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Effects of Salicylic Acid on Carotenoids and Antioxidant Activity of Saffron (*Crocus sativus* L.)

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Abstract

Saffron (*Crocus sativus* L.), the most valuable medicinal food product, belongs to the Iridaceae family, which has been widely used as a coloring and flavoring agent. The stigmas contain three major compounds; crocins (carotenoid compound responsible for color), picrocrocin (responsible for taste) and safranal (responsible for odor). It has been used for medicinal purposes, as a spice and condiment for food and as a dye since ancient times. Numerous studies have shown crocins as main carotenoids of saffron to be capable of a variety of pharmacological effects, such as protection against cardiovascular diseases and inhibition of cancer cell development. Salicylic acid is a signaling molecule and a hormone-like substance that plays an important role in the plant physiological processes. Due to the importance of saffron as a valuable product, the aim of this study is to investigate the effect of salicylic acid application (0.01, 0.1 and 1 mM) on crocin and safranal content and antioxidant activity of stigmas. The results showed that salicylic acid application at 1 mM was the most effective treatment in increasing the crocin content and stronger antioxidant activity of stigmas, but it had a negative effect on safranal content; the highest quantity of this compound was observed in the control plants.

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1. Introduction

Saffron derived from the dried stigma of *Crocus sativus* flower is used as a spice for flavouring and colouring food. As a sterile triploid plant, its propagation is vegetative, and spreads by means of corms. Saffron blooms only once a year, and the planting and harvesting of corms, along with the removal of stigmas, are done manually, making saffron the world's most expensive spice, apart from its traditional value as a food additive and traditional herbal medicine. Recent studies indicate its potential as an anticancer, antitumoural, cytotoxic, hypo-lipidaemic, antiinflammatory and oxygenation enhancement agent [1-3]. *Crocus sativus* stigmas are characterized by the presence of sugars, minerals, fats, vitamins and secondary metabolites: terpenes, flavornoids, anthocyanins and carotenoids. Among them, carotenoids are the most important molecules because they determinate color and taste of the spice [4]. Characteristic compounds of saffron include three main metabolites: 1) Crocins, the main biologically active metabolites of saffron (unusual water-soluble carotenoids due to their high glycosyl contents, which is the reason for their great application as a food colorants); 2) Picrocrocins, the main substances responsible for saffron's bitter taste; and 3) Safranal the main component of the essential oil, and responsible for the characteristic saffron aroma, which is produced under basic conditions and after the drying process from picrocrocin [3-5]. These compounds are degradation products of carotenoids, and are known as *apocarotenoids*. They are derived from the oxidative cleavage of carotenoid-zeaxanthin [6,7]. Crocin and safranal are the main components of saffron, and have many biological functions such as anti-inflammatory and antioxidant activities [3]. Since saffron is used as a spice, its carotenoid content

may considerably enhance its food value because of the antioxidant properties [8]. Several factors like biotic and abiotic stresses may affect the level of secondary metabolites in plants. Various factors, including the age of the plant, the season, microbial attack, grazing, radiation, competition, and nutritional status, have been proven to impact on the secondary metabolite profile in higher plants [9]. Carotenoid content is also influenced by different environmental conditions like drought and temperature [10]. In other study, effects of altitude and temperature on the carotenoid content of saffron have been indicated [11]. In recent years, many studies have been undertaken to find practical approaches to increase the yield of secondary metabolites under *in vitro* conditions. One approach is the use of elicitors, which dramatically enhances the biosynthesis of secondary metabolites in plant cell cultures [12]. Salicylic acid (SA) is an endogenous plant growth regulator of phenolic nature. As an elicitor, is SA capable of enhancing plant growth and yield in some plants. It plays an important role in regulating a number of plant physiological processes, including photosynthesis and production of bioactive compounds [13]. The results of previous studies showed that production of soluble carbohydrates, sugars and secondary metabolites enhanced in plants exposed to SA [14]. Also several studies have described SA to induce gene regulation related to the biosynthesis of secondary metabolites in plants [15]. Regarding the importance of saffron as a valuable product and medicinal properties of carotenoids, this study aims to investigate the effect of SA on the crocin and safranal content and antioxidant activities of stigmas in *Crocus sativus*.

2. Materials and Methods

Saffron corms were prepared from the farms located in Torbat-Heidariye, north east of Iran in May 2013. Then, intact corms were selected and washed in running water, and pre-treated for 12 h with different concentrations of SA; the concentrations of SA were 0.01, 0.1 and 1 mM. After 12 h, the corms were planted at a depth of 7 cm in pots with 15 cm diameter and 11 cm depth containing fine per lit. Then the pots were irrigated with Hoagland nutrient solution 2 times a week. Flowers were collected early in the morning and dried daily in a drying oven at 50°C for 12 h. The dried stigmas were stored in dark glass jars at 4°C until performing biochemical studies.

2.1. Extraction

Saffron stigmas (20 mg) were suspended in 1 ml of methanol-water (50:50 %v/v) and magnetically stirred for 12 h at 4°C in the dark. After extraction, the samples were centrifuged at 30000 g for 20 min to abolish plant residues; then the supernatant was collected and filtered through a nylon membrane (Acrodisc 13, 0.45 µm pore size, and 13 mm diameter) [11]. Finally, 20 µl of the extract was injected into the HPLC.

2.2. HPLC Equipment

A Knauer HPLC system (Philips, Germany) equipped with a multiple UV wavelength photodiode array detector and Shimadzu RP C18 column (250 mm× 4 mm) was used.

2.3. HPLC Analysis

External standards of crocin and safranal were used for HPLC analysis. A linear gradient of methanol:water:acetonitrile (50:42.5:7.5 %v/v) was injected as a mobile phase with a flow-rate of 1.0 ml/min for a maximum elution time of 30 min at room temperature. Crocin and safranal were detected at 440 nm and 310 nm, respectively [11].

2.4. Antioxidant activities

2.4.1. DPPH Free Radical-Scavenging Assay

Saffron extract antioxidant activity was determined on the basis of its scavenging activity on the stable DPPH free radical. 4 ml of the final mixture was prepared by addition of 100 µl methanolic extracts of the samples (100, 200 and 300 ppm) to 3900 µL of a 6×10^{-5} mol l^{-1} methanolic DPPH solution. We examined the absorbance decrease of DPPH methanolic solution at 517 nm after 30 minutes of sample addition. Sample antiradical activity was calculated by the following ratio:

(Abs control - Abs sample/Abs control)×100

where, Abs control is DPPH solution absorption, and Abs sample is DPPH solution absorption after sample addition [16]. Antioxidant activity of the samples was compared with ascorbic acid (100, 200 and 300 ppm) as a standard of antioxidant. The experiment was carried out in triplicate, and the results are reported as mean ±SD.

2.5. Statistical analysis

The experimental results were expressed as mean±standard deviation of three replicates. Data were analyzed by analysis of variance using SPSS software (ver. 22), and the means were compared by Duncan's tests. P-values < 0.05 were regarded as significant.

3. Results and Discussion

3.1. HPLC analysis of carotenoid compounds

In this research, the effects of different levels of SA on two main products of saffron, crocin and safranal, were analyzed by HPLC system. According to the obtained results, SA was found to have a significant effect on both of these compounds though its effect on crocin content was positive, and the SA-treated plants showed higher crocin content in comparison to the control untreated plants. The highest quantity of crocin with a value of 25.27 mg

g^{-1} DW was observed in 1 mM SA (Figure 1A). In contrast, SA reduced safranal content compared to the controls, and the highest amount of safranal (0.92 mg g^{-1} DW) was observed in the control plants (Figure 1B). SA plays a key role in a plant's growth, development, and defense responses, and is involved in some signal transduction systems to induce particular enzymes related to biosynthesis of secondary metabolites in plants [15,17]. As an elicitor, SA improved accumulation of compounds belonging to different structural classes, including phenolics, terpenoids and alkaloids [18]. In this regard, our results corroborate with those of Turkyılmaz et al. [19] who observed that application of SA increased chorophyll content and carotenoids content under normal field condition. In an earlier study, exogenous application of SA was reported to improve growth, yield and essential oil content in the case of basil and marjoram [20,21].

3.2. Antioxidant activity

3.2.1.DPPH assay

Numerous studies on the medicinal properties of saffron have shown that saffron has a strong antioxidant activity, which is generally due to the presence of crocin, a unique carotenoid with powerful antioxidant capacity that makes distinctive bright yellow color of the stigma. It was shown that the antioxidant properties of both the methanol and water-methanol (50:50 %vv^{-1}) extracts of *Crocus sativus* stigmas were higher than those of tomato and carrots [22, 23]. So regarding the importance of saffron as a valuable product, in this study, effect of SA on the antioxidant activity of saffron is indicated by DPPH method. This method is a simple, rapid, sensitive and reproducible assay used for evaluating the antioxidant activity of plant extracts, even in low concentrations [24,25].

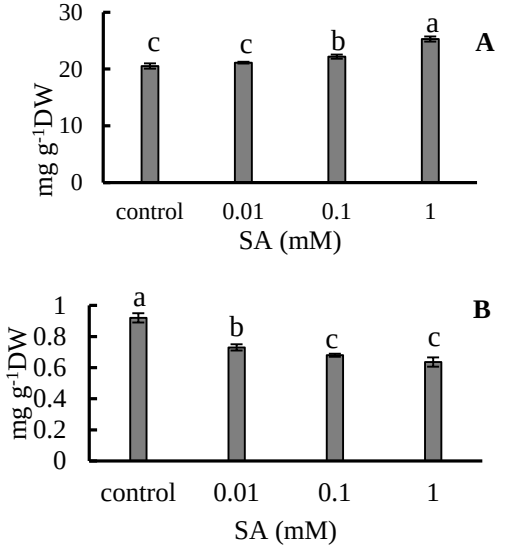

Figure 1. Effect of different concentration of SA on A: crocin B: safranal quantity in stigma of saffron. All analyses are the mean of triplicate measurement ±standard deviation; Means with different letters are significantly different at $p < 0.05$.

In the DPPH assay, an antioxidant scavenges the free radicals, and is used to measure the capacity of extracts to react with DPPH as a stable free radical. Then DPPH radicals are converted into α,α-diphenyl-β-picryl hydrazine. The value of free radical conversion is expressed as % DPPH inhibition [26]. By accepting an electron, the color of DPPH changes from purple to yellow. Discoloration degree indicates that the antioxidants possess scavenging potentials [27,28]. Figure 2 represents the results of radical scavenging activity of various saffron extracts (100, 200 and 300 ppm) under different concentrations of SA. As shown, SA had a positive effect on the antioxidant activity of various concentrations of extracts. SA with a dose of 1mM and at a concentration 300 ppm exhibited the highest radical scavenging activity among the different concentrations of SA (69%). It has been demonstrated in different plants species that SA as an elicitor enhanced secondary metabolites in plants and as a result of this compound accumulations, antioxidant activity also increased [14,18,29]. Higher antioxidant capacity in 1 mM of SA could be as a result of greater crocin content, although the safranal content was lower than control plants in this doses of SA. Considering our results, safranal content compared to crocin content was too low, so its effect on antioxidant activity should be less important. Many studies have shown a variety of pharmacyological effects of crocin; it has also been confirmed as a powerful antioxidant, stronger than α tocopherol [30,31]. Nonetheless, all values of saffron extracts were lower than those gained for ascorbic acid that used as antioxidant standard in all concentrations (83, 96 and 98%). In all the extracts examined, a concentration increase was followed by an increase in radical scavenging activity.

Overall, in this study, only the effect of SA on crocin and safranal content was considered, and other compounds of saffron were not recognized in saffron extracts. As other studies have shown, antioxidant activity of saffron could be due to the synergistic effect of bioactive constituents contained in stigmas that should not be neglected [32].

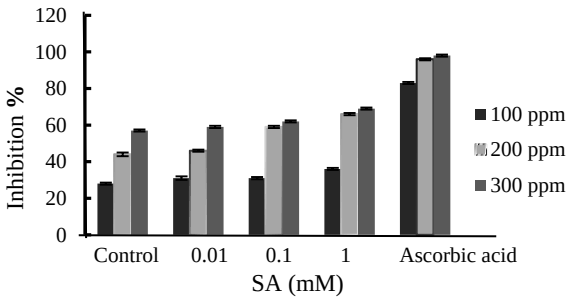

Figure 2. Free radical scavenging activity of methanolic extracts of *Crocus stigma*. Ascorbic acid was included as a positive control. Activity was measured by the scavenging of DPPH radicals and each value is expressed as the mean ± standard deviation.

4. Conclusion

SA was found to have an effect on the crocin and safranal content of saffron stigmas though the effect of SA on crocin content was positive. Unlike this, SA reduced the quantity of safranal in all concentrations, and the highest level of safranal was observed in the control plants. The result of antioxidant activity also confirmed that SA effectively increased antioxidant activity of different concentrations of saffron extracts. Among the different concentrations of SA, 1 mM treatment of SA at 300 ppm had the highest radical scavenging activity. However, the antioxidant activity of all extracts in different concentrations of SA was lower than that of ascorbic acid in all examined concentrations. Additionally, the radical scavenging activity of all extracts was concentration-dependent, and as the extract's concentration increased, the antioxidant activity was also improved.

5. Acknowledgment

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6. Conflict of interest

The authors at there is no conflict of interest.

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