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Copigmentation Effect of Some Phenolic Acids on Stabilization of Roselle (*Hibiscus sabdariffa*) Anthocyanin Extract

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Abstract The present study aimed at utilization of copigmentation phenomenon to increase the stability of anthocyanin in roselle extract during storage at 10°C for 60 days by the addition of some phenolic acids (ferulic, cinnamic and coumaric) as a copigments and investigate the possibility of using copigmented extracts as a natural food colorants instead of harmful synthetic ones. The data obtained confirmed that addition of the aforementioned phenolic acids to roselle anthocyanin extracts resulted in an increment of anthocyanin and color stability during storage comparing with the control extract. At the end of storage period, the reduction in anthocyanin content were 31.53, 20.48, 9.31 and 5.52% for control and extracts copigmented with ferulic, cinnamic coumaric acids respectively. Addition of phenolic acids to roselle extract also attributed to a hyperchromic effect and bathochromic shift in visible absorption spectra of copigmented extracts compared to the control. Roselle anthocyanin extracts treated with phenolic acids showed a noticeable antioxidant and antimicrobial activities compared with control extract. Marshmallow prepared from studied copigmented roselle extracts as a natural colorants was highly accepted by panelists.

Keywords: *anthocyanin, antimicrobial activity, antioxidant activity, copigmentation, Hibiscus sabdariffa*

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1. Introduction

Roselle (*Hibiscus sabdariffa*), red sorrel or karkade, is an annual herbaceous subshrub belonging to the family Malvaceae. This plant is native to Africa and cultivated in tropical and sub-tropical regions such as Sudan, Southern Asia, India, Indonesia, Saudi Arabia, Malaysia, Philippines, China, Vietnam, Egypt, Nigeria and México [1,2]. Fleshy calyces (sepals) are commercially important for the production of, juices, jams, beverages and syrup in the food industry. Furthermore, these calyces are a good source of natural food colorants because of their high pigment content [2]. Besides its extended consumption as a beverage and its uses in food industry, roselle is also used in animal feed, nutraceuticals, cosmetics and pharmaceuticals [3].

Hibiscus sabdariffa extract showed antioxidant, antibacterial, nephro-and hepato-protective, renal/diuretic effect, along with effects on lipid metabolism (anti-cholesterol), anti-diabetic and anti-hypertensive effects among others [4,5]. This might linked to strong antioxidant activities, inhibition of α-glucosidase and α-amylase, inhibition of angiotensin-converting enzymes (ACE), and direct vaso-relaxant effect or calcium channel modulation [4]. Phenolic acids (esp. protocatechuic acid), organic acids (hibiscus acid and hydroxycitric acid) and anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside) are likely to contribute to the reported effects [5]. Roselle is one of the most well-known sources that contain anthocyanin [4,5,6]. There are reports on the effect of anthocyanin on tumor cells, anti-inflammatory activity, anticonvulsant activity and antioxidant activity [5,6].

It has been early recognized that anthocyanin-rich plant extracts might have potential as natural food colorants, especially if suitable purified and stable materials become commercially available [7,8]. Anthocyanins are an important group of water-soluble plant pigments commonly found in various fruits and vegetables [8]. However, natural pigments are not stable due to processing and storage conditions [8,9]. A number of factors influence anthocyanin stability including oxygen, pH, enzymes, light, heat-humidity, and also the presence of sugars, ascorbic acid, sulfite salts or sulfur dioxide, metal ions and copigments [9].

Copigments are colorless or only very slightly, mainly yellowish, colored molecules occurring naturally in plant kingdom in cells alongside anthocyanin. A wide range of different molecules has been found to act as copigments. The most common structurally unrelated copigment compounds are flavonoids, and other polyphenols, amino acids, alkaloids and organic acids [10]. Copigmentation generally occurs in the following four interactions: self-association, metal complexation, intramolecular

copigmentation and intermolecular interactions. Among the four interactions, intramolecular and inter-molecular copigmentation are the most important mechanisms of copigmentation [11]. Intramolecular copigmentation, in which the central anthocyanin chromophore and aromatic acyl residues covalently linked to their glycosyl moieties is predominant in flower vacuoles [12,13]. Inter-molecular copigmentation occurs when colorless compounds are attracted to anthocyanins via weak hydrogen bonds and hydrophobic forces [11].

The European Commission Directive 1333/2008 (EC) stipulates labeling of food containing synthetic colorants with warning notices since July 2010. Hence, substitution of synthetic colorants by their natural but less stable counterparts is a major challenge. Orange, yellow, and red dyes may be replaced by natural pigments such as carotenoids, anthocyanin and betalain despite their inferior stability [14].

The present study was carried out to utilize copigmentation phenomena of some phenolic acids to increase stability of roselle (*Hibiscus sabdariffa* L.) anthocyanin water extract during storage to be more suitable for using as a natural food colorants instead of harmful synthetic ones, where it is considered a good source of anthocyanin and has a recognized antioxidant and antimicrobial activity.

2. Materials and Methods

2.1. Materials and Chemicals

Calyces of roselle (*Hibiscus subdariffa* L.) were used as a source of natural pigments in the present study. The dried calyces of roselle were purchased from a local market in Alexandria, Egypt. The dried roselle calyces were ground for 3 second using a blender (Braun, Model 2001 DL, Germany) and immediately packed in polyethylene bags and kept at low temperature (5°C) till used. All ingredients of marshmallow were purchased from local market in Alexandria, Egypt. All chemicals used for the present study were purchased from Sigma (St Louis, MO, Germany).

2.2. Anthocyanin Extraction

The aqueous extractions of anthocyanin were carried out according to [15] with slight modification. About 100 g of dried and grinded roselle calyces were mixed in blender with I liter of distilled hot water (90°C) for 15 min at room temperature (22± 2°C) and left for 24 hours. The extract was filtered using Whatman No. 4 filter paper in a Buchner funnel under vacuum and divided into four sections with and without copigments as follows-:

RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract + ferulic acid
RE+CIN: Roselle anthocyanin extract + cinnamic acid
RE+COUM: Roselle anthocyanin extract + coumaric acid

Ratio of copigments (ferulic, cinnamic and coumaric) to anthocyanin was 100:1 according to [16,17]. A part of each extract was directly used for determination the effect of copigments on antioxidant and antimicrobial activities and preparation of marshmallow and another part was stored in refrigerator at 10°C for 60 days in glass bottles to study the effect of copigments on anthocyanin content, visible absorption spectra and color stability of roselle extract during storage.

2.3. Determination of Anthocyanin

The anthocyanin content in studied roselle extracts was determined using the pH-differential method as described by [18]. Spectrophotometric measurements were carried out using a double-beam spectrophotometer (Optizen, Mecasys, Co., Ltd). Anthocyanins were expressed as cyanidin-3-glucoside (mg/L). Sample absorbance was read against a blank cell containing distilled water. The absorbance (A) of the sample was then calculated according the following formula:

$$A = \left(A_{\lambda vis} - A_{700}\right)_{pH\ 1.0} - \left(A_{\lambda vis} - A_{700}\right)_{pH\ 4.5} \quad (1)$$

Where $A_{\lambda vis}$ is the absorbance at wavelength which maximal absorbance of samples was achieved.

Anthocyanin pigments content in the original samples were calculated according the following formula

$$\text{Anthocyanin pigments } (mg/L) = A \times MW \times DF \times 10^3 / \varepsilon \times l \quad (2)$$

Where:-
MW = Cyanidin -3- glucoside molecular weight (449.2).
DF = Dilution factor
l = Path length in cm
ε = Molar extinction coefficient
10^3 =Factor for conversion from g to mg .

2.4. Determination of Antioxidant Activity

Antioxidant activities of control and copigmented roselle anthocyanin extracts were measured according to [19] by the N, N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) to determine percentage of inhibition. The antiradical activity was expressed as IC_{50} (mg/mL) whereas IC_{50} is the extract concentration required to cause a 50% DMPD inhibition and it calculated from the graph plotted inhibition percentage against extract concentration. A lower IC_{50} value corresponds to a higher antioxidant activity of studied extract [20].

2.5. Spectrophotometric Measurements

Visible absorption spectra of control and copigmented roselle anthocyanin extracts were recorded using double-beam spectrophotometer (Optizen, Mecasys, Co., Ltd) scanning the visible range from 400 to 700 nm. The change in the maximum absorbance (A_{max}) at varying wavelengths (λ_{max}) presented the change in the color intensity, and revealed a possible hyperchromic effect (ΔA_{max}) and bathochromic shift ($\Delta\lambda_{max}$), resulting from a copigmentation reaction [16].

2.6. Color Measurement

CIE – $L^* a^* b^*$ parameters were determined for control and copigmented roselle anthocyanin extracts with Hunter

Scan VIS model, colorimeter (USA). The measured parameters were L^* for lightness, b^* for yellowness and a^* for redness. With these calculation of ΔE^* for total color change according to [16] from following equations:-

$$\Delta E^* = \left[\left(\Delta L^* \right)^2 + \left(\Delta a^* \right)^2 + \left(\Delta b^* \right)^2 \right]^{1/2}. \quad (3)$$

2.7. Antimicrobial Activity

Antimicrobial activities for control and copigmented roselle anthocyanin extracts were determined according to [21] using disc diffusion assay. Colonies of *Bacillus subtilis* B505, *Escherichia coli* 0157, *Staphylococcus aureus* cultured in Luria Bertani and *Aspergillus niger* (CAIM 147) were used in the present study.

2.8. Organoleptic Properties

Color, taste, odor, texture and over all acceptability of marshmallow prepared from control and copigmented roselle anthocyanin extracts was evaluated by fifteen panelists of Food Science and Technology Department, Faculty of Agriculture, Alexandria University, Egypt, using a numerical (hedonic) rating of 1-9 (1=dislike very much, 9=like very much) as described by [22].

2.9. Technological Methods

2.9.1. Marshmallow

Marshmallow prepared according to [23] using following ingredients -:granulated sugar 1 kg, corn syrup 700 g, gelatin 100 g, roselle anthocyanin extract 200 ml and vanillin 0.5 g. The gelatin was pre-soaked in water until pliable. The sugar, corn syrup and roselle extract were placed in a steam pan, the sugar was dissolved and cooked to 115°C. The batch was transferred to a marshmallow beater; the gelatin was added and allowed to dissolve in the hot solution. The batch was beaten until well aerated, adding of vanillin was just before finishing point. The marshmallow was poured into trays to the required thickness. The sheets were cut by machine to desired shape.

2.10. Statistical Analysis

Data were statistically analyzed as Complete Block Design with three replicates according to [24] comparison between treatments means were carried out using Least Significant Differences method at 0.05 probability level ($LSD_{0.05}$). Graphs were produced using Harvard graphics software (HG, version, 5. 2003).

3. Results and Discussion

3.1. Effect of Copigments on Antioxidant Activity of Roselle Anthocyanin Extracts

Table 1 shows antioxidant activities of control and copigmented roselle anthocyanin extracts after adding phenolic acids (ferulic, cinnamic and coumaric) and before storage, expressed as IC_{50} and percentage of DMPD inhibition. The data indicated that all of roselle extracts have noticeable antioxidant activities that can be attributed to a high content of anthocyanin in all roselle extracts under investigation. Extract with coumaric acid showed the highest percentage of inhibition (44.93%) and the lowest value of IC_{50} (0.52 mg/ml) followed by the extracts with cinnamic and ferulic acids as compared with the control extract which had the lowest inhibition percentage (28.65%) and the highest IC_{50} (0.87 mg/ml). These results indicate that addition of some phenolic acids such as coumaric, cinnamic and ferulic acids resulted in elevation the antioxidant activities of the roselle extracts studied here as compared with the control one.

These data are in agreement with [25] who concluded that anthocyanin is the major source of antioxidant capacity in roselle. Also [26] concluded that the petals of *Hibiscus subdariffa* are potentially a good source of antioxidant agents as anthocyanin and ascorbic acid. In addition [27] confirmed that *Hibiscus sabdariffa* is a good source of dietary antioxidants, with its calyces containing high amounts of anthocyanin. Moreover [28,29] found that *Hibiscus sabdariffa* had high antioxidant content related to the presence of anthocyanin with potent antioxidant activity. Generally the calyces of *Hibiscus sabdariffa* are potentially a good source for antioxidant agents such as ascorbic acid and anthocyanin [30].

Table 1. Antioxidant activities of control and copigmented roselle anthocyanin extracts

Extract	IC_{50} (mg/ml)	inhibition (%)
RE	0.87^{a} ±0.01	28.65^{d} ±0.92
RE+ F	0.71^{b} ±0.02	35.00^{c} ±0.85
RE+ CIN	0.65^{c}± 0.02	37.25^{b}±0.79
RE+COUM	0.52^{d} ±0.01	44.93^{a}±0.83

Values are means of triplicates ± standard deviations
Means in a column followed by the same letter are not significantly different at ($p \leq 0.05$).
RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM: Roselle anthocyanin extract+ coumaric acid.

3.2. Effect of Copigments on Antimicrobial Activity of Roselle Anthocyanin Extracts

Table 2 represents antimicrobial activities for control and copigmented roselle anthocyanin extracts before storage. The results shown in Table 2 indicate that each of the control and copigmented roselle extracts had a noticeable antimicrobial effect against a large varieties of bacterial species (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*). Copigmented extracts showed higher antibacterial activities as compared with the control and these activities were traced against all examined bacteria species. From the data in Table 2, it could be seen that roselle extracts with each of cinnamic and coumaric acids had a higher diameter of inhibition zones for all studied bacteria species compared with the control. Diameter of inhibition zones for roselle extract with cinnamic acid reached to 5.3, 14.00 and 10.3 mm, whereas diameters for extract with coumaric acid were 8.6, 18.00

and 11.00 mm for each of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, respectively. Meanwhile, extract with ferulic acid showed noticeable antibacterial activities against all studied bacteria represented in inhibition zones being in a range between 3.00 and 7.6 mm. The control roselle extract did not show any antimicrobial activity against *Aspergillus niger* while extracts with ferulic, cinnamic and coumaric acids showed noticeable antifungal activities represented in a clear zone ranged between 3.6 to 10.3 mm in diameter. The obtained results are in agreement with [31] who reported that *Hibiscus sabdariffa* shows various inhibitory effects against Gram positive and negative bacteria. Also [32] found that *Hibiscus sabdariffa* was found to be effective at all levels in inhibiting *E. coli* isolated from food veterinary and clinical samples. Moreover [33] stated that the roselle extracts revealed greater antimicrobial activity against *Staphylococcus aureus* and *Micrococcus luteus* than *E. coli*.

3.3. Marshmallow Prepared From Control and Copigmented Roselle Anthocyanin Extracts

The results in Table 3 show organoleptic properties of marshmallow that contains each of control and copigmented roselle extracts as a natural colorant instead of synthetic ones. The data confirmed that all marshmallow samples prepared from control and copigmented extracts before storage were accepted by panelists especially those containing extracts with coumaric, cinnamic and ferulic acids, where marshmallow that contained copigmented roselle extracts had good color and good acceptability as compared with the control. Good sensory properties of marshmallow samples confirmed possibility of applying copigmented roselle anthocyanin extracts as a good natural colorant in sweets processing such as marshmallow which will be of great benefit for human health. In this regard [34] reported that *Hibiscus sabdariffa* is a tropical plant of considerable economic potential. Its calyces have been suggested as a food colorant for food industries; emulsifier for carbonated drinks, jam manufacture, juices.

3.4. Effect of Storage on Stabilization of Anthocyanin in Control and Copigmented Roselle Anthocyanin Extracts

Anthocyanin exhibit greater stability under acidic conditions, but under normal processing and storage conditions readily convert to colorless derivatives and subsequently to insoluble brown pigments [8], so the effects of studied copigments on stabilization of anthocyanin and color of roselle anthocyanin extracts during storage were investigate in this part of study.

Graphs in Figure 1 and data in Table 4 show the effect of phenolic acids (ferulic, cinnamic and coumaric) used as a copigments on the stability of roselle anthocyanin extracts compared with the control extract (without copigments) during storage for 60 days at 10°C. Graphs and data in figure and Table indicate that the control and copigmented roselle extracts contained an adequate amount of anthocyanin at zero time of storage being 161.1 mg/L, meanwhile storage of studied roselle extracts at 10°C for 60 days attributed to decreasing of anthocyanin content in all studied extracts within storage but in a varying proportion, in the control extract reduction in anthocyanin content at the end of storage period was 31.53%, meanwhile the reduction in anthocyanin content in copigmented roselle extracts after the same period were 20.48, 9.31 and 5.52% for extracts copigmented with ferulic, cinnamic and coumaric acids respectively. It was obvious that roselle extract copigmented with coumaric acid showed the lowest reduction in anthocyanin content during storage followed by extracts with cinnamic and ferulic acid.

Table 2. Antimicrobial activities of control and copigmented roselle anthocyanin extracts

	Diameter of inhibition zone (mm)			
Microorganism	**RE**	**RE+F**	**RE+CIN**	**RE+COUM**
Escherichia coli	2.3^{h}± 0.57	3.00^{g}±0.9	5.3^{f}±0.57	8.6^{d}±0.59
Staphylococcus aureus	5.3^{f}±1.52	7.6^{de}±0.57	14.00^{b}±0.73	18.00^{a}±0.98
Bacillus subtilis	4.6^{fg}±0.75	6.6^{e}±0.56	10.3^{c}±0.57	11.00^{c}±0.97
Aspergillus niger	0.00^{i}±0.0	3.6^{g}±0.55	5.7^{f}±0.87	10.3^{c} ± 0.57

Values are means of triplicates ± standard deviations
Means in a row or a column followed by the same letter are not significantly different at ($p \leq 0.05$
RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM : Roselle anthocyanin extract+ coumaric acid.

Table 3. Organoleptic properties of marshmallow prepared from control and copigmented roselle anthocyanin extracts before storage.

Sample	Taste	Color	Odor	Texture	Overall acceptability
RE	7.2^{ab}±1.8	6.4^{b}±1.3	6.4^{b}±1.3	7.8^{a}±1.3	7.00^{ab}±1.5
RE+F	7.9^{a}±1.7	7.7^{ab} ±1.1	7.8^{a}±1.2	7.6^{ab}±1.5	7.1^{ab} ±1.4
RE+CIN	7.9^{a}±1.2	7.2^{ab} ±1.5	7.1^{ab}±1.5	8.3^{a}±0.99	7.6^{ab}±1.1
RE+COUM	7.8^{a} ±1.1	8.9^{a}±1.2	7.00^{ab}±1.2	8.4^{a}±0.82	8.2^{a}±0.99

Values are means of triplicates ± standard deviations
Means in a column followed by the same letter are not significantly different at ($p \leq 0.05$).
RE : Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM: Roselle anthocyanin extract+ coumaric acid.

The aforementioned results reflected the ability of used phenolic acids (coumaric, cinnamic and ferulic) to be a good copigments to increase and enhancement the stability of roselle anthocyanin extract during storage which make it more suitable for using in food industry as a natural and safe colorant. In accordance data of [35] showed that hydroxycinnamic acid is of interest for copigmentation researchers, and has shown a prominent effect on the enhancement and stabilization of anthocyanins. Whereas [36] found that red calyces of *Hibiscus sabdariffa* L. contain high concentration of anthocyanin which can reach to 1.5 g/kg. Results of anthocyanin content presented in this study are in agreement with [37] who found that the flowers of *Hibiscus sabdariffa* are rich in anthocyanin. Also [38] found that anthocyanin were only detected in the red varieties of sorrel (*Hibiscus sabdariffa*) and ranged from 0.5 to 3.5 mg/g. The addition of copigments led to significantly lower anthocyanin losses compared to samples without copigments, this stabilizing effect may be attributed to the antioxidative properties of the copigments and the prevention of the hydration of the anthocyanin [39].

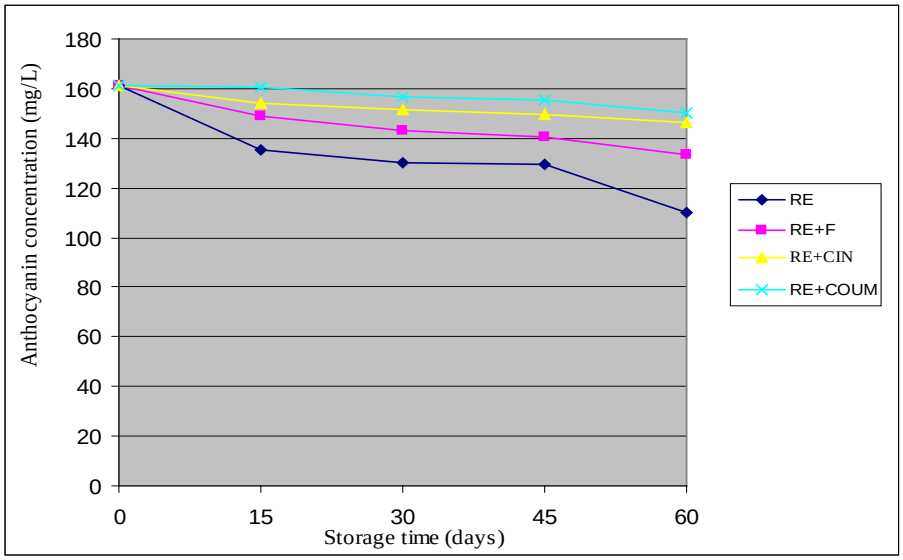
3.5. Effect of Storage on Visible Absorption Spectra of Control and Copigmented Roselle Anthocyanin Extracts

Graphs in Figure 2 show the visible absorption spectra of studied roselle anthocyanin extracts with and without copigments at zero time and after 30 and 60 days of storage at 10C°. From the graphs it could be concluded that at zero time of storage control roselle had the maximum absorption at wavelength 510- 530nm that wave length special for anthocyanin absorption. Graphs in Figure 2 reflect a hyperchromic effects (increasing in absorption intensity) and also a bathochromic shift (spectra of copigmented extracts display a shift of the visible λ_{max} toward greater wavelengths) occurred in copigmented extracts due to pigmentation reaction and formation of copigmentation complex between anthocyanin and copigments. At zero time and during storage the extracts with coumaric and cinnamic acids exhibited the highest absorbance (hyperchromic effect) followed by the extract with ferulic acid compared with the control extract. A slight decreasing in absorption in all roselle anthocyanin extracts was noticed after 30 and 60 days of storage times, due to the change in color and anthocyanin content during storage. However, the extract copigmented with coumaric acid still possessed a higher absorption followed by extracts with cinnamic and ferulic acids in all time of storage and until the end. From previous results it was found that the addition of phenolic acids induced the increase of absorbance and improve color in copigmented roselle extracts. Data presented here are in agreement with [40] who stated that copigmentation of anthocyanin is extremely important, as it is responsible for the increase in absorbance intensity (hyperchromism) and for a positive shift in the visible wavelength (bathochromism). Also [41] reported that effect of anthocyanin copigmentation reactions can be detected by a hyperchromic effect, where absorbance at the λ_{max} of the absorption spectrum increases, and/or a bathochromic shift, where the spectra of copigmented solutions display a shift of the visible λ_{max} toward greater wavelengths.

Table 4. Reduction of anthocyanin content in control and copigmented roselle anthocyanin extracts during storage (%)

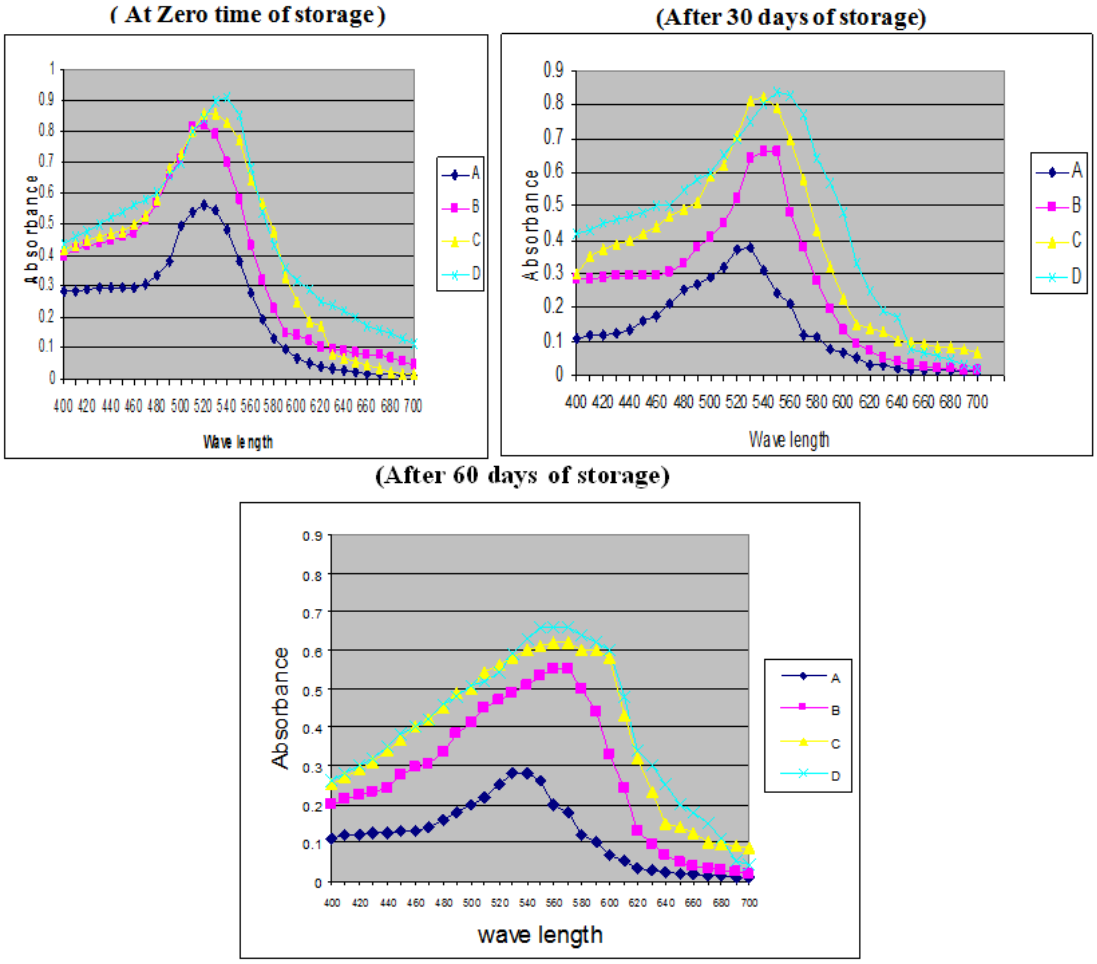
Extract	Storage period (days)			
	15	30	45	60
RE	9.62	14.50	19.128	31.53
RE+F	7.38	11.17	17.28	20.48
RE+CIN	4.22	6.08	7.32	9.31
RE+COUM	1.60	2.79	3.71	5.53

RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM : Roselle anthocyanin extract+ coumaric acid.



RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM: Roselle anthocyanin extract+ coumaric acid

Figure 1. Changes in anthocyanin concentration in control and copigmented roselle anthocyanin extracts during storage for 60 days



A : Control roselle anthocyanin extract
B : Roselle anthocyanin extract+ ferulic acid
C : Roselle anthocyanin extract+ cinnamic acid
D: Roselle anthocyanin extract+ coumaric acid

Figure 2. Visible absorption spectra for control and copigmented roselle anthocyanin extracts during storage for 60 days

3.6. Effect of Storage on Color Change of Control and Copigmented Roselle Anthocyanin Extracts

Table 5 shows changes in color values: lightness (L^*), redness (a^*), yellowness (b^*) and changes in color intensity (expressed as total color difference ΔE^* where low ΔE^* means more stabilization of color) for roselle anthocyanin extracts under study during storage at 10°C for 60 days. Data reveal that at the beginning of storage period, lightness values were 34.17, 32.03, 30.21 and 29.3 in each of the control extract and extracts with feruilc, cinnamic and coumaric acids, respectively this means that extracts with copigments were more dark than control, whereas redness values were 59.86, 58.52, 58.99 and 59.08 for the same extracts at the same time, respectively. Data illustrate that at zero time of storage each of the control roselle extract and extract with coumaric acid represented the highest values of redness (59.86 and 59.08) followed by extracts with cinnamic and ferulic acids. After 15 days of storage, extracts with coumaric and cinnamic acids possessed the highest values of redness (59.92 and 59.13) and they maintained the highest values of redness within storage. During storage, slight declines in redness and increment in lightness and yellowness were noticed for all studied extracts.

From the results in Table 5, it could be also noted that after 15 days of storage, changes in color intensity (ΔE^*) were 2.4, 1.3, 1.0 and 1.2 for each of the control extract and extracts with ferulic, cinnamic and coumaric acids, respectively, whereas extracts with copigments showed ΔE^* values less than control. Extracts with coumaric, cinnamic and ferulic acids showed lower values of ΔE^* compared with control extract within storage period which means more color retention of copigmented extracts. Results in Table 5 confirmed the effect of used copigments especially coumaric and cinnamic acids on color stability and elimination of color change in roselle anthocyanin extracts during storage as compared with the control extract.

The aforementioned data are in a good agreement with [40] who reported that a copigment alone is usually colorless, but when added to an anthocyanin solution, it greatly enhances the color of the solution. Meanwhile [42] found that there is an important color loss for cyanidin alone and significant color retention for the solution containing the anthocyanin and the copigment. Thus, the copigment effect is to reduce the production of the colorless carbinol pseudobase. Also data in present study in agreement with [43] who reported that copigmentation could be a valuable, natural tool for improving the color of anthocyanin rich food products, the color of which can be

stabilized and enhanced by the addition of different plant extracts rich in copigments. Moreover, [16] stated that copigment addition increased anthocyanin color stability in general during storage. Copigmentation is a phenomenon in which pigments and other colorless organic compounds, or metallic ions, form molecular or complex associations, resulting in an increase in the color intensity [44].

Table 5. Changes in color values and color intensity of control and copigmented roselle anthocyanin extracts during storage

		RE	RE+F	RE+CIN	RE+COUM
Zero time	L^*	34.17	32.03	30.21	29.3
	a^*	59.86	58.52	58.99	59.08
	b^*	57.52	49.52	51.99	54.37
	ΔE*	---	---	---	---
After 15 days	L^*	35.19	33.2	30.60	29. 9
	a^*	58.13	58.13	59.92	59.13
	b^*	58.9	50.16	51.88	53.30
	ΔE*	**2.4**	**1.3**	**1.0**	**1.2**
After 30 days	L^*	37.33	35.13	31.9	31.44
	a^*	55.7	57.16	58.13	58.17
	b^*	60.7	52.17	52.90	53.60
	ΔE*	**3.7**	**2.9**	**2.4**	**1.8**
After 45 days	L^*	40.14	36.01	33.7	31.11
	a^*	52.0	54.9	56.17	57.16
	b^*	60.2	53.6	54.1	54.7
	ΔE*	**4.6**	**2.8**	**2.3**	**1.5**
After 60 days	L^*	42.66	36.6	3 3.9	33.13
	a^*	49.5	53.3	54.6	55.96
	b^*	62. 9	56.3	56.2	54.9
	ΔE*	**4.4**	**3.1**	**2.6**	**2.3**

RE: Control roselle anthocyanin extract
RE+F: Roselle anthocyanin extract+ ferulic acid
RE+CIN: Roselle anthocyanin extract+ cinnamic acid
RE+COUM: Roselle anthocyanin extract+ coumaric acid.

4. Conclusion

The roselle (*Hibiscus sabdariffa*) had a relatively high content of anthocyanin pigments which are responsible for the high antioxidant activity. Addition of coumaric, cinnamic and ferulic acids as copigments to roselle anthocyanin extracts resulted in significant anthocyanin content and color stability during storage at 10°C for 60days. All copigmented roselle extracts had a noticeable antimicrobial activity against bacteria and molds. Copigmented roselle extracts could be a good and a promising source of water soluble red colorants that could be utilized as natural food colorants instead of harmful synthetic ones.

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