Antioxidant and antibacterial activity of ethanolic extract of safflower with contrasting seed coat colors

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Submitted: 19 December 2020; Accepted: 21 March 2021; Published: 2 June 2021

ORIGINAL ARTICLE

Abstract

The present study aimed to investigate total flavonoid (TFC), cyanidin-3-glucoside (Cyd-3-glu) content, and antioxidant and antibacterial activities of ethanolic seed coat extract of two safflower genotypes (genotype C111 and A82) with contrasting seed coat colors. Despite the absence of Cyd-3-glu in seed coat extracts of white-seeded genotype C111 versus black-seeded genotype A82 and equal TFC index between the two genotypes, there was no significant difference in their antioxidant activity. Also, the ethanolic extract has growth inhibitory properties in pathogenic bacteria. It seems that differences in type and level of secondary metabolites of the seed coat with different color patterns can result in the ethanolic extract's antioxidant activity. In addition, the results confirmed that seed coat color has not effect on the level (or severity) of the antibacterial properties of ethanolic seed coat extract.

Keywords: antibacterial activity; antioxidant activity; safflower; seed coat color

Introduction

Plants are always considered as one of the most important foods and medicine sources (Brizzolari *et al.*, 2019; Palacios-Rojas *et al.*, 2020). Statistics show that the effective compound of more than 50% of the medicine and a significant part of the food industry's ingredients are of plant origin (Mazzei *et al.*, 2020). Therefore, there is a growing intention to use plants in the pharmaceutical and food industries (Rezig *et al.*, 2019). The safflower (*Carthamus tinctorius L.*) is one of the first cultivated plants as a natural coloring and flavoring agent in food by using the florets (Karami *et al.*, 2017a; Zuniga-Salcedo *et al.*, 2019). Today, the primary purpose of cultivating this plant is to produce seeds (Nimrouzi *et al.*, 2020). Safflower seeds are rich in unsaturated fatty acids such as linoleic and oleic acids, biologically active compounds, and phenolic and flavonoid compounds with antioxidant properties (Sabzalian *et al.*, 2008). Therefore, these seeds are the main focus of many research types as pharmaceuticals and food additives. Safflower has high antibacterial and antioxidant activity due to its wide range of compounds with the phenolic group (Güner *et al.*, 2020). Previous studies have shown that safflower water extract has antibacterial properties against various bacteria such as *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus mycoides* (Turgumbayeva *et al.*, 2020). Numerous studies have also reported a positive effect of polyphenolic compounds, such as flavonoids, in safflower in clearing the free radicals' body (Choi *et al.*, 2018).

Many environmental and nonenvironmental factors such as genetics, physical appearance, physiological conditions, and extraction methods affect the quantity and quality of the polyphenolic compounds extracted from plants (Fang *et al.*, 2011). Seed color as one of the physical

ISSN 1757-837X online, DOI 10.15586/qas.v13i2.866

appearance factors can affect seed polyphenolic extract (Kanu, 2011). Moreover, the relationship between the seed color and the phenolic and flavonoid content of seed extract is proven in many studies (Kanu, 2011; Karami *et al.*, 2018; Sabzalian *et al.*, 2008; Shen *et al.*, 2009; Thaddi and Nallamilli, 2014). For instance, the amount of phenolic and anthocyanin compounds in the colored seed is higher than in white beans. Therefore, the colored bean has higher antioxidant activity (Akond *et al.*, 2011).

There are different color patterns of the safflower's seed coat (Karami *et al.*, 2017b). However, the color of the cultivated safflower is found to be white or cream (Karami *et al.*, 2018). Sabzalian *et al.* (2010) introduced a novel breeding line with black seed coats (A82). On the other hand, the genotype A82 has been suggested as a preferable and superior genotype for cultivation in areas infested by the safflower fly (Kanu, 2011). Also, some evidence implies that seed color and the chemical compounds of seed color are involved in reducing insect or pest injuries (Sabzalian *et al.*, 2010). Our previous study found that the concentration of flavonoid compounds detected in the black-seeded genotype's methanolic extract (A82) was significantly lower than the white/brown seeded safflower genotypes. Conversely, antioxidant activity was higher in black-seeded genotypes than in white-seed ones (Karami *et al.*, 2018). It has been shown that the type of solvent that was used for the extraction plays an important role in determining the polyphenol and flavonoid content extracted from plants and subsequently their antioxidant and antibacterial effects (Skowyra *et al.*, 2014). Despite our relatively comprehensive previous study on the identification and determination of secondary metabolites in *Carthamus* species with pigmented and nonpigmented seeds, no reports are available concerning the effect of seed color and extraction solvents on secondary metabolites and their clinical properties. Thus, the present study aimed to investigate total flavonoid (TFC), cyanidin-3-glucoside (Cyd-3-glu) content, and also antioxidant and antibacterial activities of ethanolic seed coat extract of two safflower genotypes (genotypes C111 and A82) with contrasting seed coat colors.

Materials and Methods

Plant material

For this study, two accessions of safflower were selected. One accession belonged to the cultivated species with white seed coat color (Iranian breeding lines, C111), and one novel safflower breeding line (A82) with black seed coats obtained via interspecific hybridization of C111, as the female parent, and a black-seeded genotype of a wild safflower species (*C. oxyacanthus*) following backcrossing and selfing programs (personal collection: Assoc. Prof. M. R. Sabzalian). All of the plant materials were identified by Assoc. Prof. M. R. Sabzalian, and the specimens were deposited with the Herbarium of Isfahan University of Technology, Isfahan, Iran. Harvested seeds at the maturity stage of plants were de-coated, and then seed coats were used in experiments (Figure 1).

Figure 1. Black-seeded safflower genotype A82 (left); white-seeded safflower genotype C111 (right).

Preparation of ethanolic extract

To prepare an ethanolic extract from the seed coat, the modified method of Yu *et al.* (2013) was used. For this purpose, the seed coat of the genotypes was dried at 40°C for 24 h, using a ventilated oven, up to a moisture content of 5%, and was then ground using a grinder (IKA M20, IKA, Staufen, Germany). Then, seed coat powder samples (5 g) were extracted with 100 mL of 80% ethanol (v:v) and shaken by using an orbital shaker (KS260 basic, IKA, Germany) (90 rpm, 24°C, and 24 h), and the extract was filtered through Whatman No. 3 filter paper to remove the solid debris, with each filtration repeated two times. Afterward, the resultant extracts were evaporated to remove ethanol and vacuum-dried under room temperature and were redissolved in ethanol (80%).

Total flavonoid content (TFC)

TFC of all the samples was determined according to the method described by Tohidi *et al.* (2017). Briefly, a volume of 125 µL of the filtered ethanolic extract of seed coat was mixed with 75 µL of a sodium nitrate solution (5% $NaNO_2$). The mixture was allowed to remain for 6 min, and subsequently, 150 µL of aluminum Chloride (10% $AlCl_3$) was added. Finally, after 5 min, 750 µL of sodium hydroxide solution (1 M NaOH) and 1.4 mL of distilled water were added to the mixture. The mixture's absorbance was recorded at 510 nm via a visible spectrophotometer (Novaspec II Visible Spectrophotometer; Pharmacia Biotech). The results were expressed as milligram of quercetin equivalents (QE) per 1 g of the sample's

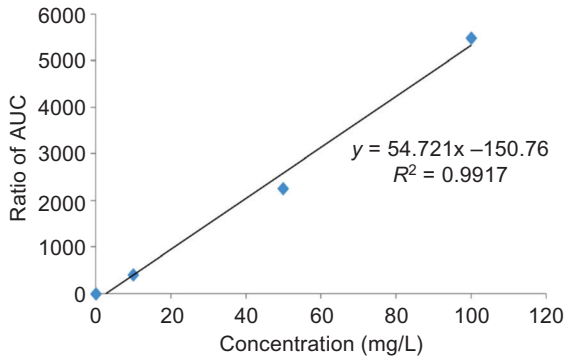

Figure 2. Spiked calibration curve for Quercetin.

dry weight. Figure 2 shows the spiked calibration curve for quercetin as a representative.

Cyanidin-3-glucoside content (Cyd-3-glu)

The Cyd-3-glu content in the seed coat of each genotype was measured using the pH difference method with two buffer systems, including potassium chloride (pH = 1 and 0.025 m) and sodium carbonate (pH = 4.5 and 0.4 m), according to guidelines (Giusti and Wrolstad, 2001) and a 1% HCl solvent system in ethanolic at 530 and 700 nm (Siegelman and Hendricks, 1958).

Antioxidant activity assay by DPPH (2,2-diphenyl-1-picrylhydrazyl)

The strength of extracts of each genotype in trapping the DPPH free radicals was measured according to the Hatemnia *et al.* (2014) method at a wavelength of 515 nm. DPPH free radical removal activity, which is an indicator of the antioxidant activity rate of plant extracts, was calculated according to equation 1:

$$\text{RSA } (\%) = \frac{A0 - A1}{A0} \times 100 \quad (1)$$

In this regard, A0 is the control absorption, A1 is the absorption sample, and RSA (Radical Scavenging Activity Assay) is the percentage of free radical removal activity. To better evaluate this activity, synthetic antioxidant Butylated Hydroxy Anisole (BHA) was used as a positive control.

Chromatographic separation of phenolic and flavonoid Compounds

Components using HPLC (high-performance liquid chromatographic) Analysis (Shimadzu, Tokyo, Japan) was used to separate the effective compounds (chlorogenic acid, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, quercetin, and apigenin) in the studied genotypes. The resultant extracts were evaporated to remove ethanol and vacuum-dried under room temperature. The residues were redissolved in ethanol (80%). The HPLC evaluation procedure used by Lin and Harnly was followed, with some modifications. HPLC was conducted using a Shimadzu chromatographic system (Shimadzu, Tokyo, Japan). The injection volume was 50 µL, and article chromatograms were acquired at 260 and 330 nm. Solutions of available pure known compounds were chromatographed as external standards. All standards were dissolved in HPLC grade ethanol before injection in the analytical column for analysis. Identification of phenolic and flavonoid compounds of seed coat extracts was performed depending on the retention time (RT) of each one compared with those of pure standards. The results were expressed as milligram per 100 g of sample dry weight. According to Lin and Harnly (2010), after performing sloping chromatography at a speed of 1 mm per min, the chromatogram was obtained by HPLC manager software, and then the curves were calculated and interpreted.

The measurement of antibacterial activity

Standard strains including *Pseudomonas aeruginosa* (PTCC 1707), *Escherichia coli* (PTCC 1763), *Klebsiella pneumonia* (PTCC 1290), and *Salmonella typhi* (PTCC 1609) as gram-negative bacteria and *Staphylococcus aureus* (PTCC 1431) as gram-positive bacteria were obtained in the lyophilized form from Scientific and Industrial Research Organization of Iran.

To investigate the antibacterial properties of the extracts, the micro-dilution method was used. The bacterial strains were cultured twice in the Mueller–Hinton agar medium and then incubated for 24 h at 37°C. To obtain a half McFarland concentration (10^8 CFU/mL), each of the cultured strains was re-cultured in the Mueller–Hinton agar medium and incubated for 18 h at 37°C. Various dilutions of each genotype's extract were prepared in the liquid Mueller–Hinton agar medium to calculate the minimum inhibitory concentration (MIC). The microbial suspension of half McFarland was then added to each dilution (per mL of liquid) and incubated for 24 h at 37°C. For each genotype, the last dilution in which no turbidity was observed was considered the MIC.

Statistical analysis

According to a *t*-test procedure, data were analyzed with 3 replicates in each treatment, using SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Total flavonoid content (TFC)

Comparing the average TFC between the seed coat extracts (Table 1) showed no significant difference between the studied genotypes, which is not consistent with our previous study results (Karami *et al.*, 2018). In our previous study, seed coat extract of black-seeded genotype (A82) had significantly lower TFC content than white-seeded genotypes. One explanation could be the type of solvent used for the extraction. In our previous study, methanol was used for extraction, while in this study, ethanol was used. Several studies have shown that the type of solvent used to extract plays an important role in determining the plant's extracted polyphenolic content (Skowyra *et al.*, 2014). In the Kermani *et al.* (2019) study, the TFC index of seed methanolic extract in black sesame seeds was higher than in white seeds, which is probably due to the effect of the species on flavonoid content

Cyanidin-3- glucoside content (Cyd-3-glu)

The results of Cyd-3-glu measurement in the studied genotypes showed that unlike the black-seeded genotype (A82), the seed coat extract of white-seeded genotype (C111) lacked this compound (Table 1). These results are consistent with the results of our previous study (Karami *et al.*, 2018). However, the concentration of Cyd-3-glu in the methanolic extract was higher than ethanolic extract, which may be related to the extracted solvent's effect. The association between anthocyanin content and seed color has previously been reported in some oilseeds, cereals, and legumes. Choung *et al.* (2001) compared black, green, and yellow seed soybeans and found that anthocyanins only exist in black seed soybeans, and the green and yellow seed soybeans lack the anthocyanin. On the other hand, the absence of anthocyanin in the seed coat extract of white-seeded safflower genotypes can be attributed to the lack of expression of some genes in biosynthesis pathway flavonoids (Karami *et al.*, 2018).

Antioxidant activity assay by the DPPH method

The result of the *t*-test analysis indicated that no significant differences between the two genotypes with respect to their antioxidant activity (Table 1). Antioxidant activity depends on the number of phenols, flavonoids, and anthocyanins in a plant, and these compounds have a high ability to remove the free radicals. Therefore, genotypes' ability to remove free radicals can be attributed to differences in the secondary metabolites content. As mentioned above, the genotype C111 with a white seed coat exhibited a similar level of radical scavenging activity compared with the black-seeded genotype A82, despite the absence of Cyd-3-glu in its seed coat. Thus, it seems that other compounds such as phenolics and/or flavonoids possibly contribute to the antioxidant activities of this genotype.

Afterward, the HPLC results confirmed this hypothesis. They revealed that despite the equal concentrations of chlorogenic, *p*-coumaric, and caffeic acid in the two genotypes, it seems that due to the higher concentrations of gallic acid, rutin, ferulic acid, quercetin, and apigenin in the white-seeded genotype C111 (Table 2), the above compounds are highly associated in antioxidant activity and compensate for the absence of Cyd-3-glu in this

Table 1. Total flavonoid content, cyanidin-3-glucoside and antioxidant activity of ethanolic seed coat extract of the studied safflower genotypes.

Samples	Seed coat color	Total Flavonoid Content (mgQUE.g^{-1}DW)	Antioxidant activity (%)	Cyanidin-3 glucoside (mgcyd-3-glu.g^{-1}DW)
A82	Black	3.38 ± 0.13^{a}	72.26 ± 2.53^{a}	13.96 ± 0.34^{a}
C111	White	3.06 ± 0.11^{a}	68.8 ± 3.05^{a}	0.00^{b}

Similar letters indicate no significant difference at the 0.05 level in the column.
DW: dry weight.

Table 2. Polyphenolic compounds identified in ethanolic extract of seed coat extract in the studied safflower genotypes based on HPLC analysis (mg/100 g WD).

Samples	Seed coat color	Gallic acid	Chlorogenic acid	Caffeic acid	*p*-Comaric acid	Rutin	Ferulic acid	Quercetin	Apigenin
A82	Back	0.25	1.46	6.15	6.03	7.89	4.31	2.80	4.47
C111	White	0.51	1.45	6.26	6.05	11.63	7.58	3.41	6.69

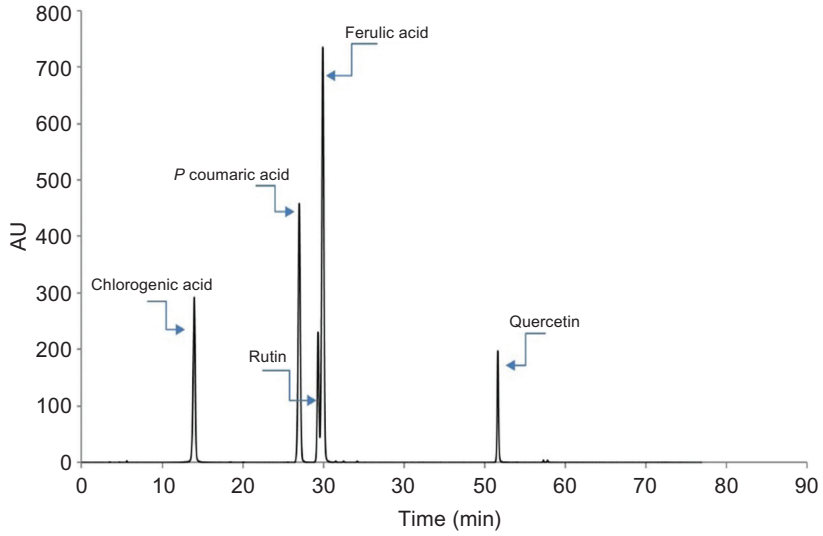

Figure 3. A chromatogram obtained for the polyphenolic compounds.

genotype. An HPLC chromatogram of polyphenolic compounds analyzed in the safflower is shown in Figure 3.

In line with this hypothesis, Salami *et al.* (2016) reported that the content of total phenol and phenolic compounds compared to the TFC and flavonoid compounds showed more free radical removal activity in different fenugreek genotypes (*Foeniculum vulgare*). The present study results were different from the previous study (Karami *et al.*, 2018). As in our previous study, white-seed safflower genotypes showed a lower ability to remove free radicals than black-seeded genotypes. One explanation for these differences detected between the two studies is the difference in the type of solvent used for extraction. In a study by Shen *et al.* (2009), black-seeded rice indicated higher free radical removal potency levels than other rice colors (Shen *et al.*, 2009). Kermani *et al.* (2019) also showed that black sesame had higher antioxidant properties than white sesame.

Antibacterial activity

The results of the antibacterial effect of different genotype seed coat extracts on the studied bacteria are given in Table 3. There was no significant difference in the MIC index between the two genotypes. The results also exhibited that *P. aeruginosa* and *K. pneumonia* bacteria's susceptibility to the seed coat extracts of both genotypes was much higher than other studied bacteria. Sabah *et al.* (2015) reported that the phenolic extract of safflower oil had an excellent inhibitory effect on *S. aureus* and *E. coli* bacteria. In another study, Abdel Moneim *et al.* (2018) showed that safflower methanolic and water extracts have a high potency in inhibiting the growth of *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* bacteria, and their inhibitory power varies depending on the type of bacteria (Abdel Moneim *et al.*, 2018). The highest inhibitory effect was observed on *K. pneumonia*, which is consistent with the results of our study. Qazi *et al.* (2013) investigated the bacteria isolated from clinical samples (*E. Coli*, *B. subtilis*, *S. agalactiae*) and showed that safflower ethanolic flower extract has lower antibacterial effects at low concentrations and the antibacterial effects also depend on the type of bacteria. In another study, Salami *et al.* (2016) reported a moderate antibacterial effect of leaf extract of fenugreek genotypes (*Foeniculum vulgare*) on *S. aureus*, *E. Coli*, and *P. aeruginosa* bacteria. The antibacterial activity of plant extracts is more related to the presence of polyphenolic compounds. These compounds inhibit bacteria growth through various mechanisms such as cell wall destruction, cell membrane destruction, and intracellular bacterial matrix destruction. Bacterial type, cell wall structure, and quantitative and qualitative content of extract compounds are among the determinants of the extract's antibacterial potency (Mazzei *et al.*, 2020).

Table 3. Minimum inhibitory concentration (MIC) of ethanolic extract of seed coat in the studied safflower genotypes (mg/mL).

Samples	Seed coat color	*E. coli*	*K. pneumonia*	*S. typhi*	*St. aureus*	*P. aeruginosa*
A82	Black	500	125	500	500	250
C111	White	500	125	500	500	250

Therefore, the observed difference in the results of different studies could be related to these factors.

Conclusion

The safflower genotype A82 with a unique seed color pattern (black) is known as a novel breeding line and a promising safflower fly-resistant safflower genotype; however, a limited assay was only performed on this novel breeding line. Thus, the present study aimed to investigate secondary metabolites' content and also antioxidant and antibacterial activities of ethanolic seed coat extract of two safflower genotypes (genotype C111 and A82) with contrasting seed coat colors. Based on the results obtained, the ethanolic extract of the safflower seed coat has appropriate antioxidant and antibacterial properties. Also, despite the differences detected in polyphenolic compounds of black-seeded genotypes (A82) and white-seeded genotypes (C111), there was no significant difference between them in antibacterial and antioxidant properties. On the other hand, the results of ethanolic seed coat extract (the present study) with methanolic seed coat extract (previous studies) showed that the type of solvent used for extraction has a significant effect on the quantity and quality of polyphenolic compounds in each genotype, and this can affect the antioxidant properties.

Acknowledgments

The authors are grateful to Professor Sabzalian M.R. for providing safflower varieties (specimen_voucher = personal collection: Mohammad R. Sabzalian).

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

This study does not involve any human or animal testing.

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