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Extraction of phenolic and flavonoid compounds and evaluation of their antioxidant activity in saffron anthers (*Crocus sativus* L.)

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Abstract

Saffron (*Crocus sativus* L.) is the most expensive spice in the world and rich in a variety of bioactive compounds including phenolic acids, flavonoids, and vitamins. Saffron is used in food, cosmetics, perfumery, and dye industries due to its color, taste, aroma, and medicinal properties. The extraction of bioactive compounds imposes a constant search for economically and environmentally viable extraction strategies for higher yields including the solvent type. The research was carried out to evaluate total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of *C. sativus* L. anthers using different extraction solvents including ethanol, methanol, and distilled water. The results showed that ethanol was the most effective extraction solvent type exhibiting the highest TPC (7.29 mg GAE g^{-1} DW), TFC (3.77 mg QE g^{-1} DW), and antioxidant activity of flavonoids (88%) and ascorbic acid (76.07%). Flavonoids proved to be stronger antioxidants than ascorbic acid, irrespective of solvent type, however the ethanolic extracts showed significantly higher antioxidant activity. A Reverse Phase High-Performance Liquid Chromatography method revealed that gallic acid (3.1 mg g^{-1}), syringic acid (0.2 mg g^{-1}), and vanillic acid (0.13 mg g^{-1}) were the main phenolic compounds detected in the dried anther ethanolic extracts, while quercetin (2.13 mg g^{-1}), pyrogallol (1.73 mg g^{-1}), kaempferol (1.2 mg g^{-1}), rutin (0.2 mg g^{-1}), and tricin (0.1 mg g^{-1}) the main flavonoid compounds. Thus, apart from the spice, constituted by the red stigmas, the production of other flower parts such as the anthers, so far considered agricultural waste can constitute a rich source of bioactive compounds of high antioxidant potential by setting quality standards for new products' development and ensuring better valorization of saffron's bioresidues in Mashhad region of Iran.

Keywords: DPPH free radical scavenging; extraction solvents; medicinal plant; reversed-phase HPLC; saffron flower parts; secondary metabolites

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Introduction

Pharmaceutical, cosmetic, and food industries use medicinal and aromatic plants rich in bioactive compounds, mainly polyphenols, vitamins, and enzymes that have specific properties (Lachguer *et al.*, 2023). Saffron (*Crocus sativus* L.) is the most fascinating and intriguing species, among the 85 species in total, which belongs to the genus *Crocus* (Fernandez, 2007). *C. sativus* has been cultivated and used in herbal medicine as early as 1550 years BC, nevertheless not much and detailed information is available about its' spread worldwide. Several authors trace back its origin to Central Asia, Middle East or southwestern islands of Greece. After that, saffron was expanded to India, China, Middle East countries, and the Mediterranean basin (Mzabri *et al.*, 2021a). There are a few countries like Iran, Greece, Morocco, Spain, Italy, Turkey, France, Switzerland, Pakistan, China, Japan, and Australia where this spice is cultivated and exported to other countries, with Iran first in saffron production covering almost the 80% of the world demand (Kumar *et al.*, 2022).

Saffron (*C. sativus* L.) (Iridaceae) is a sterile geophyte with autumnal flowering and asexually reproduced only through corms. It has been reported that *C. sativus* grows well into various environmental conditions from temperate to subtropical climates and from arid to semi-arid regions (Mzabri *et al.*, 2021b), exhibiting higher yields under rainy autumn, mild winter, and warm summer climatic conditions (Ahmad *et al.*, 2013). In major saffron producing countries, particularly in Iran and India, there are more than 95,000 farm families associated directly or indirectly with the crop. There are several production constraints including large area under rain fed cultivation, inadequate seed replacement rate, lack of high yielding cultivars adapted to diverse growing conditions, biotic and abiotic stresses, inadequate technical support, poor infrastructure and institutional support on crop management, limited policy directives and incentives, and inefficient technology delivery system. Measures need to be taken care off as an offset to restrict or even eliminate the aforementioned problems so as the saffron industry to become more profitable (Ahmad *et al.*, 2013).

Saffron or red gold or golden condiment is recognized as a profitable economic product across the globe because of the red dried stigmas of its flower used as a very expensive, luxurious, and valuable spice and dye in food. Other uses of saffron include enhancer of product shelf-life, fortifying agent in developing different functional food products, additive in perfumes and cosmetics, all the way to medicinal purposes (Maqbool *et al.*, 2022). It is highly appreciated for its color (crocin), fragrance (picrocrocin), and flavor (safranal) due to over 150 volatile and aromatic compounds it contains (Serghini *et al.*, 2017).

The intense, demanding and laborious harvest and postharvest (dehydration and storage) processes raise the saffron price (Koocheki, 2020). For 1 kg of stigma production, around 1000 kg of flowers are needed, which corresponds to 220,000-260,000 flowers (Ghanbari *et al.*, 2019), therefore the minimization of losses and efficient waste management of other plant parts (by-products) (Lahmass *et al.*, 2017) such as sepals, petals, anthers, stamens, leaves, etc. that contain compounds with sensorial properties or biological activity (Caser *et al.*, 2020) are essential to offset the low biomass production of the red stigmas (Lahmass *et al.*, 2017). The raising interest of the various saffron flower parts and more efficient biological waste management may improve small-scale farmers' income through the development of innovative products (Jadouali *et al.*, 2019).

Phenolic acids (*i.e.* chlorogenic acid, caffeic acid, methylparaben, gallic acid, pyrogallol), flavonoids, vitamins, monoterpenes, minerals, alkaloids, phytosterols, carotenoids, glycosides, aldehydes, picrocrocin and anthocyanins, amino acids, proteins, starch, and gums are the main constituents isolated and discovered in different flower saffron extracts including the red stigmas (Fernadez, 2007; Gismondi *et al.*, 2012; Abu-Izneid *et al.*, 2022; Maqbool *et al.*, 2022). Hydroxybenzoic acids (flavonoid biosynthesis precursors) have been identified in several parts of *C. sativus* and hydroxycinnamic acids such as vanillic acid in petals (Termentzi and Kokkalou, 2008). Significant amounts of vitamins (A, B1, B2, B6, and C) have been discovered in antioxidant compounds of the Moroccan *C. sativus* L. flower parts (Jadouali *et al.*, 2019).

Many researchers have demonstrated the relation between the antioxidant activity of plants and the presence of bioactive compounds such as phenolic acids and flavonoids, among others (Rashmi and Negi, 2020). Saffron flower by-products have higher total polar phenols content, exerting higher antioxidant activity, towards other spices (Chichiricco *et al*., 2019). Several studies have investigated whether food products (*i.e.* wheat flour pasta, fresh ovine cheese), supplemented with saffron extract enhanced the antioxidant and sensory properties of the final product (Aktypis *et al*., 2018; Armellini *et al*., 2018). With the discovery of saffron's new therapeutic properties against several health problems (cancer, depression, hypertension, Alzheimer, diabetes, Parkinson, psychological) (Rahimi, 2015), the demand for stigmas as the most consumed part of the plant and other flower parts as by-products has increased the last decade.

A continuous search for economically and environmentally viable extraction strategies have been carried out to isolate bioactive compounds from saffron. The traditional extraction procedures are slow and require a large amount of solvent, nevertheless improved extract quality and higher yield can be achieved using novel extraction strategies that require less time and limited solvent amount (Rahaman *et al*., 2021). The use of coextraction techniques, selection of appropriate solvent type, time, temperature, and consideration of solvent-solute affinity are factors that influence the whole extraction process (Ozkan *et al*., 2021). Water and organic solvents, such as methanol, are the most used for the extraction of many bioactive constituents from saffron stigmas (Rahaiee *et al*., 2015). Nevertheless, water is adequate as an extraction solvent for polar compounds, while organic solvents are efficient for the extraction of only polar and weak polar compounds (Turrini *et al*., 2019), however, their application is limited due to toxicity, environmental hazardous, high cost, and low biodegradability (Rahaiee *et al*., 2015). It has been proven that ethanolic and aqueous extracts of saffron serve as strong antioxidants, since they inhibit malondialdehyde formation and lipid peroxidation in red blood cells, prevent the activation of reactive oxygen species by boosting cell viability through apoptotic pathway inhibition, in specific, ethanolic extracts scavenge hydroxyl radicals and accelerate deoxyribose breakdown (Rodrigues-Ruiz *et al*., 2016).

Although there is a plethora of published articles related to TPC, TFC, and antioxidant activity in different saffron flower parts including stigmas, sepals, petals and stamens, the anthers are tissues that have not studied extensively so far, highlighting the need for *C. sativus* anthers to be explored further for their benefits. Taking into account the augmented scientific and industrial interest for saffron by-products, the aim of this study was to apprehend the chemical composition of anthers from *C. sativus* cultivated in Iran (Mashhad province). The samples were evaluated for their TPC, TFC, antioxidant activity, and major compounds in phenolics and flavonoids. The specific objectives were (1) to determine TPC and TFC of saffron anthers using different extraction solvents (ethanol, methanol, distilled water), (2) to evaluate the antioxidant activity of flavonoids in the anther extracts by various assays (DPPH, FRAP) and solvents, in comparison to that of ascorbic acid as a standard antioxidant, and (3) to evaluate qualitatively and quantitatively the major phenolic and flavonoid compounds by RP-HPLC.

Materials and Methods

Plant and chemical materials

Saffron anthers were purchased from Novin Saffron Co. (Mashhad, Iran). Ethanol, methanol and all reagents and solvents HPLC grade were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of crude extract

Three methods were tested to extract saffron: 10 g of *C. sativus* dried anthers were extracted with three different solvents [aqueous ethanol (50% v/v), aqueous methanol (50% v/v), and distilled water]. Then the aqueous extract from each step, was evaporated (Heidolph, model: Rota vacvario power unit No: 11-300-004-

33-3, Germany) at 40 °C to separate alcoholic solvent. The stock solutions of each sample were prepared in methanol (1 mg L^{-1}) and stored at 0 °C in darkness. They were further diluted in water to 2 and 50 mg L^{-1} for Spectrophotometry and HPLC analysis, respectively. For saffron anthers extract, solution was centrifuged at 13000 rpm and supernatant was kept and used as the stock (Hadizadeh *et al*., 2010).

Total phenolic content

The total phenolic content (TPC) of *C. sativus* anther extracts was calculated using the Folin-Ciocalteau (FC) technique. 5 ml of FC reagent (i.e., 1:10, 1 ml of anther extract and 0.9 ml of FC reagent) and 4 ml of sodium carbonate were added to the anther extracts (7.5%). After 30 min of incubation at 20 °C, the absorbance was measured spectrophotometrically at 765 nm. The results were compared to a gallic acid calibration curve that was used previously. The extracts' total phenolic content was calculated as mg gallic acid equivalent g^{-1} dry weight (mg GAE g^{-1} DW) (Suvarchala *et al*., 2020). The experiment was repeated three times.

Total flavonoid content

The total flavonoid content (TFC) of *C. sativus* anther extracts was determined using the aluminum trichloride technique. Total flavonoid content was calculated using quercetin as a standard. The anther extracts (1 mg ml^{-1}) were then mixed with 75 ml of sodium nitrate (5% $NaNO_2$) solution and 150 ml of 10% aluminum chloride solution and allowed to stand for 5 min. By adding 500 ml of 4% NaOH, the resulting reaction mixture was diluted with distilled water to a final volume of 2.5 ml. The absorbance of the blank, standard, and anther extracts were all measured at 510 nm. The extracts' total flavonoid concentration was calculated as mg quercetin equivalent g^{-1} dry weight (mg QE g^{-1} DW) (Suvarchala *et al*., 2020). The experiment was done in triplicates.

Antioxidant activity of flavonoids

The extracts' free radical scavenging activities were measured using the method described by Karimi *et al*. (2010), with some modifications. Three replicates of each determination were performed. Higher free radical scavenging activity was demonstrated by lower absorbance values in the reaction mixture. The percentage of inhibition of the free radical scavenging activities of the examined samples was estimated using the following equation by Karimi *et al*. (2010): DPPH activity inhibition in percent (%) = $[(A0 - A1) / A0)] \times 100\%$ where A0 is the absorbance value of the blank sample or control reaction and A1 is the absorbance value of the test sample. The concentration of the sample required for 50% inhibition was obtained by plotting a percent inhibition or percent scavenging effect with sample concentrations. The inhibition curve at 50%, or IC50, was used to represent the value for each test sample.

Antioxidant activity of ascorbic acid

Ferric reduces antioxidant activity (FRAP). The extracts' ferric reducing ability was evaluated using an assay developed by Karimi *et al*. (2010). The experiment was done three times. The standard antioxidant was ascorbic acid.

Determination of phenolic and flavonoid compounds by HPLC

A reversed-phase high performance liquid chromatography (RP-HPLC) methodology based on the method reported by Karimi *et al*. (2010) was used to quantify the phenolic and flavonoid components in saffron. Gallic acid, syringic acid, vanillic acid, salicylic acid, and caffeic acid were used as phenolic compound standards, while apigenin, kaempferol, uteolin, quercetin, tricin, rutin, and pyrogallol were employed as flavonoid compound standards. The ethanolic extract was fed into an Agilent-1200 series high-performance liquid chromatography (HPLC) system with a UVVis photodiode array (DAD) detector, binary pump, vacuum degasser, auto sampler, and an analytical column (Intersil ODS-3 5 m 4.6 150 mm Gl Science Inc).

Deionized water and acetonitrile were utilized as solvents, and the pH of the water was lowered to 2.5 with trifluoroacetic acid. At 280 nm, phenolic compounds were detected, while flavonoid compounds were detected at 350 nm. The column was equilibrated with 85% solvent A and 15% solvent B. The ratio of solvent B was increased to 85% in 50 min, then reduced to 15% in 55 min. At a flow rate of 0.6 ml min^{-1}, this ratio was maintained until the 60th min of the next analysis.

Statistical analysis

Analysis of variance (ANOVA) was performed with the SPSS 17.0 statistical package and mean separation with Duncan's Multiple Range Test at a significance level of 5% ($p < 0.05$). The experimental layout was completely randomized. The experiment related to different solvents effect on TPC and TFC consisted of three treatments (3 replicates × 3 samples/ replicate = 9 samples/ treatment) (one-way ANOVA). The experiment regarding the antioxidant activity was a 3 × 2 factorial one with three solvents (ethanol, methanol, distilled water) and two bioactive compound groups-analytical methods (flavonoids-DPPH assay, ascorbic acid-FRAP assay), thus included six treatments (3 replicates × 3 samples/ replicate = 9 samples/ treatment). The main effect of factors (solvent type, antioxidant activity of flavonoids vs ascorbic acid) and their interaction was determined by General Linear Model/ 2-way ANOVA. One-way ANOVA was used for means comparison derived from the three solvents for each antioxidant activity evaluation assay (flavonoids-DPPH, ascorbic acid-FRAP), separately. The means for the major phenolic and flavonoid compounds identified by RP-HPLC were subjected to one-way ANOVA.

Results and Discussion

A recent study conducted on saffron reports that TPC of flower extracts was found higher (130.4 mg GAE g^{-1} DW) in the case of hydro-ethanol, lower in water (104.8 mg GAE g^{-1} DW), and intermediate (114.7 mg GAE g^{-1} DW) in methanol as a solvent (Lachguer *et al.*, 2023). In this study and among the three extraction solvents used, ethanol proved to be the most effective one exhibiting the highest TPC (7.29 mg GAE g^{-1} DW) and TFC (3.77 mg QE g^{-1} DW), the distilled water displayed the lowest TPC and TFC values, whereas methanol gave intermediate results as compared to the other solvents (Table 1). As regards the effectiveness of the three solvents used herein, the descending order of quantitative content for both bioactive compound groups in dried saffron anther extracts was ethanol > methanol > distilled water (Table 1).

Table 1. Effect of extraction solvent on total phenolics (TPC) and total flavonoids content (TFC) from anthers of *C. sativus* L

Solvent	TPC (mg GAE g^{-1} DW)	TFC (mg QE g^{-1} DW)
Ethanol	7.29 ± 0.01 a	3.77 ± 0.07 a
Methanol	6.56 ± 0.01 b	2.60 ± 0.12 b
Distilled water	5.10 ± 0.12 c	1.87 ± 0.03 c
p-values (one-way ANOVA)	0.000***	0.000***

Means (n = 3) ± standard deviation (SD) with different letters in each column (TPC, TFC) between solvents denote significant differences (Duncan's test, $p < 0.05$). *** $p \le 0.001$

Consistent with the findings presented in this study, ethanol has been reported to be the most appropriate solvent type for higher extraction yields of total phenols in spring saffron (Horozic *et al.*, 2019) and *C. sativus* L. waking corms (Esmaeili *et al.*, 2011) among different solvents (water, acetone, methanol) using the Folin-Ciocalteu method. Accordingly, Rahaiee *et al.* (2015), Jadouali *et al.* (2017), and Yousuf *et al.* (2018) report the greater suitability of ethanol solvent for obtaining better qualitative and quantitative data and higher extraction yields of total phenolics from different saffron flower part (style, petals, stamens, stigmas) extracts as

compared to other solvent sources (methanol, water, ethyl acetate, diethyl ether, hexane). In contradiction with the results herein, the higher amounts of TPC in the methanolic extract of the saffron anthers than in the water extract, are not in agreement with their higher polarity as previously stated (Sun *et al*., 2020). Our findings regarding TPC and TFC differ from data obtained by Karimi *et al*. (2010) who found that methanol was the most efficient extraction solvent followed by ethanol, whereas both studies agree with the postulation that water is the least effective solvent.

Different amounts of TPC have been recorded in the different flower parts of *C. sativus* dried extracts including 11.8 mg g^{-1} DW (Moradi *et al*., 2018) and 0.04 mg quercetin g^{-1} in the style (Fardaghi *et al*., 2021), 1.38 mg caffeic acid g^{-1} DW in petals (Termentzi and Kokkalou, 2008), 21.36 mg GAE g^{-1} in the spath, 16.63 mg GAE g^{-1} (Lahmass *et al*., 2017), 1.83 mg quercetin g^{-1} (Fardaghi *et al*., 2021), and 6.5 mg GAE g^{-1} DW in stigmas (Karimi *et al*., 2010). Besides extraction solvent type and flower part sample analyzed, the geographical origin and cultivation year and site of saffron from which samples are taken play a significant role on TPC and TFC. Indeed, the saffron produced in the north west Italian Alps exhibited a very high TPC ranging between 8.8 and 36.4 mg GAE g^{-1} DW with no differences between years or sites (Caser *et al*., 2020), TPC of saffron cultivated in Lebanon was lower (1.6 mg GAE g^{-1} DW) (Makhlouf *et al*., 2021), while TPC of Iranian (0.02 mg g^{-1} DW) (Karimi *et al*., 2010) and Greek saffron (0.01 mg g^{-1} DW) is reported to be significantly lower (Proestos *et al*., 2005). Accordingly to TPC, TFC also varies depended on saffron plant parts and extraction solvents used as evidenced by several studies including 0.5 mg rutin equivalent g^{-1} DW in style calli (Moradi *et al*., 2018), 5.8 mg rutin equivalent g^{-1} DW in stigmas (Karimi *et al*., 2010), 60.6 mg CE g^{-1} DW in petals (methanolic extract) (Jadouali *et al*., 2017), 0.03-0.09 mg CE g^{-1} in tepals, and 0.03-0.06 mg CE g^{-1} in stamens (Bellachiona *et al*., 2022b), and 6.4 mg CE g^{-1} DW in methanolic stamen extracts (Jadouali *et al*., 2017). In the present study, irrespective of extraction solvent, the amounts of TPC (5.1-7.29 mg GAE g^{-1} DW) in *C. sativus* anther extracts (Mashhad region, Iran) were considerably higher than the respective amounts of TFC (1.87-3.77 mg QE g^{-1} DW) (Table 1). Similarly, other studies report higher TPC in different saffron flower parts [petals: 25-65 mg GAE g^{-1} DW (Jadouali *et al*., 2019)/ tepals: 36 mg GAE g^{-1} DW (Bellachiona *et al*., 2022a)] as compared to TFC [petals: 12-60 mg CE g^{-1} DW (Jadouali *et al*., 2019)/ tepals: 2.4 mg CE g^{-1} DW (Bellachiona *et al*., 2022a], in particular petals compared to styles (Jadouali *et al*., 2019) and tepals compared to stigmas (Bellachiona *et al*., 2022a).

Comparison of the antioxidant activity of flavonoids (DPPH) vs ascorbic acid (FRAP)

Several parameters have been reported to affect the antioxidant activity of samples such as the extraction method, solvent type, genotype, environmental conditions, plant part sample, season of the year, analytical method used, pH, and metal ions, among others (Kalinowska *et al*., 2020). The antioxidant activity of flavonoids (DPPH assay) was significantly higher (88%) in ethanolic extracts, lower (73.23%) in the case of distilled water as a solvent, while methanol performed intermediate value (81.17%) differing from the other solvents (Table 2). Accordingly, ethanol was the most appropriate solvent resulting in the highest antioxidant activity of ascorbic acid (FRAP assay) (76.07%) differing significantly from methanolic (70.53%) and distilled water (71.20%) (Table 2), a result that differs from the findings of Karimi *et al.* (2010) who observed the highest antioxidant activity (FRAP assay) in the methanolic saffron extracts (79%) followed by the aqueous (69%) and ethanolic (51%) extracts. The antioxidant activity of flavonoids and ascorbic acid in saffron dried anther extracts herein was maximized with ethanol as a solvent (Table 2). Different solvents have different polarities thus a shift in polarity modifies the solvent capacity to take apart a selected group of antioxidant compounds affecting the antioxidant activity assessment (Rahaiee *et al*., 2015). Consistent with our results, the ethanolic extract of saffron stigmas showed the highest IC_{50} value (Baba *et al*., 2015) and the aqueous extract of the Italian saffron very low IC_{50} (Gismondi *et al*., 2012). There are numerous studies, however pointing out the greater effectiveness of methanol, as the best solvent type, for higher antioxidant activity of bioactive

compounds in different saffron flower part extracts (*i.e.* whole flowers, stamens, stigma, petals, styles) followed by water and ethanol (Karimi *et al*., 2010; Jadouali *et al*., 2017; Lachguer *et al*., 2023). It becomes evident from the results obtained herein that the descending order of quantitative antioxidant activity of flavonoids was ethanol > methanol > distilled water (DPPH) while that of ascorbic acid was ethanol > distilled water ≥ methanol (FRAP) (one-way ANOVA) (Table 2).

In the present study, the antioxidant activity of flavonoids (DPPH) was significantly higher than the antioxidant activity of ascorbic acid (FRAP) comparing the two IC_{50} values for each solvent type separately (2-way ANOVA) (Table 2), showing clearly that flavonoids are foreseen as stronger antioxidants than ascorbic acid, an outcome that comes in contradiction with the findings of Lachguer *et al*. (2023) in the same species as presented herein, where the antioxidant activity of ascorbic acid was found considerably higher than that of quercetin as a flavonoid component. Antioxidant activity of ascorbic acid was similar when methanol and distilled water were used as solvents without a significant difference (2-way ANOVA) (Table 2), therefore, among the three solvents and the two bioactive compounds, flavonoids ethanol-extracted proved to be the strongest antioxidant source. Horozić *et al*. (2019) found that ethanol displayed the strongest antioxidant capacity in spring saffron and water the weakest one, while Karimi *et al.* (2010) found that the free radical scavenging (68.2%) and ferric reducing power activities (78.9%) of saffron stigmas were higher in the methanolic extract in relation to those of boiling water and ethanol. The weakest antioxidant capacity of ascorbic acid or vitamin C herein could be ascribed either to its pro-oxidant activity (Kosar *et al*., 2011) or to the fact that is soluble in water, thus some of its amounts were depleted and the available remaining quantity left was not enough for the process of reducing fatty acid oxidation, leading to bleaching of β-carotene (Hossein Goli *et al*., 2012). The positive influence of plant extracts on the antioxidant DPPH radical scavenging activity can be owing to their competence to provide hydrogen, which lessens the stable purple DPPH free radical to the yellow non-radical DPPH-H form (Xie *et al*., 2015).

Table 2 Antioxidant activity of flavonoids (DPPH assay) of anthers of *C. sativus* L. compared with antioxidant activity of ascorbic acid (FRAP assay) using different solvents

Solvent	Antioxidant activity of flavonoids (%)	Antioxidant activity of ascorbic acid (%)
Ethanol	88.00 ± 0.30 a (A)	76.07 ± 0.03 a (C)
Methanol	81.17 ± 0.27 b (B)	70.53 ± 0.33 b (E)
Distilled water	73.23 ± 0.24 c (D)	71.20 ± 0.35 b (E)
p-values (one-way ANOVA)	0.000***	0.000***
p-values (2-way ANOVA / General Linear Model)		
Solvent type (A): 0.000***		
Flavonoids vs Ascorbic acid (B): 0.000***		
(A)*(B): 0.000***		

Means (n = 3) ± SD with different small letters in each column denote significant differences (Duncan's test, $p < 0.05$, one-way ANOVA). Means (n = 3) ± SD with different capital letters in parenthesis in the two columns denote significant differences (Duncan's test, $p < 0.05$, 2-way ANOVA, General Linear Model). ***$p \leq 0.001$

In this study, the descending order of quantitative antioxidant activity between flavonoids-ascorbic acid and different solvents was: flavonoids-ethanol > flavonoids-methanol > ascorbic acid-ethanol > flavonoids-distilled water > ascorbic acid-methanol ≥ ascorbic acid-distilled water (Table 2). A higher TPC and TFC has been correlated with the higher antioxidant activity of saffron extracts that can be used as antioxidant food supplements (Karimi *et al*., 2010; Menghini *et al*., 2018; Wang *et al*., 2018). In saffron dried anther extracts herein, there was a linear relationship between TPC, TFC, antioxidant activity of flavonoids and antioxidant activity of ascorbic acid since all four parameters were simultaneously maximized in ethanolic extracts, which might be explained by the application of different antioxidant evaluation methods (DPPH, FRAP), the higher

content in phenolics and particularly in flavonoids, which are affected by solvent nature, either hydrophilic or lipophilic, and it's ability to separate different compounds (Karimi *et al*., 2010). Specifically, phenolic compounds have free radical scavenging ability facilitated by their hydroxyl groups, while flavonoids, suppress reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species, and up-regulate and protect antioxidant defences (*i.e.* breaking down of linkages between phenolics and flavonoids with their corresponding glycosides due to hydrolytic extraction) (Agati *et al*., 2012). According to Terpinc *et al*. (2012), a conflict may occur in the correlation between TPC and antioxidant compounds in saffron extracts either synergistic or antagonistic, seeing that several molecules of non-phenolic origin and non-free radical scavenging activity may arise due to possible reaction with the Folin–Ciocalteu reagent.

Phenolic/ flavonoid compounds in ethanolic extract of C. sativus L. anthers using RP-HPLC

The main bioactive components of the phenolic group identified in the dried saffron anther ethanolic extracts were gallic acid (3.10 mg g^{-1} DW), syringic acid (0.20 mg g^{-1} DW), and vanillic acid (0.13 mg g^{-1} DW), however, caffeic acid and salicylic acid were not detected (Table 3; Figures 1A-E). In accordance with the findings presented herein, the predominant phenolic compound detected in the methanolic saffron extract was gallic acid (1.82 mg g^{-1} DW) (Karimi *et al*., 2010). The main bioactive components of the flavonoid group identified in the dried saffron anther ethanolic extracts were quercetin (2.13 mg g^{-1} DW), pyrogallol (1.73 mg g^{-1} DW), kaempferol (1.20 mg g^{-1} DW), rutin (0.20 mg g^{-1} DW), and tricin (0.10 mg g^{-1} DW), however, apigenin and luteolin were not detected (Table 3; Figures 1F-L).

Table 3 Phenolic and flavonoid compounds content in the ethanolic extract of *C. sativus* L. anthers using RP-HPLC (injection volume: 0.1 ml, dilution: 1, area: 100%, height: 100%, W 05: 0.20 min)

Phenolic compound	Content (mg g^{-1} DW)	Retention time (min)	Area (mAUs)	Height (mAU)
Gallic acid	3.10 ± 0.06 a	2.28	620.15	521.44
Vanillic acid	0.13 ± 0.03 b	5.90	600.14	714.22
Syringic acid	0.20 ± 0.06 b	4.09	823.98	788.10
Caffeic acid	Not detected	3.88	951.46	744.05
Salicylic acid	Not detected	8.00	521.19	741.56
p-value (one-way ANOVA)	0.000***	-	-	-
Flavonoid compound	**Content (mg g^{-1} DW)**	**Retention time (min)**	**Area (mAUs)**	**Height (mAU)**
Kaempferol	1.20 ± 0.06 c	7.78	632.59	520.19
Quercetin	2.13 ± 0.03 a	8.83	900.14	750.12
Tricin	0.10 ± 0.00 d	11.15	632.08	655.98
Pyrogallol	1.73 ± 0.07 b	9.32	587.49	622.00
Rutin	0.20 ± 0.00 d	7.36	628.79	598.68
Apigenin	Not detected	10.00	522.69	700.12
Luteolin	Not detected	5.89	412.88	610.23
p-value (one-way ANOVA)	0.000***	-	-	-

Means (n = 3) ± SD with different letters in the content column for each compound group (phenolics, flavonoids) separately denote significant differences (Duncan's test, $p < 0.05$). *** $p \leq 0.001$

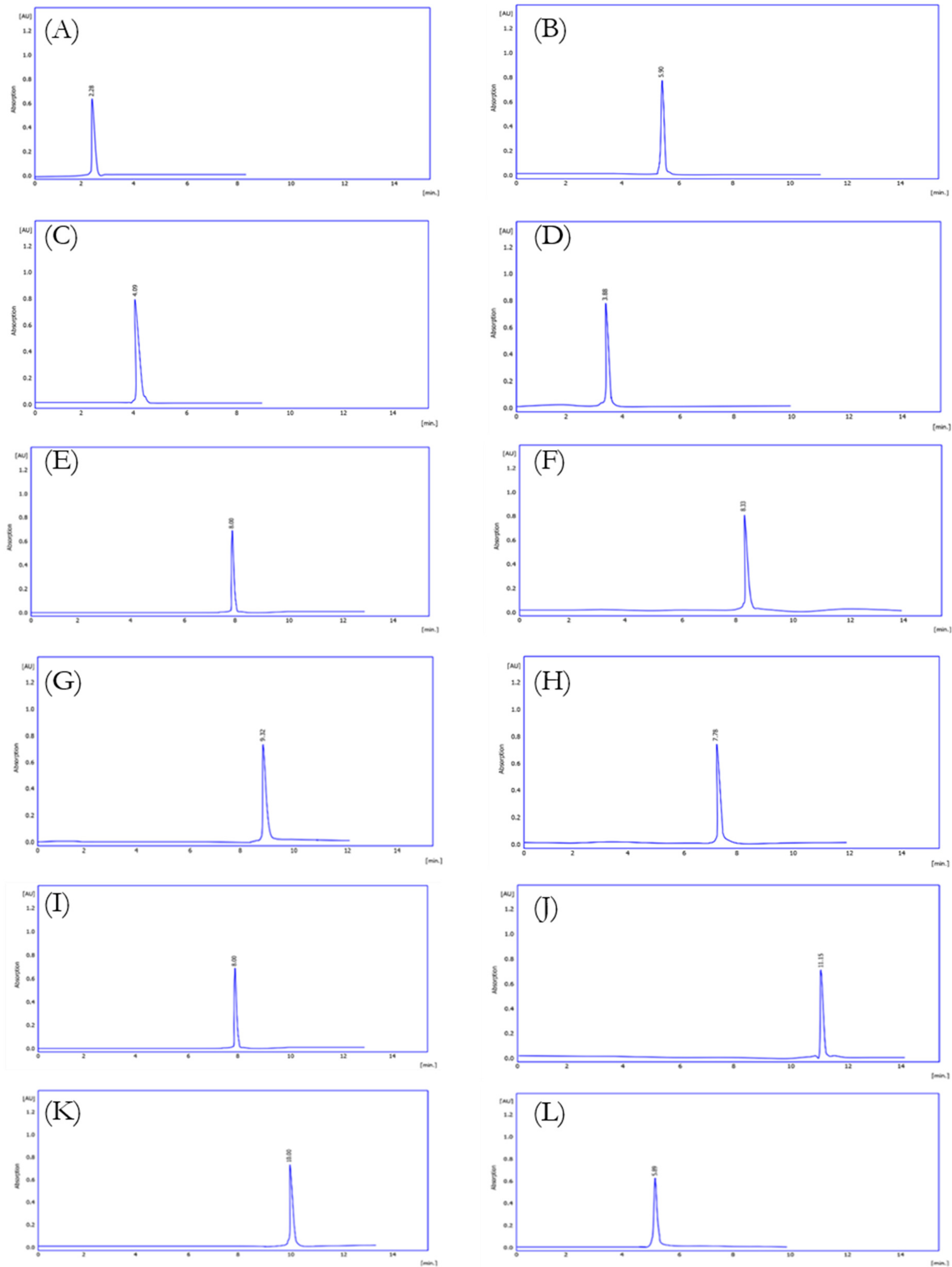

Figure 1. Chromatographs of the five phenolic (A-E) and the seven flavonoid compound standards (F-L), each at 10 ppm, identified in the dried anther ethanolic extracts of *C. sativus*; (A) Gallic acid; (B) Vanillic acid; (C) Syringic acid; (D) Caffeic acid; (E) Salicylic acid; (F) Quercetin; (G) Pyrogallol; (H) Kaempferol; (I) Rutin; (J) Tricin; (K) Apigenin; (L) Luteolin

A similar study in saffron anther extracts revealed that rutin 0.1 mg g^{-1}, vanillic acid 0.02 mg g^{-1}, gallic acid 0.09 mg g^{-1} (3.10 mg g^{-1} herein), and quercetin 0.01 mg g^{-1} were among the main constituents detected (Menghini et al., 2018), similarly to this study (Table 3). Three out of the 11 in total phenolic compounds detected in saffron corms including vanillin, syringic acid, and gallic acid (Esmaeili *et al*., 2011), were also identified in the studied saffron ethanolic anther extract (Table 3). In this study, the predominant phenolic and flavonoid compounds with the significantly highest amounts were gallic acid and quercetin, respectively (Table 3). Therefore, the descending quantitative order of the three major phenolic and the five major flavonoid compounds was gallic acid > syringic acid ≥ vanillic acid (one-way ANOVA) (Table 3; Figures 1A-E) and quercetin > pyrogallol > kaempferol > rutin > tricin (one-way ANOVA) (Table 3; Figures 1F-L), respectively. Pyrogallol (1.4 mg g^{-1} DW) was identified as the major flavonoid compound in methanolic saffron extract (Karimi *et al*., 2010), kaempferol 3-O-sophoroside in water extract (Gigliobianco *et al*., 2021), isoquercitrin, quercitrin, rutin, and vitamin C among others in tepal water-methanol extracts (Caser *et al*., 2020; Stelluti *et al*., 2021), vanillic acid, kaempferol- and quercetin glucosides as a rich source of flavonols (6-10 mg g^{-1}) in sepals, petals, and stamens with potential to be used as quantity and quality marker compounds (Hashemi Gahruie *et al*., 2020; Sun *et al*., 2020). Even though the abundance of gallic acid, quercetin and pyrogallol in anther extracts herein exhibited antioxidant activity, the existence of other compounds with high bioactive potential such as β-carotene, lycopene, vitamin E, ascorbic acid and other organic acids may also contribute as antioxidant sources (Ghasemzadeh *et al*., 2010).

Conclusions

The present study demonstrated that TFC, TFC, and antioxidant activity of flavonoids in saffron dried anthers (Mashhad region, Iran) were significantly higher in ethanolic extracts. Ethanol was the most effective solvent type followed by methanol, whereas the distilled water gave the lowest yields. These phytochemical contents were positively correlated with DPPH scavenging capacity and FRAP reducing power antioxidant activities. Regardless extraction solvent, the antioxidant activity of flavonoids was higher to that of ascorbic acid. Gallic acid was the predominant phenolic compound detected in ethanolic anther extracts followed by vanillic and syringic acids. Quercetin was the flavonoid compound identified in the highest amount followed by pyrogallol and kaempferol. The results of this study support further the sustainable utilization and valorization of derived high-quality floral bio-residues such as anthers, commonly shredded as waste during the processing of saffron stigmas as a spice, towards high value-added products in a sustainable way for high yields of strong antioxidant compounds (phenolics, flavonoids) with potential applications in agriculture, nutraceutical, pharmaceutical, cosmetic, and food industries. Yet, more research is required to clarify the different antioxidant mechanisms and scrutinize the isolation, quantification and identification of molecular profile of plant bioactive compounds detected in different saffron flower parts.

Authors' Contributions

Conceptualization: HEM and AAD; Methodology: HEM and AAD; Software: HEM, AAD, VS and TTT; Validation: HEM, AAD and VS; Formal analysis: HEM, AAD and VS; Investigation: HEM and AAD; Data curation: HEM, AAD and VS; Writing—original draft preparation: HEM, AAD, VS and TTT; Writing—review and editing: VS; Visualization: HEM, AAD and VS. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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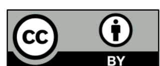

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