Biochemical and molecular characterization of three colored types of roselle (*Hibiscus sabdariffa* L.)

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ABSTRACT: Roselle (*Hibiscus sabdariffa* L.) is a plant which has a considerable industrial, pharmaceutical and economic values in Egypt and many other countries around the world, mainly for its pleasant sepals. There are many colored types of Roselle depends on sepals color. The biochemical and molecular characterization of three roselle types, green (G), light red (LR) and dark red (DR), were studied. RAPD-PCR patterns for their genomic-DNA were significantly different. The total protein electrophoretic profile of their seeds was similar except for some inter-individual variation in band density. Their total protein contents were 46.0, 66.5 and 68.1 mg/g seed, respectively. In addition to the water-soluble antioxidant capacity, the total polyphenolic-content and the antioxidant activity of 12 roselle extracts, three colored types in 2 solvent systems (aqueous, A and 30% ethanolic, E) and 2 extraction temperatures (hot, H and cold, C), were determined by Folin-Ciocalteu reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods, respectively. The ability of these roselle extracts to inhibit the formation of nitrous acid-induced tyrosine nitration decreases in the order of LREC > DREC > LREH > GEC > DREH > GEH > GAC > DRAH > LRAC > GAH > LRAH > DRAC.
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KEYWORDS: Antioxidant activity; DPPH; HPLC; molecular characterization; polyphenol content; reactive nitrogen species (RNS); sepals, Roselle (*Hibiscus sabdariffa* L.).

1. Introduction

Roselle (*Hibiscus sabdariffa* L.) belongs to *Malvaceae* family and generally regarded as safe (GRAS) as foodstuff. It is commonly used to make jelly, jam, juice, wine, syrup, gelatin, pudding, cake, ice cream and flavors. The brilliant red color and unique flavor make it a valuable food product [(1)]. Many medicinal applications of this plant have been developed around the world. In China, it is used to treat hypertension, pyrexia, and liver damage [(2)]; and lately its sepal extract has been used as an effective treatment against leukemia, due to its high content in polyphenols, particularly protocatechic acid [(3)]. Roselle seeds, which until now do not have any commercial applications, are a source of a vegetable oil that is low-cholesterol and rich in other phytosterols and tocopherols, particularly *β*-sistosterol and *γ*-tocopherol. The global characteristics of roselle seed oil allow important industrial applications for this oil. These characteristics represent an added value for the culture of this plant [(4)].

The fleshy calyces of roselle flowers (sepals) have a pleasant acid taste and very attractive red color, for which roselle is used as a beverage crop in many countries. The beverage drink is known in Egypt as Karkadi and recommended as a mild laxative in the form of acidulous drink. The anthocyanins (**Figure 1**) are responsible for the red color, while the acid taste is due to the presence of some organic acids. Sepals' acidity may also contribute to their color variation. The dark red colored type has the highest content of anthocyanins followed by the light red colored type, while the green colored type has no or just traces of anthocyanins [(5)].

Many chemical components present in roselle have potential health benefits and support the ethnomedicinal use of roselle in promoting cardio-vascular health and preventing hypertension [(2)]. The red varieties of roselle had greater overall antioxidant and cyclooxygenase inhibitory activity than the white variety, and therefore they would potentially have greater health benefits. However, the white variety could also be used in antihypertensive applications, since its ethyl acetate extract had similar cyclooxygenase inhibitory activity as aspirin and Ibuprofen [(6)]. Nevertheless, further *in vivo* research is required to confirm the specific health benefits in biological systems, before increased consumption of any of the three varieties of roselle could be recommended.

There are few studies on roselle at the molecular level. In this study, we present a molecular characterization based on total protein and DNA level of three colored types of roselle [green (G), light red (LR) and dark red (DR)] (**Figure 2**). The variability of antioxidant capacity of these three colored types of roselle using different biochemical approaches was determined.

2. Materials and Methods

2.1. Plant Material

Seeds and sepals from three colored types of roselle were collected from previous experiments of our research group at the National Research Center, Egypt.

2.2. Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co., USA. All reagents and other chemicals were of high analytical grade. The buffers were prepared according to Gomorie [7] and Blanchard [8] and the final pH was checked by pH meter (Hanna, pH 211 Microprocessor pH meter). *Taq* polymerase and PCR reagents were from Fermentas, Latvia.

2.3. DNA Extraction and PCR Amplification Conditions

Genomic DNA of *H. sabdariffa* was extracted from fresh young leaves using the CTAB (cetyltrimethylamonium bromide) extraction method [9]. PCR was carried out using a BioCycler TC-S thermal cycler from HVD, Austria. The PCR reactions were developed in a total volume of 50 µl with the following components: 5 µl of 10X reaction buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$ and 0.001 % bovine serum albumin), 2 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM/each dNTP mix, 2.5 µl of 20 mM of each primer, 1 µl of *Taq* DNA polymerase (1 U/µl), and template DNA. The volume was completed to 50 µl with deionized, diethylpyrocarbonate (DEPC), water. The following PCR program was used: an initial denaturation of DNA was carried out at 94°C for 2 min followed by 40 cycles including denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 12 min. Template DNA was prepared from fresh young roselle leaves. The tubes containing PCR product were stored at -20°C till the next use. For RAPD analysis, the following random primers were used: B05 (5′-TGCGCCCTTC-3′), B08 (5′-GTCCACACGG-3′), B09 (5′-TGGGGGACTG-3′), B11 (5′-GTAGACCCGT-3′), B14 (5′-TCCGCTCTGG-3′) and C15 (5′-GACGGATCAG-3′).

2.4. Protein Determination

2.4.1. Protein Extraction

Protein extraction from *H. sabdariffa* seeds was carried out according to Rao and Pernollet [10] with minor modifications: 1 g of different types of seeds each was incubated overnight in 10 ml of 0.15 M phosphate-buffered saline (PBS) and 1 mM disodium EDTA, pH 7.2, containing cocktail protease inhibitors (1 mM ethylenediamine tetraacetic acid, ethylene glycol bis *N*, *N*, *N*, *N*-tetraacetic acid, *N*-ethylmaleimide and phenylmethylsulphonyl fluoride). The mixture was homogenized, filtered, sonicated and then centrifuged at 15,000 × *g* for 60 min. at 4°C. Supernatants were collected as roselle seed extracts.

2.4.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA). Preparation of gels, samples and electrophoresis was performed according to Laemmli [11].

2.4.3. Estimation of Protein Concentration

Protein concentration was determined according to Bradford [12], using bovine serum albumin as standard protein.

2.5. Determination of Total Polyphenol Content (TPC)

The content of total phenolic compounds in each of the 12 extracts was determined according to the Folin-Ciocalteu method [13] with some modification to minimize the volume of the reactants used to microlitres. An aliquot of 10 µl of each extract (1 mg/ml) was mixed with 50 µl of Folin–Ciocalteu phenol reagent (10 × dilutions) and allowed to react for 5 min. Then 40 µl of 20% saturated Na_2CO_3 solution was added and allowed to stand for 1 h in the dark before the absorbance of the reaction mixture was read at 725 nm using a microplate ELISA reader (BioRad). A gallic acid standard curve was obtained for the calculation of phenolic content. The total polyphenol content (TPC) of each extract was expressed as mg gallic acid equivalents per gram of plant material on a dry-weight basis.

2.6. Antioxidant Activity Determination

2.6.1. Determination of Water-Soluble Antioxidant Capacity (HACT)

Water-soluble antioxidants were extracted as follows: Plant sepals were frozen in liquid nitrogen and later, they were crushed in a hand mortar until obtaining a fine powder, to which a volume of distilled water was added (10 ml/3 g). The homogenized suspension was transferred to polypropylene tubes then shaken for 1 h at room temperature in the dark. The suspension was transferred to Eppendorf tubes and centrifuged at 6000 × *g* for 10 min; the supernatant was transferred to new Eppendorf tubes and filtered through a centricon (5 kDa cut-off) to eliminate proteins and other macromolecules from the water extract. Later, the resulting supernatant was kept at 4°C until analysis.

Samples of water extracts (5 to 200 µL) were supplemented with 1 ml phosphomolybdenum reagent, homogenized then incubated at 95°C for 90 min. Pure water was used in control reactions. Finally, absorbance at 695 nm was measured. HACT is expressed as equivalents of L-ascorbic acid. Standard curves were constructed with different amounts of L-ascorbic acid dissolved in water. An average extinction coefficient of 213 μM^{-1} (r^2 = 0.9996) was used for quantitation. Total HACT per gram of wet plant material was obtained with the following formula:

HACT (μmol L−ascorbic acid/g) = $A_{695} \times \epsilon^{-1} RV \times SV^{-1} \times EV \times M^{-1}$

where A_{695} is the absorbance at 695 nm, ϵ^{-1} is the inverse of the extinction coefficient (213 μM^{-1}), RV is the overall reaction volume, SV is the sample volume used in the reaction, EV is the volume of solvent used in the extraction of the plant material analyzed and M is the amount of fresh plant material extracted in grams. When the assays were performed at 37°C, the total antioxidant capacity was

obtained due to strong water-soluble antioxidants, HACT[37] [14]. All determinations were done in triplicate.

2.6.2. *Preparation of the Extracts and Standard*

A weighed quantities of three colored roselle sepals (G, LR and DR) were extracted with 30% aqueous ethanol (E) and distilled water (A) either at room temperature (C) or boiling temperature (H). This gave 12 samples to analyze: GEH, GEC, GAH and GAC; LREH, LREC, LRAH and LRAC; DREH, DREC, DRAH and DRAC (**Table 1**). A solution of ascorbic acid in water was used as a standard in this study. Serial dilutions of all these solutions were done with their respective solvents.

2.6.3. *DPPH Radical Scavenging Assay*

The antioxidant activity of the 12 roselle extracts and standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radical [15]. 10 µl of each extract or standard (from 0.0 to 100 µg/ml) was added to 90 µl of a 100 µM methanolic solution of DPPH in a 96-well microtitre plate (Sigma-Aldrich Co., St. Louis, MO, US). After incubation in the dark at 37°C for 30 min, the decrease in absorbance of each solution was measured at 520 nm using an ELISA micro plate reader (Model 550, Bio-Rad Laboratories Inc., California, USA). Absorbance of blank sample containing the same amount of either water or 30% aqueous ethanol and DPPH solution was also prepared and measured. All experiments were carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. Radical scavenging activity was calculated by the following formula:

% Reduction of absorbance = $[(A_B - A_A) / A_B] \times 100$, where: A_B – absorbance of blank sample and A_A – absorbance of tested extract solution (t = 30 min) [16].

2.6.4. *Reaction of Roselle Extracts with Reactive Nitrogen Species*

The abilities of different roselle extracts to inhibit nitrous acid-mediated tyrosine nitration were investigated. Equimolar concentrations (400 µM) of tyrosine and $NaNO_2$ were co-incubated with each extract (0 to 300 µg) in 0.5 M HCl at 37°C for 4 h [17]. Snap freezing reaction mixtures prior to HPLC analysis successfully terminated reactions. We monitored the formation of 3-nitrotyrosine (3-NT) from nitrous acid-mediated tyrosine nitration by HPLC analysis with photodiode array detection (see below). 3-NT formed was characterized and quantified by use of an authentic standard (retention time and unique spectral characteristics). Trolox was used as positive control. Artefactual formation of 3-NT during the preparation stage was found to be negligible.

2.6.5. *HPLC Analysis*

Reaction mixtures were analyzed using reverse-phase HPLC. Analysis was performed on an Agilent 1100 system with a Zorbax ODS C18 column (150 × 4.6 mm i.d., 4 µm) and guard column (15 × 4.6 mm i.d., 4 µm). Mobile phase A consisted of methanol/water/5 N HCl (5/94.9/0.1, v/v/v) and mobile phase B of acetonitrile/water/5 N HCl (50/49.9/0.1, v/v/v). The following gradient system was used (min/% acetonitrile): 0/0, 5/0, 40/50, 60/100, 65/100, and 65.1/0 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm for 3-NT measurements with spectra of products obtained over the 220–600 nm range.

2.7. *Statistical Analysis*

All experiments were conducted in triplicate (n=3). All the values were represented as mean ± SD. Significant differences between the means of parameters as well as IC_{50} were determined by probit analysis using SPSS software program (SPSS Inc., Chicago, IL); with p values < 0.05 considered statistically significant.

Correlation coefficients (R) to determine the relationship between two variables (radical scavenging tests and content of total phenolic compounds) were calculated using MS Excel software (CORREL statistical function).

3. Results and Discusion

3.1. *RAPD Analysis of Roselle Types*

PCR was used for genotypes analysis of roselle (**Figure 3**). The PCR products, which amplified using the random primer B05, were around 1 kb for genotypes green (G) and light red (LR) (**Figure 3A**) and dark red (DR) (**Figure 3B**). The primer B05 showed more specific and distinct bands in case of LR and DR genotypes. LR genotype showed a specific band with molecular size around 700 bp while the DR genotype showed a band around 1.5 kb and two faint amplification products at 800 and 700 bp, respectively. The other random primers B08, B09, B11, B14 and C15 used in this study, to differentiate between roselle genotypes, did not show any visible amplification products.

3.2. *Determination of Protein Content of Roselle Seed Extracts*

The protein content of three colored roselle seeds revealed that the DR-colored type has the highest protein content (68.1 mg/g) followed by the LR-colored type (66.5 mg/g), while the G-colored type has the lowest protein content (46.0 mg/g).

3.3. *Characterization of Protein Content of Roselle Seed Extracts*

Protein electrophoretic profiles of roselle seed extracts were very similar, except for some quantitative inter-individual variations. The SDS-PAGE analysis showed approximately 14-15 major protein bands, with relative molecular masses ranging from 14 to 100 kDa (**Figure 4**).

3.4. *Total Polyphenol Content (TPC)*

As plant polyphenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, the total amount of phenolic compounds in the 12 roselle extracts was determined using the Folin-Ciocalteu method. The Folin–Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of phenolic compounds present in an extract. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the

reagent. Phenolic compounds respond differently to the Folin–Ciocalteu reagent, depending on the number of phenolic groups they have [18]. As shown in **Table 1**, all extracts contain high amount of polyphenolics, to which their antioxidant activity may be ascribed. DREC, GEC, DREH, GEH and DRAC extracts which showed the highest antioxidant activities also had the highest amount of polyphenols. GAC, DRAH, LREH, LREC and GAH extracts which showed moderate antioxidant activities, had moderate TPC as well. LRAC and LRAH extracts showed the lowest TPC and the lowest antioxidant activities. Further studies are in progress to isolate and elucidate the structure of main components present in each extract.

3.5. *Determination of Antioxidant Activity of Extracts*

3.5.1. *Water-Soluble Antioxidant Capacity*

Water-soluble antioxidant capacity was determined by the phosphomolybdenum method. HACT[37] and HACT represented strong water-soluble antioxidants and total water-soluble antioxidants, respectively [14]. It is known that roselle sepals are very rich in vitamin C, anthocyanins, polyphenols and other water-soluble antioxidants [19]. In Egypt, however, roselle farmers discard the roselle G-colored type plants, because roselle consumers prefer and demand the DR-colored type sepals [20, 21]. As shown in **Figure 5**, sepals of DR type showed the highest levels of total water-soluble antioxidant capacity followed by G type then LR type. These results are consistent with recent study on roselle sepals' water-soluble antioxidant capacity [4].

On the contrary, G type showed the highest levels of strong water-soluble antioxidant capacity, followed by LR then DR types. These results are in agreement with the study of Rady et al. [22]. A high level of strong water-soluble antioxidant capacity in sepals of G type suggests that, there are abundant levels of vitamin C, and other strong water-soluble antioxidants, this fact would support the recommendation of consuming both DR- and G-colored types of roselle sepals.

3.5.2. *DPPH Radical Scavenging Activity*

The stable DPPH radical has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 520 nm. Radical scavenging activities of the 12 roselle extracts were measured using model colorimetric test, DPPH radical scavenging test. The results are summarized in **Table 2**. GEH, DREH, DREC, GEC and DRAC showed the highest DPPH free radical scavenging activities. GAH, LREH, LREC, DRAH and GAC also showed relatively high DPPH free radical scavenging activities. Both LRAH and LRAC showed the lowest DPPH free radical activities. These results are in agreement with both HACT[37] and HACT results.

3.5.3. *Reaction of Different Roselle Extracts with Reactive Nitrogen Species (RNS) Derived from Nitrous Acid*

Acidification of nitrite is known to generate a number of RNS, including N_2O_3, which is capable of nitrosating secondary amines and amides. The gastric formation of N-nitrosocompounds has been suggested to be a major source of human exposure to this class of environmental carcinogens [23]. It has been established that compounds such as α-tocopherol and ascorbic acid react with nitrite, and hence are able to inhibit endogenous nitrosation [24]. More recently, the ability of certain polyphenols to inhibit tyrosine nitration mediated by nitrite in acid has been demonstrated in vitro [25]. This prompted our interest in studying the potential of 12 different roselle extracts as potential scavengers of RNS derived from nitrous acid. In order to monitor the potential of different extracts to react with RNS derived from nitrous acid, a model system was employed. In this study the ability of 12 different roselle extracts, to inhibit the formation of nitrous acid-induced tyrosine nitration, was investigated, for the first time, under conditions akin to those in the gastric milieu of humans. Exposure of tyrosine to nitrous acid in the presence of different extracts resulted in a significant inhibition of 3-NT formation in a dose-dependent manner (**Figure 6**). The IC_{50}'s for the inhibition of 3-NT formation by different extracts are shown in **Table 2**. LREC, DREC, LREH and GEC extracts are very effective at a relatively low concentration. DREH, GEH, GAC and DRAH extracts showed moderate inhibition. However, LRAC, GAH, LRAH and DRAC extracts showed relatively low inhibition activities.

3.6. *Statistical Analysis*

With reference to Table 1, the correlations of TPC against the antioxidant activity based on the DPPH assay involving all 12 extracts were very high reflected by the high correlation coefficient (0.86), confirming that phenolic compounds contribute to the radical scavenging activity of these extracts. while, there were no correlations of TPC against IC50 of 3NT-inhibition and DPPH against IC50 of 3NT-inhibition reflected by their negative correlation coefficients (– 0.49 and – 0.42, respectively).

In Summery, the molecular studies on three colored types of roselle (*Hibiscus sabdariffa*) presented in this article suggested that the total protein electrophoretic profiles of these three types of roselle seeds were similar, while, their RAPD-PCR patterns were significantly different. The biochemical studies on these three roselle colored types revealed that, all three types could be used as a considerable source of strong antioxidants. The sepal extracts of these three types of roselle showed important abilities to react with the biologically relevant reactive nitrogen species (RNS) by inhibiting nitrous acid-mediated tyrosine nitration, using different solvents and extraction temperatures. The characteristics of the green sepals roselle type revealed that, it could not be less important than the other roselle colored types in the industrial and pharmaceutical applications. These characteristics support the ethnomedicinal use of roselle in Africa and the Caribbean and suggest that all the three types of roselle could potentially provide health benifits. In addition, our findings present helpful information to food markets and small farmers and may help increasing the roselle

marketability and profitability. Further studies are required to evaluate the contribution of these three types of roselle to the specific health benefits in biological systems, before increased consumption of any of these three colored types could be recommended.

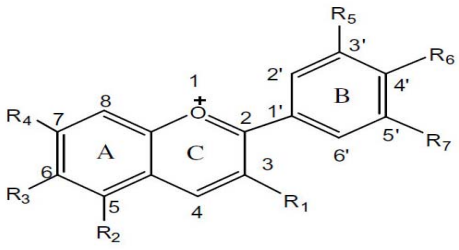
Table 1. Full description of the abbreviations used in the text.

Abbreviation	Description
DREH	Dark red roselle sepals (DR) extracted with 30% aqueous ethanol (E) at boiling temperature (H).
DREC	Dark red roselle sepals (DR) extracted with 30% aqueous ethanol (E) at room temperature (C).
DRAH	Dark red roselle sepals (DR) extracted with distilled water (A) at boiling temperature (H).
DRAC	Dark red roselle sepals (DR) extracted with distilled water (A) at room temperature (C).
LREH	Light red roselle sepals (LR) extracted with 30% aqueous ethanol (E) at boiling temperature (H).
LREC	Light red roselle sepals (LR) extracted with 30% aqueous ethanol (E) at room temperature (C).
LRAH	Light red roselle sepals (LR) extracted with distilled water (A) at boiling temperature (H).
LRAC	Light red roselle sepals (LR) extracted with distilled water (A) at room temperature (C).
GEH	Green roselle sepals (G) extracted with 30% aqueous ethanol (E) at boiling temperature (H).
GEC	Green roselle sepals (G) extracted with 30% aqueous ethanol (E) at room temperature (C).
GAH	Green roselle sepals (G) extracted with distilled water (A) at boiling temperature (H).
GAC	Green roselle sepals (G) extracted with distilled water (A) at room temperature (C).
HACT	Hidrosoluble Antioxidant Capacity Total

Table 2. Antioxidant activity, total polyphenol content and IC50 of roselle sepal extracts. Antioxidant activity was based on the ability to scavenge DPPH free radicals. Total phenolic content (TPC) was determined by the Folin-Ciocalteu method and IC50 was determined by the inhibition of nitrous acid-mediated 3-nitrotyrosine formation.

IC50 (µM) Means ± SD (n = 3)	TPC mg/g plant extract Means ± SD (n = 3)	Decease of DPPH absorbance Means % ± SD (n = 3)	Extract name
84.801 ± 1.796*	29.363 ± 0.597	64.678 ± 4.546	DREH
33.864 ± 0.986*	32.691 ± 1.873	63.761 ± 3.032	DREC
139.583 ± 2.553*	21.763 ± 1.509	48.165 ± 7.392	DRAH
191.065 ± 1.926*	26.867 ± 2.316	61.926 ± 3.301	DRAC
38.655 ± 1.224*	20.683 ± 1.438	48.969 ± 2.547	LREH
29.011 ± 0.967*	21.377 ± 1.702	48.323 ± 4.893	LREC
180.451 ± 2.893*	17.354 ± 0.639	29.893 ± 3,179	LRAH
163.836 ± 1.859*	19.604 ± 1.692	29.012 ± 2,972	LRAC
86.412 ± 1.132*	28.201 ± 2.641	65.596 ± 4,013	GEH
60.645 ± 2.219*	31.033 ± 1.205	62.385 ± 4.065	GEC
174.586 ± 5.262*	20.035 ± 2.724	53.758 ± 3.891	GAH
107.173 ± 5.663*	22.042 ± 1.481	48.073 ± 8.078	GAC
--	--	83.027 ± 1.079	ascorbic acid
--	--	81.651 ± 2.098	rutin
15.260 ± 1.280*	--	--	trolox

*** = P < 0.001**



R_1 = O-Sugare (glucose, arabinose, galactose)
R_2, R_4, R_6 = OH
R_3 = H and R_5, R_7 = H, OH, OCH_3
Figure 1. Structure of anthocyanins

Figure 2. Sepals of three colored types of roselle (*H. sabdariffa*). Green (G), light red (LR) and dark red (DR).

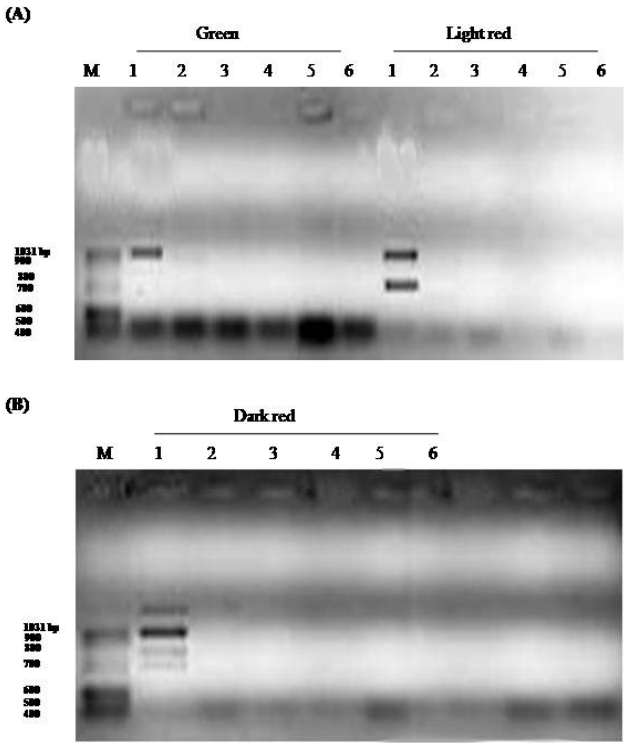


Figure 3. RAPD-PCR amplification products of three colored types of roselle genomic DNA. (A) G and LR genotypes and (B) DR genotype. 1 to 6 refers to the random primers (B05, B08, B09, B11, B14 and C15, respectively). M is the 100 bp DNA ladder.

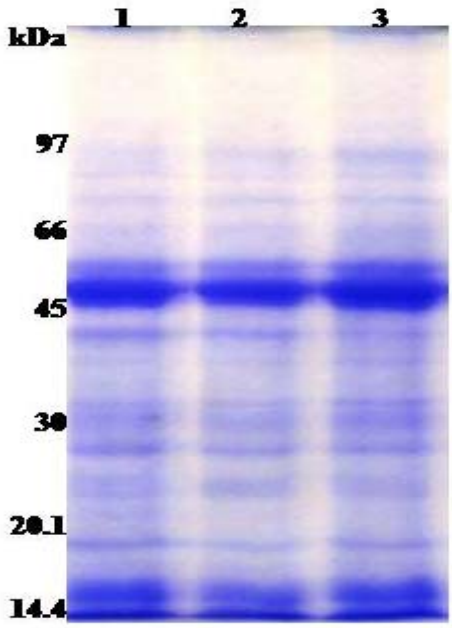


Figure 4. SDS-PAGE analysis of different protein extracts from roselle seeds. Gel was stained with Coomassie Brilliant blue R-250. The different lanes correspond with the following samples: G (1); LR (2) and DR seeds (3).

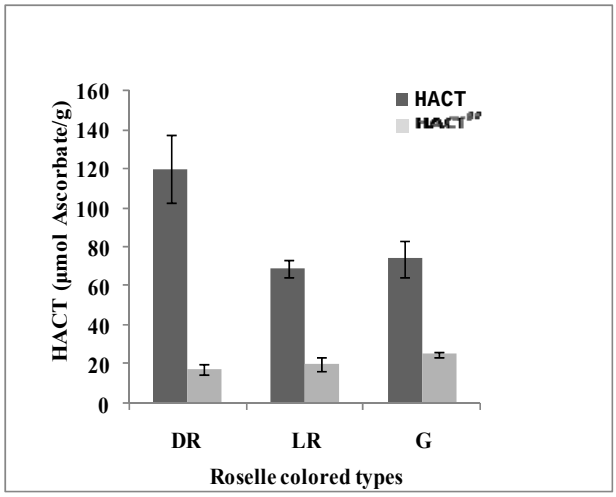


Figure 5. Water-soluble antioxidant capacity. Samples of three colored types of roselle plant sepals were extracted with water, and aqueous extracts were analyzed for their water-soluble antioxidant capacity (HACT and HACT[37]) by the phosphomolybdenum method. Data are the average of 3 independent determinations. Standard deviation was always below 10%.

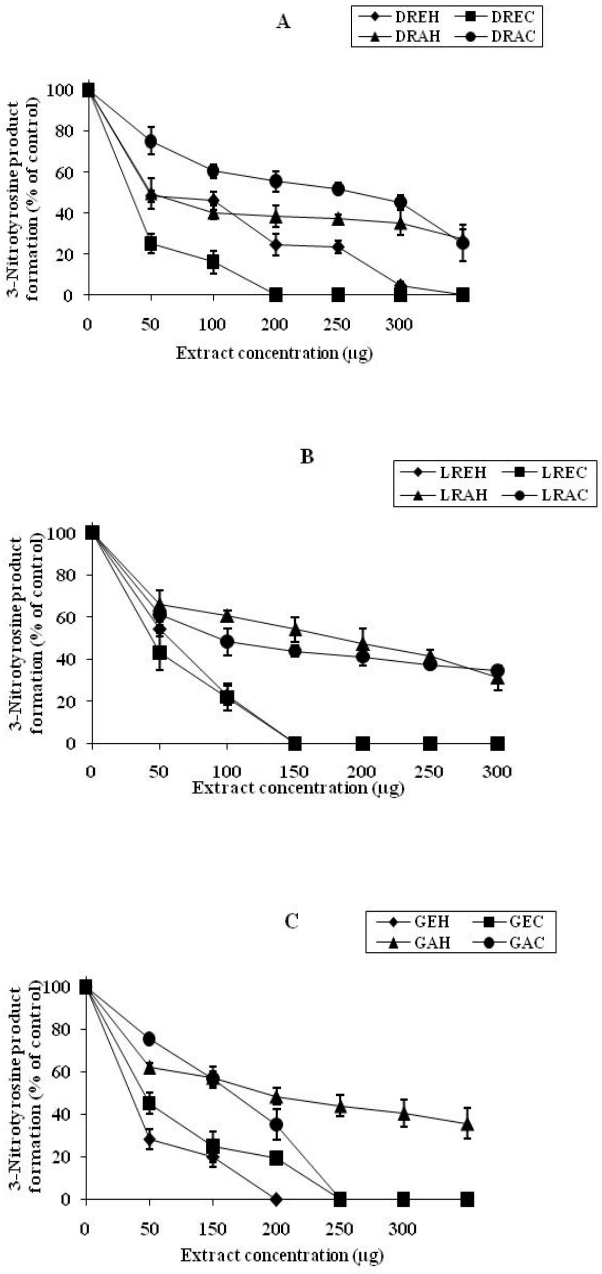


Figure 6. Inhibition of nitrous acid-induced formation of tyrosine nitration by 12 roselle extracts. Each roselle extract (0 to 300 µg) was exposed to 400 µM nitrite in 0.5 N HCl for 4 h at 37 °C in the presence of tyrosine (400 µM) to mimic the environment of the stomach. Results are expressed as percentage inhibition of 3-NT formation and were obtained from three independent experiments performed in triplicate and presented as means ± SD.

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