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Variation in Phenolic Composition and Antioxidant Activity during Flower Development of Safflower (*Carthamus tinctorius* L.)

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ABSTRACT: This work was aimed to study the effect of extraction solvent system with varying polarities on polyphenol, flavonoid and proanthocyanidin contents and DPPH scavenging activity. Obtained results showed that phenolic contents and antioxidant activities varied considerably as function of solvent polarity. The extraction with acetone/water (2:8) showed the highest flower polyphenol content (15.09 mg GAE/g DW). Moreover, antiradical capacities against DPPH, chelating power and lipid peroxidation assay were maximal in acetone/water (2:8) of flower extract. Significant variation in antioxidant properties was observed between different development stages of *Carthamus tinctorius* flowers; the highest antioxidant activity was observed at stage III (full flowering) while phenolic composition reached its maximum at stage II (flower formation). Gallic acid was the most abundant phenolic compound in *C. tinctorius* orange flowers, accounting for about 102.57 (μg/g DW). Findings underline the potential health benefits as a result of consuming *C. tinctorius* flowers and suggest that it could be used as valuable flavor with functional properties for food or nutraceutical products on the basis of the high polyphenol contents and antioxidant activities.

KEYWORDS: *Carthamus tinctorius* L., flower development, antioxidant activities, phenolics

INTRODUCTION

The demand for nontoxic, natural preservatives has been rising with increased awareness and reports of the ill effects of synthetic chemicals present in foods.[1] Medicinal plants are the source of natural antioxidants thanks to their main secondary metabolites such as polyphenols. Nowadays, polyphenols are the target of numerous studies since their intake has been associated with decreased risks of cancers, cardiovascular diseases and neurodegenerative disorders. In fact, depending on their structures, it has been observed that they exhibit analgesic, antiallergic or anti-inflammatory effects.[2] The prevalent understanding at this time is that these biological activities seem to be related to their strong antioxidant properties.[3] However, production of these natural compounds in plant tissues is a highly ordered process and varies during the growing season. Therefore, determination of the optimum harvesting time is important to obtain maximum natural production and assess the viability of a medicinal plant as a potential crop.[4] However, there is not a standard method of phenolic extraction. Extraction yield is dependent on the solvent and the method of extraction. For instance, different solvent systems have been used for the extraction of polyphenols from plant material.[5] Water and aqueous mixtures of ethanol, methanol and acetone are commonly used in plant extraction.[6] Consequently, the solubility of phenolic compounds is actually governed by the type of solvent used and the degree of polymerization of phenolics, as well as by the interaction of phenolics with other food constituents and formation of insoluble complex.[7]

Among the sources of these natural antioxidants, safflower (*Carthamus tinctorius*. L), a member of Asteraceae family, is one of the important aromatic and medicinal plants. It is a branching, thistle-like herbaceous annual plant with numerous spines on leaves and bracts.[8] Safflower is grown almost exclusively for its flowers, which are used in treatment of cardiovascular, cerebrovascular and gynecological diseases.[9] More than 200 compounds were isolated from *C. tinctorius* where chalcone flavonoids are the main compounds in the water extract. These natural pigments have achieved industrial significance in many fields. They are used in food, pharmaceutics, cosmetics and various other commodity preparations. Hydroxysafflor yellow A is the main active component of safflor yellow and has been demonstrated to have good pharmacological activities of antioxidation and myocardial and cerebral protection.[10] Despite the wealth of studies focusing on natural colorants and dyes in safflower florets,[11] there is unavailable data on the antioxidant activity and the content of phenolic compounds of safflower florets during flower development. Thus, the purpose of this work was to characterize *C. tinctorius* flower extracts through its phenolic composition and antioxidant capacities at different stages of flower development in order to determine the optimal period of pigment production and to try to valorize *C. tinctorius* flowers as a source of natural colorants.

The aim of the present study was (i) to ascertain the potential effects of several pure and mixture extracting solvents on *C. tinctorius* flower phenolic contents and their antioxidant capacity. (ii) After choosing the efficient solvent for antioxidant extraction, phenolic composition and antioxidant capacities at different stages of flower development were evaluated. (iii) Finally, the profiles of the main phenolic compounds in safflower flowers were identified and quantified during flowering. Such a study will be helpful to enlarge our insight of the flowering behavior in finding out suitable indicator(s) of various physiological processes which may help to valorize *C. tinctorius* flowers as source of natural colorants.

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Table 1. Physical Characteristics of *C. tinctorius* during Flower Development[13] [a]

days since emergence	50	75	100
stage	bud formation	flower formation (petal, stamen, pistil and pollen)	full flowering
flower color	yellow (+++), red (+)	yellow (++), red (++)	red (+++), yellow (+)

[a] +: weak intensity of red and yellow color. ++: moderate intensity of red and yellow color. +++: high intensity of red and yellow color.

MATERIALS AND METHODS

Reagents. All solvents used in the experiments (acetone, ethanol and methanol) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH), chlorhydric acid (HCl), disodium hydrogen phosphate (Na_2HPO_4), sodium monobasic phosphate ($NaH_2PO_4H_2O$), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), butylated hydroxytoluene (BHT), β-carotene, linoleic acid, ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), iron(II) tetrahydrate, iron(II), iron(III), 1,1-diphenyl-2-picrylhydrazyl (DPPH), polyvinyl polypyrolidone Folin−Ciocalteu reagent, potassium ferricyanide ($K_3Fe(CN)_6$) and aluminum chloride ($AlCl_3$) were purchased from Sigma−Aldrich (Steinheim, Germany).

Authentic standards of phenolic compounds were purchased from Sigma and Fluka which were phenolic acids (gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, syringic acid, vanillic acid, *p*-coumaric acid, ferulic acid, trans-2-hydroxycinnamic acid, trans-cinnamic acid) and flavonoids (gallate epicatechin, catechin, rutin, quercetrin, quercetin, apigenin, amentoflavone and flavone). Stock solutions of these compounds were prepared in HPLC-grade methanol. These solutions were wrapped in aluminum foil and stored at 4 °C. All other chemicals used were of analytical grade.

Plant Material. *C. tinctorius* flowers were harvested from plants grown in the National Institute of Agricultural Research Tunisia (latitude 36° 50′ 29.68″ (N), longitude 10° 12′ 19.44″ (E), 3 m elevation) in summer, 2008 at floral budding (I), flower formation (II) and full flowering (III) stages. The color of florets changes gradually from yellow to red during stages of flower development. The harvested plants were identified by Prof. A. Smaoui (Biotechnology Center in Borj-Cedria Technopark, Tunisia) according to the Tunisian flora,[12] and a voucher specimen was deposited at the herbarium of the Laboratory of Bioactive Substances at the Biotechnology Center. After that, the samples were freeze-dried, then ground to fine powder by an electric mill and conserved in a desiccator at room temperature (~25 °C) in darkness for further uses. Full details of flower characteristics data are provided in Table 1.[13]

Preparation of Plant Extracts. Sample extracts were obtained by stirring 0.2 g of dry *C. tinctorius* flower powder with 6 mL of solvent test for 30 min at 120 rpm using a magnetic stirrer plate. Three solvents with increased polarity at different ratios were used: ethanol, acetone and methanol (0−100%). Extracts obtained were kept for 24 h at 4 °C, filtered through a Whatman no. 4 filter paper and freed of solvent under reduced pressure at 45 °C, using a rotary evaporator and then lyophilized. The dried crude concentrated extracts were stored at −20 °C, until used for analyses. All extractions were performed in triplicate for each test solvent.

Determination of Total Polyphenol Contents. Colorimetric quantification of total phenolics was determined, as described by Dewanto et al.[14] Briefly, 125 μL of suitable diluted sample extract were dissolved in 500 μL of distilled water and 125 μL of Folin−Ciocalteu reagent. The mixture was shaken, before adding 1250 μL of Na_2CO_3 (7 g/100 mL), adjusting with distilled water to a final volume of 3 mL, and mixed thoroughly. After incubation for 90 min at 23 °C in darkness, the absorbance versus a prepared blank was read at 760 nm. Total phenolic contents of flower were expressed as mg gallic acid equivalents per gram (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 50−400 mg/mL (R^2 = 0.99). All samples were performed in triplicate.

Determination of Flavonoid Contents. Total flavonoid contents were measured according to Dewanto et al.[14] An aliquot of diluted sample was added to 75 μL of $NaNO_2$ solution (5%), and mixed for 6 min, before adding 0.15 mL of $AlCl_3$ (10%). After 5 min, 0.5 mL of 1 M NaOH solution was added. The final volume was adjusted to 2.5 mL, thoroughly mixed, and the absorbance of the mixture was determined at 510 nm. Total flavonoid contents of flower extracts were expressed as mg catechin equivalents (CE) per gram of dry weight respectively (mg GAE/g DW) through the calibration curve ranging from 50 to 500 mg/mL (R^2 = 0.85).

Quantitative Determination of Condensed Tannins. In the presence of concentrated HCl, condensed tannins were transformed by the reaction with vanillin to anthocyanidols.[15] 50 μL of the extract appropriately diluted was mixed with 3 mL of vanillin (4%) and 1.5 mL of HCl. After 15 min, the absorbance was measured at 500 nm. Condensed tannin contents of flowers were expressed as mg catechin equivalents (CE) per gram of dry weight through the calibration curve with catechin. The calibration curve range was 50−600 mg mL^{-1} (R^2 = 0.85).

Analysis of Individual Phenolic Compounds by Analytical RP-HPLC. Phenolic compound analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC) coupled with an UV−vis multiwavelength detector. The separation was carried out on a 250 × 4.6 mm, 4 μm Hypersil ODS C18 reversed phase column. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulfuric acid (solvent B). The flow rate was kept at 0.5 mL/min. The gradient program was as follows: 15% A/85% B 0−12 min, 40% A/60% B 12−14 min, 60% A/40% B 14−18 min, 80% A/20% B 18−20 min, 90% A/10% B 20−24 min, 100% A 24−28 min. The injection volume was 20 μL, and peaks were monitored at 280 nm. Peaks were identified by congruent retention times compared with those of authentic standards. Phenolic compound contents were expressed in micrograms per gram of dry plant material weight.

DPPH Assay. The electron donation ability of the flower extracts was measured by bleaching of the purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato.[16] One half milliliter of 0.2 mM DPPH methanolic solution was added to flower extracts of *C. tinctorius* (2 mL, 10−1000 μg mL^{-1}). After an incubation period of 30 min at room temperature, the absorbance was read against a blank at 517 nm. The inhibition percentage of free radical DPPH (IP%) was calculated as follows:

$$\text{IP } \% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of plant extract. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the regression equation prepared from the concentration of the extracts and the inhibition percentage. BHT was used as a positive control (R^2 = 0.93).

Reducing Power. The method of Oyaizu[17] was used to assess the reducing power of *C. tinctorius* flower extracts. These extracts (1 mL) were mixed with 2.5 mL of a 0.2 M sodium phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$) and incubated in a water bath at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture that was centrifuged at 650*g* for 10 min. The supernatant (2.5 mL) was then mixed with 2.5 mL of distilled water

Table 2. Total Polyphenol, Flavonoid, Condensed Tannin Contents Extracted by Three Different Solvents and DPPH Scavenging Activity in *Carthamus tinctorius* Flower Extracts[a]

	phenolic contents (mg GAE·g^{-1} MS)			flavonoid contents (mg EC·g^{-1} MS)			tannin contents (mg EC·g^{-1} MS)			DPPH$^{\bullet}$ scavenging act. (IC_{50} μg/mL)		
% organic solvent	acetone	MeOH	ethanol	condensed	MeOH	ethanol	acetone	MeOH	ethanol	acetone	MeOH	ethanol
20	15.09 ± 0.03 a	13.19 ± 0.26 de	12.80 ± 0.09 e	4.76 ± 0.22 a	4.07 ± 0.18 bc	2.35 ± 0.12 f	0.85 ± 0.11 de	0.69 ± 0.08 fg	0.94 ± 0.17 cd	2.50 ± 0.56 f	30.00 ± 1.95 c	10.00 ± 0.09 e
40	14.94 ± 0.09 a	11.90 ± 0.22 f	13.90 ± 0.30 c	4.23 ± 0.35 b	2.12 ± 0.07 fg	1.15 ± 0.16 h	0.74 ± 0.08 ef	0.75 ± 0.10 ef	0.55 ± 0.04 gh	33.00 ± 1.95 c	10.50 ± 2.03 e	89.00 ± 0.93 a
60	14.23 ± 0.07 bc	14.59 ± 0.26 b	13.80 ± 0.10 cd	3.93 ± 0.07 c	4.55 ± 0.16 a	1.89 ± 0.11 g	0.66 ± 0.10 gh	1.02 ± 0.04 c	0.57 ± 0.07 gh	30.00 ± 1.99 c	5.50 ± 0.56 ef	90.00 ± 1.13 a
80	12.63 ± 0.89 e	11.61 ± 0.57 fg	11.07 ± 0.01 g	3.06 ± 0.20 e	3.47 ± 0.26 d	3.97 ± 0.14 bc	0.54 ± 0.03 h	1.39 ± 0.16 b	1.54 ± 0.07 a	10.00 ± 1.95 e	3.00 ± 0.96 f	33.00 ± 0.91 c
100	2.40 ± 0.93 j	5.00 ± 0.30 i	6.26 ± 0.47 h	0.52 ± 0.06 i	1.02 ± 0.08 h	2.00 ± 0.06 g	0.15 ± 0.05 i	0.13 ± 0.02 i	0.79 ± 0.09 ef	35.50 ± 0.56 c	20.33 ± 1.84 d	41.00 ± 1.13 b

[a] Data are reported as means ± SD and compared to control (C). ANOVA followed by Duncan multiple range test ($P < 0.05$). Values with different superscripts (*a-i*) are significantly different at $P < 0.05$.

and 0.5 mL of 0.1% iron(III) chloride solution. The intensity of the blue−green color was measured at 700 nm. The extract concentration at which the absorbance was 0.5 for the reducing power (EC_{50}) was obtained from the linear regression equation prepared from the concentrations of the extracts and the absorbance values. High absorbance indicates high reducing power. Ascorbic acid was used as a positive control ($R^2 = 0.97$).

Metal-Chelating Power. According to Zhao,[18] 0.1 mL of flower extracts was added to 0.05 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.1 mL of 5 mM ferrozine and 2.75 mL of distilled water. The mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was then measured at 562 nm. The scavenging activity was calculated as follows:

$$\text{IP } \% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of plant extract. IC_{50} was calculated from the plot of inhibition percentage against extract concentration. EDTA was used as a positive control ($R^2 = 0.88$).

β-Carotene−Linoleic Acid Assay. Antioxidant capacity of *C. tinctorius* extracts was evaluated by β-carotene linoleic acid assay. The β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants.[19] First, β-carotene (0.2 mg) was dissolved in 1.0 mL of chloroform. Afterward, 200 μL of linoleic acid and 0.2 mL of Tween 80 were added and the mixture was left at room temperature for 15 min. After evaporation of chloroform, 50 mL of oxygenated distilled water was added and the mixture was shaken to form an emulsion (β-carotene−linoleic acid emulsion). Aliquots of 3.0 mL of this emulsion were transferred into test tubes containing 0.2 mL of different extract concentrations. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance (A_0) was measured at 470 nm using a spectrophotometer. A second absorbance (A_1) was measured after 120 min. A blank, without β-carotene, was prepared for background subtraction. Lipid peroxidation (LPO) inhibition was calculated using the following equation:[20]

$$\text{LPO inhibition } (\%) = A_1/A_0 \times 100$$

The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. BHT was used as standard ($R^2 = 0.93$).

Statistical Analysis. All analyses were performed in triplicate, and the results are expressed as mean values ± standard deviations (SD). The data were subjected to statistical analysis using statistical program package STATISTICA.[21] The one-way analysis of variance (ANOVA) followed by the Duncan multiple range test was employed and the differences between individual means and each solvent used were deemed to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Optimization of Phenol Extraction. The composition of the extracts was found to depend on the plant species, the plant part and the extraction technique.[22] Solvent nature is the most controversial one that influences antioxidant potentialities in plant analysis.[23] Therefore, in the first part of this study, three kinds of solvents with different polarities such as acetone, methanol and ethanol and their mixtures with water (at different ratios) were assessed.

Solvent Effect on Phenolic Extraction. Phenolic quantification revealed a wide range of flower polyhenols contents as function of the used solvent as shown in Table 2.

The extraction with acetone 20% showed the highest flower polyphenol content (15.09 mg GAE/g DW), followed by acetone 40% (14.94 mg GAE/g DW) and MeOH 60% (14.59 mg GAE/g DW). The latest group included acetone, MeOH and ethanol extracts which exhibited the lowest content (2.4 to 6.26 mg GAE/g DW). These results demonstrate clearly the influence of the solvent on the extractability of phenolics that is in agreement with a previous study[24] showing that the use of pure solvents such as ethanol had the lowest polyphenol content. However, results of Sultana et al.[25] showed that, among all the solvent extracts tested, the aqueous methanol and aqueous ethanol extracts had the highest phenol content. This may be due to the fact that phenolics are often extracted in higher contents in more polar solvents such as aqueous methanol/ethanol as compared with absolute methanol/ethanol.

As for total phenolics, flavonoid and proanthocyanidin (condensed tannins) contents also varied depending on the solvent extraction with maximum values of 4.76 and 1.54 mg EC/g DW, respectively (Table 2). The effect of solvent on flavonoid solubility showed approximately the same classification as phenolics, while differing for tannins. In fact, flower extracts had higher tannin content (1.54 mg EC/g DW) in ethanol 80%, followed by MeOH 80% (1.39 mg EC/g DW) and acetone 20% (0.85 mg EC/g DW). These results are in agreement with those of Chavan et al.[26] for procyanidins and those of Trabelsi et al.[24] who showed that water addition to the solvent improved flavonoid extraction and that ethanol/water (80:20, v/v) was the performant solvent system giving the optimal flavonoid and proanthocyanidin extraction.

Effect of Solvent on Antioxidant Activities. In order to choose the adequate solvent for antioxidant capacity, the antiradical activity (DPPH test) was evaluated using the same pure and solvent systems. Results demonstrate that, in the same way, *C. tinctorius* extracts exhibited a variable activity to quench DPPH radical as a function of the solvent type (Table 2). Flower extracts obtained with acetone 20% displayed the highest ability to reduce DPPH, with an IC_{50} value about 2.5 μg/mL, followed by MeOH 80% (3 μg/mL) and ethanol 80% (33 μg/mL). This scavenging effect of safflower extracts was stronger than that of BHT (Table 2). It was stronger than the antiradical activity reported in other species of Asteraceae family with various antioxidant activities of methanolic flower extracts (*Ambrosia artemisiifolia* (27.6 μg/mL), *Achillea ligustica* (47.2 μg/mL), and *Helichrysum* (37.52 μg/mL).[27−29]

The significant differences in antioxidant potential between the three solvent systems used in this experiment was essentially due to the difference in polarity, and thus different extractability of the antioxidative compounds.[23] Acetone, methanol or ethanol alone does not constitute a good extracting solvent, because the hydrophilic compounds required a little water. The efficiency of *C. tinctorius* extracts of the three solvent systems (acetone 20%, methanol 80% and ethanol 80%) was tested by their lipid peroxidation inhibition assay and metal-chelating power, in order to select the performant extracting system.

Effect of Selected Solvents on Lipid Peroxidation Inhibition Assay and Metal-Chelating Powers. In order to choose the appropriate solvent systems of extraction for *C. tinctorius* extracts, these three aqueous extracts were assessed for complementary antioxidant activities (lipid peroxidation inhibition assay and metal-chelating powers).

As it can be seen in Figure 1, the extraction with acetone 20% is distinguishable from the two other solvent systems and it

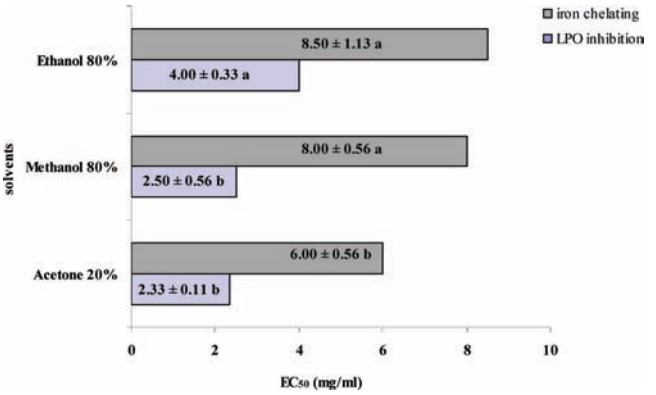

Figure 1. Iron chelating ability and LPO inhibition assay in *C. tinctorius* flower extracts prepared in three different solvents (acetone 20%, methanol 80% and ethanol 80%). Data are reported as means ± SD and compared to control (C). ANOVA followed by Duncan multiple range test ($P < 0.05$) and compared to control (C). Values with different superscripts (*a*-*b*) are significantly different at $P < 0.05$.

presents the highest chelating power activity showing the minimal EC_{50} (6 mg/mL) as well as the lipid peroxidation assay (EC_{50} = 2.33 mg/mL). However, methanol and ethanol extracts showed the higher EC_{50} ranging from 2.5 mg/mL to 4 mg/mL for chelating power and from 8 mg/mL to 8.5 mg/mL for lipid peroxidation assay, respectively, explaining weak antioxidant activities with these two solvent systems.

This chelating power activity was weak, in fact, compared to the standard EDTA (97.8 μg/mL) also for lipid peroxidation assay (BHT = 400 μg/mL). Hence, acetone 20% will be used for ulterior experiments. Our results are partially in agreement with those of Trabelsi et al.[24] and Zhao et al.[18] which found that the addition of water to the solvent improved the extracting power and antioxidant activity especially acetone/water.

Influence of Flower Development on Phenolic Contents. Petals are the floral organs that primarily determine the longevity of flowers, and as a consequence much attention has been given to the physiological, biochemical and genetic processes that occur during their development.[30]

Quantitative and qualitative changes of the antioxidant compounds during growth and development hold great significance from both dietary and nutritional points of view.[31] Among these natural compounds, yellow and red quinochalcone pigments are contained in safflower petals and are used as alternatives to synthetic dyes and shown to be safe for use in processed foods and soft drinks.[8] In our case, different flower colors (yellow, orange and red) are on the same plant and the intensity color of flowers is yellow just after flowering and gradually changes to red during flower development. Phenolic extracts obtained from safflower flowers harvested at three stages of its development (budding, fully opened and full flowering stages) and grown at the same geographical region in Tunisia (National Institute of Agricultural Research in Tunis) were quantified and are reported in Table 3.

Quantitative differences in polyphenol, flavonoid and proanthocyanidin contents in *C tinctorius* flowers during their development have been observed. In fact, polyphenolic contents reached a maximum in fully opened flowers (stage II) with values of 31.55 mg GAE·g^{-1} and 16.22 mg GAE·g^{-1}, respectively, for

Table 3. Total Polyphenol, Flavonoid, Condensed Tannin Contents and DPPH Scavenging Activity in *Carthamus tinctorius* Flower Extracts[a]

stages of flower development	total phenolic (mg GA equiv g^{-1})	total flavonoid (mg CE equiv g^{-1})	condensed tanins (mg CE equiv g^{-1})
	Orange Flowers		
I	16.37 ± 0.09 b	5.16 ± 0.33 b	4.31 ± 0.10 c
II	31.55 ± 0.43 a	7.25 ± 0.15 a	11.44 ± 0.13 a
III	30.86 ± 0.24 a	3.82 ± 0.05 c	5.19 ± 0.08 b
	Yellow Flowers		
I	8.65 ± 0.25 a	2.05 ± 0.10 a	1.31 ± 0.01 a
II	7.79 ± 0.11 b	2.16 ± 0.09 a	1.34 ± 0.03 a
III	6.83 ± 0.13 c	2.58 ± 0.04 a	1.30 ± 0.02 a
	Red Flowers		
I	16.22 ± 0.40 a	4.01 ± 0.06 b	3.53 ± 0.07 b
II	15.69 ± 0.28 b	5.98 ± 0.05 a	8.05 ± 0.34 a
III	8.48 ± 0.34 c	3.25 ± 0.12 c	2.45 ± 0.09 c

[a] (I) Bud formation, (II) flower formation, (III) full flowering. Data are reported as means ± SD and compared to control (C). ANOVA followed by Duncan multiple range test ($P < 0.05$). Values with different superscripts (*a-c*) are significantly different at $P < 0.05$.

total and red flowers whereas, they decreased sharply at stage III with 30.86 mg GAE·g^{-1} and 8.48 mg GAE·g^{-1}, respectively. These contents decreased slowly in yellow flowers with the major value at the first stage (8.65 mg GAE·g^{-1}).

A similar trend was also found in flavonoid and proanthocyanidin concentrations that reached a maximum value of 7.25 mg CE·g^{-1} and 5.98 mg CE·g^{-1} for flavonoids and 11.44 mg CE·g^{-1} and 8 mg CE·g^{-1} for proanthocyanidins at stage II in orange and red flowers, respectively. Similar findings were observed during development of the florets of *Chrysanthemum morifolium* Ramat where flavonoid concentrations reached a maximum in the half-open flower and then decreased sharply.[32] Bellflower (*Campanula isophylla* Moretti) is another example in which total anthocyanin (family of flavonoids) content peaked approximately 5 days after anthesis followed by a slow decline. The highest total flavone content was reached at anthesis, after which it remained almost constant.[33] In agreement with Marin et al.,[34] flavonoid contents followed the same trend as phenol ones, decreasing with fruit maturity of sweet pepper (*Capsicum annuum* L.). Also, a decrease in the anthocyanin content with age was found in *Chrysanthemumenus* genus (Asteraceae),[32] but in disagreement with the petals of 'Masquerade' rose (orange-yellow when freshly opened and turn deep red upon full flowering) where more than a 10-fold increase in anthocyanin level was measured during the period of full flowering).[35] This increase in anthocyanin formation with wilting is one of the typical post-pollination phenomena in *Cymbidium orchids*.[36] However, we noted a constant content in yellow flowers for condensed tannins (1.31 mg CE·g^{-1}) while a slight increase in flavonoid contents ranging from 2.05 mg CE·g^{-1} to 2.58 mg CE·g^{-1} during flower development was noted (Table 3). The polyphenol content in *C. tinctorius* flowers was higher than in several species of the same family cited in the literature, flowers of *Cynara cardunclus*, 6.96 mg GAE·g^{-1},[37] *Achillea wilhelmsii*, 36 μg GAE·g^{-1} and *Helichrysum arenarium* subsp., 56 μg GAE·g^{-1} [38] and compared to some other organs from Asteraceae family such as leaves of *Hieracium karagoellense* (31.78 mg/g DW). However, it was lower than other spices (Helichrysum species) where the total phenolic content ranged from 73.70 to 160.63 mg GAE/g.[29] Djeridane et al.[39] found that this abundance is characteristic of the Asteraceae family. This may be related to the hard climate conditions of Asteraceae's usual habitat (hot temperature, high solar exposure, drought, salinity), which stimulate the biosynthesis of secondary metabolites such as polyphenols. Indeed, in terms of their biosynthesis, the key enzyme phenylalanine ammonia-lyase (PAL) is especially relevant as it can be induced by different environmental conditions. These include intrinsic (genetic) and extrinsic (environmental, handling and storage) factors that phenolic contents of a plant which may depend on.[40] The phytochemical changes that occur during flower development and the resultant effect on total phenolic content of *C. tinctorius* flowers hold a great significance that may affect the consumption of safflower flowers. Additionally, we determined how changes in chemical composition, in response to flower development, influenced antioxidant activity.

Effect of Flower Development on Antioxidant Activities. Antioxidants have been widely used as food additives to provide protection against oxidative degradation. Spices used in different types of food to improve flavors, since ancient times, are well-known for their antioxidant properties.[41] There are different methods to evaluate the antioxidant capacities of compound mixture.[42] For this reason, in the present work, five different complementary methods were selected for the evaluation of the antioxidant activity and differ from each other in terms of substrates, probes, reaction conditions, and quantification methods.

Free radical scavenging properties of acetonic extracts from *C. tinctorius* flowers are presented in Table 4. $ABTS^{+}$ and DPPH are popular free radicals for use in assessing radical scavenging or antioxidant activities. Lower IC_{50} value indicated higher antioxidant activity. All extracts of safflower flower showed higher scavenging ability on DPPH radicals (IC_{50} = 4 μg/mL) for orange flowers, (IC_{50} = 5 μg/mL) for yellow flowers and (IC_{50} = 2.5 μg/mL) for red flowers. In addition, DPPH scavenging ability of the acetonic flower extracts was higher than that of synthetic antioxidant BHT (IC_{50} = 16 μg/mL). However, these extracts showed moderate to high levels of scavenging activity on ABTS radical and lower than Trolox (Table 4). Radical scavenging activity also showed an increasing trend from bud to full flowering stages in orange and red flowers ranging

Table 4. Antioxidant Properties against $ABTS^{\bullet+}$, $DPPH^{\bullet}$ Radicals (IC_{50} in μg/mL) and Chelating, Reducing, Lipid Peroxidation Inhibition Power (EC_{50} mg/mL) of *C. tinctorius* Flower Extracts Compared to That of Authentic Standards (BHT, Trolox, EDTA and Ascorbic Acid)[a]

stages	IC_{50} (μg/mL) on DPPH	IC_{50} (μg/mL) on ABTS	EC_{50} (mg/mL) on reducing power	IC_{50} (mg/mL) on chelating power	EC_{50} (mg/mL) on LPO inhibn
			Orange Flowers		
I	4.00 ± 0.04 a	438.00 ± 0.65 a	1.10 ± 0.97 b	7.33 ± 0.04 b	5.90 ± 0.04 a
II	3.60 ± 0.09 b	440.00 ± 0.87 b	2.50 ± 0.68 a	14.00 ± 0.03 a	6.00 ± 0.04 a
III	3.20 ± 0.09 b	440.00 ± 0.77 b	0.70 ± 0.95 c	6.00 ± 0.01 c	6.20 ± 0.04 a
			Yellow Flowers		
I	5.00 ± 0.14 a	700.00 ± 0.04 a	0.70 ± 0.04 b	>10.00 ± 0.54 a	7.50 ± 0.09 a
II	4.80 ± 0.24 a	460.00 ± 1.04 b	1.20 ± 0.03 a	>10.00 ± 0.44 a	7.50 ± 0.05 a
III	5.00 ± 0.34 a	120.00 ± 0.99 c	0.25 ± 0.01 c	>10.00 ± 0.09 a	6.90 ± 0.04 b
			Red Flowers		
I	5.00 ± 0.06 a	360.00 ± 0.54 a	0.32 ± 0.02 a	>15.00 ± 0.01 a	7.00 ± 0.09 a
II	2.50 ± 0.08 b	320.00 ± 0.55 b	0.37 ± 0.04 a	>10.00 ± 0.14 b	5.50 ± 0.03 b
III	2.50 ± 0.04 b	310.00 ± 0.92 b	0.25 ± 0.04 b	8.00 ± 0.64 c	2.80 ± 0.04 b
Trolox		242.30 ± 20.36			
BHT	16.00 ± 1.89				0.08 ± 0.02
ascorbic acid			0.04 ± 0.00		
EDTA				0.10 ± 0.01	

[a] (I) Bud formation. (II) Flower formation. (III) Full flowering. Data are reported as means ± SD and compared to control (C). ANOVA followed by Duncan multiple range test ($P < 0.05$). Values with different superscripts (*a-c*) are significantly different at $P < 0.05$.

from (IC_{50} = 4 μg/mL) to (IC_{50} = 3.2 μg/mL) in orange flowers and from (IC_{50} = 5 μg/mL) to (IC_{50} = 2.5 μg/mL) in red flowers for DPPH radicals. However, in yellow flowers the DPPH radical scavenging activity is similar for all stages of flower development. Concerning ABTS radical cation scavenging activity, an increasing trend was reported and the values varied from (IC_{50} = 700 μg/mL) to (IC_{50} = 120 μg/mL) in yellow flowers. However, constant levels were shown for orange and red flowers among three development stages of safflower (Table 4). Acetonic extracts of *C. tincorius* showed significantly ($P < 0.01$) greater antiradical scavenging activity compared to those of species belonging to Asteraceae family reported in the literature (*Tessaria fastigiata*, IC_{50} = 53.2 μg/mL; *Tagetes maxima Kuntze*, IC_{50} = 53.2 μg/mL; *Pluchea sagittalis*, IC_{50} = 17 μg/mL;[43] *Echinacea purpurea*, IC_{50} = 65.48 μg/mL;[44] and *Helichrysum* species IC_{50} from 7.95 μg/mL to 53.10 μg/mL.[29] The antioxidant activity of acetonic extracts from flowers was also evaluated by reducing power assay (Table 4). Although safflower extract showed lower antioxidant activity than ascorbic acid, it decreased from stage I to II and increased from stage II to III for all types of flowers with a high level of reducing power (EC_{50} = 0.7 mg/mL for orange flowers; EC_{50} = 0.25 mg/mL; for yellow and red flowers). Concerning chelating ability and β-carotene-linoleate bleaching assays (Table 4), *C. tinctorius* flower presented a very low ability to chelate irons and prevent the bleaching of β-carotene with IC_{50} ranging from 6 to 10 mg/mL and with EC_{50} ranging from 2.8 to 6.9 mg/mL, respectively, since their antioxidant values were approximately 100-fold higher than those of BHT and ascorbic acid. As for antiradical scavenging and reducing power methods, flower extracts showed an increase in antioxidant levels during flower development (Table 4). At first glance, it can be thought as a contradiction between the higher radical scavenging or the increase in antioxidant activities during flower development and the lower phenolic content because a linear correlation between radical scavenging activity and polyphenolic concentration has been reported in an extensive range of vegetables, fruits and beverages.[45] In our study, the antioxidant activity did not show correlation with phenol contents. In fact, the synergistic interactions between the antioxidants in the mixture of phenolic compounds make the antioxidant activity dependent not only on the concentration but also on the nature and the interactions between the antioxidants.[7] Therefore, content of phenolic compounds could not be the major criterion in the assessment of antioxidant activity. The cause of the high activity of *C. tinctorius* flower remains unclear and can possibly be due to quinochalcone compounds which are characteristic flavonoid pigments of safflower[46] and have a unique structure with a C-glycosylated cyclohexanonedienol moiety that occurs only in this plant.[47] However, little information about the major bioactive components was mentioned except for hydroxysafflor yellow A. This latter has been demonstrated to have important pharmacological and antioxidant activities and myocardial, cerebral protective and neuroprotective effects in *C. tinctorius* florets.[9,48] Hydroxysafflor yellow A is the main chemical component of the safflower yellow pigments[48] and may be responsible for the high antioxidant activity for yellow flowers in this study (Table 4). Concerning red flowers, the high antiradical activity could be explained by their high content of carthamin. Recently, Hiramatsu et al.[49] found that water extract of *C. tinctorius* flower has free radical scavenging activity against DPPH radicals and the component responsible for this activity is especially carthamin with IC_{50} of 1.5 μg/mL of dry flower water extract. Antioxidant activity of carthamin is almost the same as that of vitamin C, and the radical scavenging activity in safflower yellow is ten times lower than in carthamin. *C. tinctorius* flower contains lipid-soluble carthamin and water-soluble safflower yellow, suggesting that it might protect membrane functions against free radicals,[49] which may explain the high scavenging activity of our extracts (Table 4).

Table 5. Contents of Individual Phenolic Compounds (μg/g DW) of *C. tinctorius* Flowers at Three Stages of Flower Development (I, II, III)[a]

flower development stages	gallic acid	chlorogenic acid	syringic acid	rutine trihydrate	quercetin-3-galactoside	naphthoresorcinol
			Orange Flowers			
I	54.96 ± 0.09 c	25.25 ± 0.04 a	4.83 ± 0.21 a	0.56 ± 0.09 b	10.71 ± 0.13 b	1.98 ± 0.19 b
II	88.41 ± 0.07 b	24.20 ± 0.10 b	2.56 ± 0.11 b	0.24 ± 0.04 c	10.53 ± 0.15 b	1.48 ± 0.03 c
III	102.57 ± 0.05 a	17.76 ± 0.10 c	1.13 ± 0.23 c	1.38 ± 0.02 a	17.01 ± 0.12 a	2.23 ± 0.05 a
			Yellow Flowers			
I	207.74 ± 0.32 a	32.26 ± 0.23 a	9.78 ± 0.15 a	6.85 ± 0.11 a	47.42 ± 0.10 a	9.69 ± 0.09 a
II	44.71 ± 0.24 c	11.37 ± 0.16 b	2.70 ± 0.04 c	2.33 ± 0.19 b	9.50 ± 0.22 b	4.57 ± 0.06 b
III	58.21 ± 0.17 b	9.64 ± 0.17 c	3.18 ± 0.01 b	2.40 ± 0.13 b	7.87 ± 0.21 c	2.01 ± 0.11 c
			Red Flowers			
I	48.98 ± 0.09 b	3.15 ± 0.04 a	18.71 ± 0.06 a	20.80 ± 0.01 a	11.50 ± 0.13 c	5.21 ± 0.35 a
II	192.63 ± 1.09 a	75.99 ± 0.45 a	2.44 ± 0.56 a	12.70 ± 0.60 b	29.10 ± 0.89 a	1.93 ± 0.01 b
III	64.80 ± 0.15 c	21.70 ± 0.97 a	1.80 ± 0.44 a	0.58 ± 0.99 c	15.63 ± 0.09 b	0.58 ± 0.12 c

[a] Each value represents mean ± standard deviation of three replicates; different letters in the same row mean significant difference ($P < 0.05$). (I) Bud formation. (II) Flower formation. (III) Full flowering. Data are reported as means ± SD and compared to control (C). ANOVA followed by Duncan multiple range test ($P < 0.05$).

Effect of Flower Development on the Contents of Different Phenolic Compounds. For a clear understanding of metabolic changes in phenolics during flower development, it is necessary to investigate the profiles of individual phenolic compounds in *C. tinctorius* flowers. To the best of our knowledge, this is the first report on phenolic compound contents during *C. tinctorius* flower development. Changes of six main phenolic compounds in *C. tinctorius* flowers were different during flower development (Table 5). Gallic acid was the most abundant phenolic compound, accounting for about 102.57 (μg/g DW), and a linear correlation between the gallic acid content and antioxidant capacities during flower development for orange flowers (Table 5), indicating that gallic acid could be the main compound responsible for its antioxidant power.[50] As previously reported, a study of vitamin C equivalent antioxidant capacity (VCEAC) in relation with its molecular structure indicated that gallic acid has the highest antioxidant capacity among all the phenolic compounds tested.[51] Govindarajan et al.[52] showed that the major compound of *Anogeissus latifolia* extract was gallic acid (0.95%), which was responsible of antioxidant potential of this plant. Rutin, quercetin-3-galactoside and naphthoresorcinol followed also the same profile of gallic acid (Table 5). For instance, this acid has been widely used as additive to avoid the degradation of foods and is known to have anti-inflammatory, antimutagenic and anticancer activities.[53] Also antioxidant activity was found for rutin.[54] Therefore, rutin and gallic acid may contribute greatly to the high antioxidant activities of the species. Further studies are needed to support this hypothesis.

Nevertheless, no correlation was found between chlorogenic and syringic acids and antioxidant properties in the extract of orange flowers and phenolic compounds of yellow and red flowers during three stages of flower development. This fact may be explained in numerous ways. Phenolics may not incorporate all the antioxidants and their radical scavenging activity might mostly depend on their molecular structure. This latter depends on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation.[55] Chlorogenic acid and rutin were also isolated from aerial parts of *Carthamus lanatus* in Bulgaria.[54] However, Zheng et al.[56] in research on phenolic compounds in *C. tinctorius* flowers, have determined only two phenolic acids including *p*-coumaric and ferulic acids with levels of 69.6 μg/g DW and 9.67 μg/g DW, respectively. Thus, the types and contents vary with cultivar, flower development and processing.[57] Changes in flavonoid contents of safflower leaf during growth were also studied and results obtained were comparable with the data of the present work. In fact, levels of five flavonoid glycosides in the leaf increased progressively over time according to growth stages, reached a maximum and then decreasing sharply, Moreover, those of three flavonoid aglycons increased greatly at the early stage of growth, reached a peak and then decreased rapidly.[58]

These results suggest that *C. tinctorius* flower harvested at late stage of its development may be a good source of bioactive components as natural colorants.

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