikhan saffron (Crocus sativus L.) prepared by ultrasonic extraction. Fresenius Environ. Bull. 2018: 9557-9563 10.1021/acs.jafc.5b03194.
6. Baba S.A., Malik A.H., Wani Z.A., Mohiuddin T., Shah Z., Abbas N., Ashraf N. Phytochemical analysis and antioxidant activity of different tissue types of Crocus sativus and oxidative stress alleviating potential of saffron extract in plants, bacteria, and yeast. S Afr J Bot .2015; 99:80-7. https://doi.org/10.1016/j.sajb.2015.03.194.
7. Caballero-Ortega H., Pereda-Miranda R., Riverón-Negrete L., Hernández J.M., Medécigo-Ríos M., Castillo-Villanueva A., Abdullaev F.I. Chemical composition of saffron (Crocus sativus L.) from four countries. Acta horticulturae. 2004:321-6. https://doi.org/10.1007/s12010-022-03984-8.
8. Cirillo N.A., Quirrenbach C.G., Corazza M.L., Voll F.A. Enzymatic kinetics of cetyl palmitate synthesis in a solvent-free system. Biochem. Eng. J. 2018; 137:116-24. 10.1016/j.bej.2018.05.021
9. Drioiche A., Ailli A., Handaq N., Remok F., Elouardi M., Elouadni H., Al Kamaly O., Saleh A., Bouhrim M., Elazzouzi H., El Makhoukhi F. Identification of Compounds of Crocus sativus by GC-MS and HPLC/UV-ESI-MS and Evaluation of Their Antioxidant, Antimicrobial, Anticoagulant, and Antidiabetic Properties. J. Pharm. 2023; 16(4):545. https://doi.org/10.3390/ph16040545.
10. Falahatpishe H., Mazloumi M.T., Komili Phonoud R. Seyed Ahmadian F. Chromatography method (HPLC) for determination of saffronol as quality characteristic of Iranian saffron and comparison with spectrophotometry method. J. Food Sci. Tech. 2004; 1(3): 57-66.
11. Fan J.C., Ren R., Jin Q., He H.L., Wang S.T. Detection of 20 phthalate esters in breast milk by GC-MS/MS using QuEChERS extraction method. Food Addit. Contam: Part A. 2019; 36(10):1551-8. 10.1080/19440049.2019.1646435.
12. Gomathi D., Kalaiselvi M., Ravikumar G., Devaki K., Uma C. GC-MS analysis of bioactive compounds from the whole plant ethanolic extract of Evolvulus alsinoides (L.) L. JFST. 2015; 52:1212-7. 10.1007/s13197-013-1105-9.
13. Habib M.R., Karim M.R. Antimicrobial and cytotoxic activity of di-(2-ethylhexyl) phthalate and anhydrosophoradiol-3-acetate isolated from Calotropis gigantea (Linn.) flower. Mycobiology. 2009;37(1):31-6. 10.4489/MYCO.2009.37.1.031.
14. Hadizadeh F., Mahdavi M., Emami S.A., Khashayarmanesh Z., Hassanzadeh M., Asili J., Seifi M., Nassirli H., Shariatimoghadam A., Noorbakhsh R. Evaluation of ISO method in saffron qualification. InII Int Symposium on Saffron Biol Technol 739. 2006; 28: 405-410.
15. Hoshyar R., Bathaie S.Z., Etemadikia B. Quantitative and comparative analysis of major metabolites (crocin, picrocrocin and safranal) in different packages of Iranian saffron by HPLC. Pathobio. Res. 2010; 13(2):63-71.http://mjms.modares.ac.ir/article-30-1974-en.html.
16. Huang L., Zhu X., Zhou S., Cheng Z., Shi K., Zhang C., Shao H. Phthalic acid esters: Natural sources and biological activities. Toxins. 2021; 13(7):495. 10.3390/toxins13070495.
17. Kametani T., Furuyama H. Synthesis of vitamin D3 and related compounds. Med. Res. Rev. 1987;7(2):147-71.10.1002/med.2610070202.
18. Kangsamaksin T., Chaithongyot S., Wootthichairangsan C., Hanchaina R., Tangshewinsirikul C., Svasti J. Lupeol and stigmasterol suppress tumor angiogenesis and inhibit cholangiocarcinoma growth in mice via downregulation of tumor necrosis factor-α. PloS one. 2017;12:12(12):e0189628. https://doi.org/10.1371/journal.pone.0189628.
19. Kavitha A., Prabhakar P., Vijayalakshmi M., Venkateswarlu Y. Production of bioactive metabolites by Nocardia levis MK-VL_113. J. Appl. Microbiol. 2009;49(4):484-90. 10.1111/j.1472-765X.2009.02697.x.
20. Kumari N., Menghani E., Mithal R. GCMS analysis & assessment of antimicrobial potential of rhizospheric Actinomycetes of AIA3 isolate. Indian J. Tradit. Knowl. (IJTK). 2019; 19(1):111-9. 10.56042/ijtk.v19i1.30849.
21. Lage M., Cantrell C.L. Quantification of saffron (Crocus sativus L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. Sci Hortic. 2009; 121(3):366-73. https://doi.org/10.1016/j.scienta.2009.02.017.
22. Li N., Lin G., Kwan Y.W., Min ZD. Simultaneous quantification of five major biologically active ingredients of saffron by high-performance liquid chromatography. J Chromatogr. A. 1999; 849(2):349-55. 10.1016/s0021-9673(99)00600-7.
23. Liu M., Li S. Nitrile biosynthesis in nature: how and why?. Nat. Prod. Rep. 2024; 41(4):649-71. https://doi.org/10.1039/d3np00028a.
24. Lotfy M.M., Hassan H.M., Hetta M.H., El-Gendy A.O., Mohammed R. Di-(2-ethylhexyl) Phthalate, a major bioactive metabolite with antimicrobial and cytotoxic activity isolated from River Nile derived fungus Aspergillus awamori. J. Basic Appl. Sci. 2018;7(3):263-9. https://doi.org/10.1016/j.bjbas.2018.02.002.
25. Magdouli S., Daghrir R., Brar S.K., Drogui P., Tyagi R.D. Di 2-ethylhexylphtalate in the aquatic and terrestrial environment: a critical review. J. Environ. Manage. 2013; 127:36-49. https://doi.org/10.1016/j.jenvman.2013.04.013.
26. Moraga Á.R., Rambla J.L., Ahrazem O., Granell A., Gómez-Gómez L. Metabolite and target transcript analyses during Crocus sativus stigma development. phytochem. 2009; 70(8):1009-16. 10.1016/j.phytochem.2009.04.022.
27. Mosaviniya M., Kikhavani T., Tanzifi M., Yaraki M.T., Tajbakhsh P., Lajevardi A. Facile green synthesis of silver nanoparticles using Crocus Haussknechtii Bois bulb extract: Catalytic activity and antibacterial properties. Colloids Interface Sci. Commun. 2019;33:100211. https://doi.org/10.1016/j.colcom.2019.100211.
28. Mousavi S.M., Khoshkam M., Feizi J. Comparison of metabolic profile in different saffron samples based on their geographical origin using gas chromatography-mass spectroscopy techniques (GC-MS). Saffron Agron Technol. 2021; 9(2):177-191. https://doi.org/10.22048/jsat.2021.245568.1408.

29. Mutlu V.N., Yilmaz S. Esterification of cetyl alcohol with palmitic acid over WO3/Zr-SBA-15 and Zr-SBA-15 catalysts. Appl. Catal. A-Gen. 2016; 522:194-200. https://doi.org/10.1016/j.apcata.2016.05.010.
30. Mykhailenko O., Kovalyov V., Goryacha O., Ivanauskas L., Georgiyants V. Biologically active compounds and pharmacological activities of species of the genus Crocus: A review. Phytochem. 2019; 162:56-89. https://doi.org/10.1016/j.phytochem.2019.02.004.
31. Namayandeh A., Nemati Z., Kamelmanesh M.M., Mokhtari M., Mardi M. Genetic relationships among species of Iranian crocus (Crocus spp.). Crop Breed. 2013; 3(1): 61-67. 10.22092/CBJ.2013.100451.
32. Nanda S., Madan K. The role of Safranal and saffron stigma extracts in oxidative stress, diseases and photoaging: A systematic review. Heliyon. 2021;7(2). https://doi.org/10.1016/j.heliyon.2021.e06117.
33. Newill H., Loske R., Wagner J., Johannes C., Lorenz R.L., Lehmann L. Oxidation products of stigmasterol interfere with the action of the female sex hormone 17β-estradiol in cultured human breast and endometrium cell lines. Mol. Nutr. Food Res. 2007; 51(7):888-98. https://doi.org/10.1002/mnfr.200700025.
34. Ordoudi S.A., Tsimidou M.Z. Saffron quality: Effect of agricultural practices, processing and storage. InProduction Practices and Quality Assessment of Food Crops Volume 1: Preharvest Practice. Dordrecht: Springer Netherlands. 2004; 209-260. 10.1007/1-4020-2533-5_8.
35. Pandita D. Saffron (*Crocus sativus* L.): Phytochemistry, therapeutic significance and omics-based biology. In Medicinal and aromatic plants. Academic Press. 2021; 325-396. https://doi.org/10.1016/B978-0-12-819590-1.00014-8.
36. Radjabian T., Saboora A., Naderimanesh H., Ebrahimzadeh H. Comparative Analysis of Crocetin and Its Glycosyl Esters from. J. Food Sci. 2001; 38(4):324-8. Thiemann T. Isolation of phthalates and terephthalates from plant material–natural products or contaminants?. Open Chem. J. 2021; 8(1). 10.2174/1874842202108010001.
37. Ramu R., Shirahatti P.S., Nayakavadi S., Vadivelan R., Zameer F., Dhananjaya B.L., Prasad N. The effect of a plant extract enriched in stigmasterol and β-sitosterol on glycaemic status and glucose metabolism in alloxan-induced diabetic rats. Food Funct. 2016; 7(9):3999-4011. 10.1039/c6fo00343e.
38. Ranjbar R., Shayanfar P., Maniati M. In vitro antileishmanial effects of saffron compounds, crocin and stigmasterol, on iranian strain of Leishmania major (MHOM/IR/75/ER). Iran. J. Parasitol. 2021;16(1):151. https://doi.org/10.18502/ijpa.v16i1.5535.
39. Rezaee R., Hosseinzadeh H. Safranal: from an aromatic natural product to a rewarding pharmacological agent. Iran. J. Basic Med. Sci. 2013; 16(1):12. 10.22038/IJBMS.2013.244.
40. 24. Romeh A.A. Diethyl phthalate and dioctyl phthalate in Plantago major L. Afr. J. Agric. Res. 2013;8(32):4360-4. https://doi.org/10.5897/AJAR2013.7242.
41. Santos M.L., Albini E., Corazza M.L., Krieger N., Voll F.A. Kinetics of enzymatic cetyl palmitate production by esterification with fermented solid of Burkholderia contaminans in the presence of organic solvent. React. Kinet. Mech. Catal. 2021; 132:139-53. https://doi.org/10.1007/s11144-020-01889-3.
42. Shafeian E., Ghavam Mostafavi P., Moridi Farimani M., Mashinchian Moradi A., Nazemi M. Extraction and investigation of biological activities of dioctyl phthalate and dibutyl phthalate from marine sponge Haliclona (Soestella) caerulea Larak Island, Persian Gulf. Iran. J. Fish. Sci. 2022; 21(5):1141-55..10.22092/IJFS.2022.127710.
43. Shahinfar F., Taghikhah-Khomami S., Fallah S.F., Afshar-Mohammadian M., Bakhshi D. The stamen and stigma of some species of wild saffron (Crocus sp.) as a rich source of antioxidants. Journal of Plant Process and Function. 2021;10(42):1-2. 20.1001.1.23222727.1400.10.42.8.0.
44. Sujata V., Ravishankar G.A., Venkataraman L.V. Methods for the analysis of the saffron metabolites crocin, crocetins, picrocrocin and safranal for the determination of the quality of the spice using thin-layer chromatography, high-performance liquid chromatography and gas chromatography. J. Chromatogr. A. 1992; 624(1-2):497-502. https://doi.org/10.1016/0021-9673(92)85699-T.
45. Tajik S., Zarinkamar F., Bathaie Z. Quantification of crocin, picrocrocin and safranal components of saffron (Crocus sativus L.) in Ghaen and Tabas regions. Iranian J bio. 2012;25(3):423-429. 10.1002/pca.3047.
46. Tammaro F. Notizie storico-colturali sullo zafferano (Crocus sativus L. Iridaceae) nell'area mediterranea. Micol. Veget. Medit. 1987; 2:44-59.https://doi.org/10.1021/acsomega.3c03342.
47. Tarantilis P.A., Tsoupras G., Polissiou M. Determination of saffron (Crocus sativus L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. J. Chromatogr. A. 1995; 699(1-2):107-18. 10.1016/0021-9673(95)00044-n.
48. Thiemann T. Isolation of phthalates and terephthalates from plant material–natural products or contaminants?. Open Chem. J. 2021; 8(1).
49. Umaru I.J., Badruddin F.A., Umaru H.A. Phytochemical screening of essential oils and antibacterial activity and antioxidant properties of Barringtonia asiatica (L) leaf extract. Biochem. Res. Int. 2019; 2019(1):7143989. 10.1155/2019/7143989.
50. Zhang H., Hua Y., Chen J., Li X., Bai X., Wang H. Organism-derived phthalate derivatives as bioactive natural products. Iran. J. Environ. Health Sci. Eng. Part C. 2018; 36(3):125-44. 10.1080/10590501.2018.1490512.
51. Zwane B.N., Kamatou G.P., Viljoen A.M., Betti G., Schmidt M. Variation in headspace volatiles of saffron determined by GC × GC-ToF-MS. Nat. Prod. Commun. 2020; 15(11):1934578X20967612. https://doi.org/10.1177/1934578X20967612.