[HSM03] Antioxidative activity of the crude extract and anthocyanins isolated from *Hibiscus sabdariffa,* L. (roselle)

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Introduction

Reactive oxygen species (ROS) are produced *in vivo* during the normal body metabolism. In the excess of ROS, it causes oxidative damage *in vivo* to components such as lipids, proteins, and DNA. This damage is believed to be strongly associated with carcinogenesis, ageing (Ames & Shigenaga, 1992), atherosclerosis (Vendemiale *et al*., 1999), diabetes (Vendemiale *et al*., 1999) and neurodegeneration diseases (Beckman & Ames, 1998). In addition, antioxidants are used in food to minimize free radical damage to lipids and other substances.

There is increasing interest in the protective biochemical function of natural antioxidants contained in dietary plants, which are candidates for the prevention of oxidative damage caused by oxygen free radical species. (Tsuda *et al*., 1996).There is also a growing interest in the use of 'natural' antioxidants for food preservation. *Hibiscus sabdariffa* L., or commonly known as roselle have been reported to have high contents of vitamin C, β-carotene, anthocyanins and other phenolic compounds, thus making it a potential source of natural antioxidant. Roselle is one of the most well-known sources that contain anthocyanins. There are report on the effect of anthocyanins on tumor cells, anti-inflammatory activity, anticonvulsant activity and antioxidant activity (Gracia *et al*., 1997). The pigments may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems (Tsuda *et al*., 1996).

In the present study, the anthocyanins content of the calyces of roselle were analyzed through chromatographic separation in HPLC. The antioxidative activity of the crude extract and the anthocyanins isolated from roselle were determined by different systems; the structural-activity of the anthocyanins is also discussed.

Materials and Methods

Materials

Dried calyces of *Hibiscus sabdariffa* L. (roselle) were obtained from a commercial plantation in Terengganu, Malaysia. All solvents used (Analytical and HPLC grades) were obtained from Fisher Scientific, (USA). All other materials were of reagent grade or the highest available grade from Sigma Chemicals (USA), Merck (Germany) or from BDH (UK).

High-performance liquid chromatography (HPLC) separation of anthocyanins

1g of dried roselle calyces was extracted with 10ml of 1% HCl in methanol overnight at 0°C. The slurry was filtered through filter paper. Analytical HPLC was carried out according to the method described as follows: Column: Genesis C18, 4μ (25cm X 4.6mm). Solvent: (A) 4% phosphoric acid; (B) 100% acetonitrile. Program: Linear gradient of 10% to 100% acetonitrile in 30 min; flow rate 1.2 ml/min; room temperature operation; primary detection at 520nm; injection volume 15μL.

Extraction and isolation

The dried calyces of roselle were extracted with 0.01% HCl in methanol solution for 3 days at room temperature. The extract was concentrated at 40°C using a rotary evaporator. The concentrated extract was introduced onto the 1mm thick TLC plates (20X20) cm as a continuous streak using a capillary tube and was developed ascendingly in the upper phase of butanol:acetic acid:water (4:1:5,BAW). Each pigmented band was scraped separately from the TLC plates. The cellulose residues which contained the separated compounds were soaked in methanol: acetic acid: water (90:5:5, MAW) overnight at 4°C, filtered and left to evaporate. The above procedure was repeated many times to accumulate the quantity for further purification. Each pigment was then further

purified with acetic acid: water (15:85, 15%HAc), BAW, 15%HAc in that order.

The Rf values (distance run by sample/ distance of solvent front) of the isolated pigments were determined by spotting the pigments on 0.25 mm cellulose layers (Merck, Germany) in a ascending direction in the 5 common solvent systems and the values were compared with some of the available known pigments (Du&Francis, 1973;Harborne,1967). The absorption curves of the isolated pigments were recorded in MeOH containing 0.01% HCl with a Shimadzu Model UV-160A Spectrophotometer. The wavelength from 200-600nm were scanned. The isolated pigments were hydrolyzed by heating approximately 2ml pigment solution with 2ml of 2N HCl in a water bath at 100°C for 40 minutes. The anthocyanidins was extracted with amyl alcohol and were then identified by comparison of Rf values with standard aglycones in BAW, formic acid: HCl: water (5:2:3,Formic) and acetic acid: HCl: water (30:3:10,Forestal). The standards used were red rose extract for cyanidin and extract from egg plant skin for delphinidin. As for the aqueous fraction, analytical TLC was carried out to compare Rf values with those of authentic sugars.

Antioxidative assays

Antioxidative activity in a linoleic acid system

The antioxidative activity of the samples (4mg) in the linoleic acid oxidation system was measured as described by Osawa & Namiki (1981). The degree of oxidation was measured by the TBA method and monitored daily for seven days. All test data are the averages of triplicate analyses.

Scavenging Effect on 1,1-Diphenyl-2-Pircrylhydrazyl (DPPH) Radical

The effect of the samples (0.025-0.1mg/ml methanol) were studied on the free radical, 1,1-Diphenyl-2-Pircrylhydrazyl (DPPH) according to the method of Hatano *et al*., (1988). The absorbance of the resulting solution was measured spectrophotometrically at 517nm.

Reducing Activity

The reducing activity of the samples (0.2-1.0mg) was determined according to the method of Yen *et al*., (1995). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing action of the extracts tested.

Deoxyribose assay to assess reaction with hydroxyl radical (OH•)

2-Deoxyribose is oxidized by OH• that is formed by the Fenton reaction and degraded to malondialdehyde. The assay was conducted as described in Takara *et al*., (2002). The percentage inhibition was determined from the means of 3 independent experiments.

Statistical Analysis

The results were reported as means (standard deviations from three repeated determinations). Statistical differences were analyzed according to Student's t-test wherein the differences were considered to be statistically significant at $P < 0.05$.

Results and Discussion

Analysis and isolation of anthocyanins

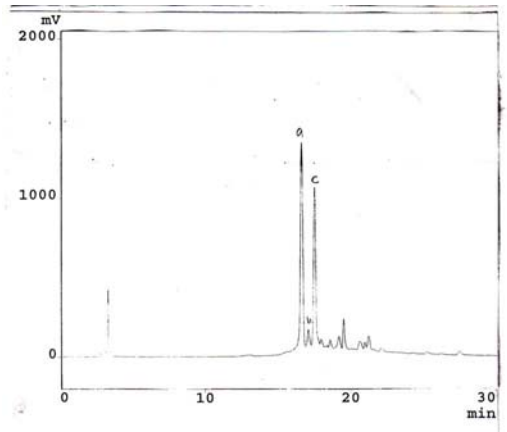


FIGURE 1 HPLC chromatogram of roselle anthocyanins detected at 520nm. Peak identification: a, delphinidin-3-sambubioside; b, delphinidin-3-glucoside; c, cyanidin-3-sambubioside.

The HPLC profile of the acidified methanol extract of the calyces of roselle detected at 520nm showed three major peaks (a,b&c) and several minor anthocyanins (Figure 1). The peaks were identified on the basis of UV-VIS spectral data and Rf values. TLC on cellulose precoated plate of the crude extract in BAW (6 hours), revealed 5 coloured bands, namely P, P0, P1, P2 and P3. Numbering of bands

starts from the fastest moving band upwards. It was noticed that the first 2 bands (P&P0) appeared as trace quantity and present at a very low concentration. These 2 bands faded after a while, and caused difficulties in the separation and purification procedure. Band P1, P2 and P3 were further purified with 15% HAc and BAW in order to obtain cleanly separated individual pigments. P1 was the second most abundant pigment and was pink in colour. P2 was the most abundant pigment, with an intense reddish-violet colour. P3 was a minor pigment and was purple in colour. The identities of these 3 pigments were based on the Rf, UV-visible spectral values and its products produced by acid hydrolysis. The Rf values of P1, P2 and P3 were compared with those reported by Du & Francis (1973) and Harborne, (1967) in 5 different solvent systems. The Rf values of P1 suggested that it is a cyanidin-3-sambubioside (C-3-S), P2 as delphinidin-3-sambubioside (D-3-S) and P3 as delphinidin-3-glucoside (D-3-G). This was further supported by the results of the UV-visible spectral properties (Figure 2) that were agreeable with those of Du & Francis (1973). Additionally, the hydrolysis products of the pigments further confirms the structures. The end products of hydrolysis for P1 (C-3-S) were identified as cyanidin, glucose and xylose. P2 (D-3-S) yielded delphinidin, glucose and xylose as the end products of acid hydrolysis. Acid hydrolysis of P3 (D-3-G) showed the presence of delphinidin and glucose as the end products. The chemical structure of the pigments isolated from roselle are shown in Figure 3.

Inhibition of lipid peroxidation

Lipid peroxidation is an *in vitro/ in vivo* chain reaction that produces highly reactive toxic secondary products to cell membranes and biological compounds. The crude methanolic *Hibiscus sabdariffa* extract (HSC) and the isolated anthocyanin pigments (D-3-S and C-3-S) from *Hibiscus sabdariffa* was tested in a linoleic acid oxidation system. The data of linoleic acid peroxidation, monitored daily for 5 days after 3 days of incubation at 40°C after the addition of HSC, C-3-S, D-3-S and BHA respectively are plotted in Figure 4. HSC extract was the most effective among all the samples tested and was more effective than the synthetic antioxidant, BHA. However, the percentage of inhibition between HSC and BHA were not significantly different ($P<0.05$). The antioxidant activity decreased in the order of HSC>BHA> D-3-S>C-3-S.

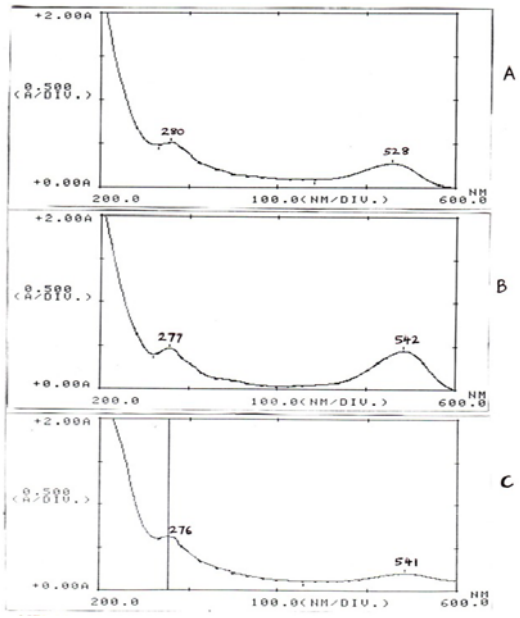
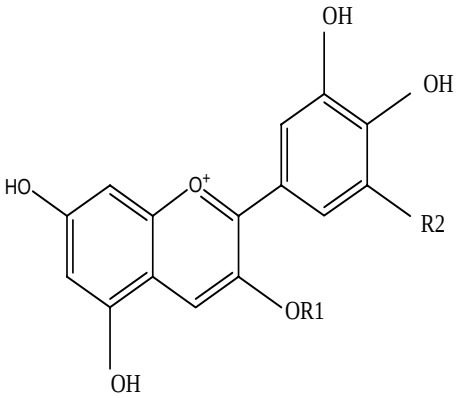


FIGURE 2 UV-visible spectra of the 3 pigments isolated from roselle. (A)cyanidin-3-sambubioside;(B)delphinidin-3-sambubioside; (C) delphinidin-3-glucoside



Delphinidin-3-sambubioside,R1=sambubiose, R2 = OH; Delphinidin-3-glucoside,R1= glucose, R2 = OH; Cyanidin-3-sambubioside,R1=sambubiose, R2=H

FIGURE 3 Anthocyanin pigments isolated from roselle extract

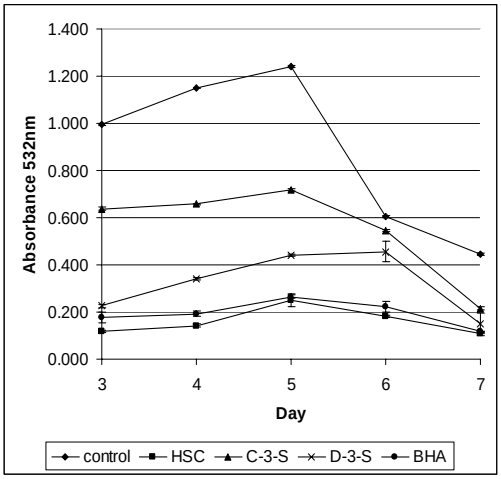


FIGURE 4 Antioxidative activity of HSC, C-3-S, D-3-S and BHA measured by the inhibition of linoleic acid peroxidation. Each value represents the mean of 3 separate experiments. HSC= *Hibiscus sabdariffa* crude; C-3-S= Cyanidin-3-sambubioside;D-3-S=Delphinidin-3-sambubioside; BHA= Butylated hydroxyanisole

Scavenging Effect on DPPH Radicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) has been used as a free radical to evaluate antioxidative activity of natural sources. Antioxidants are believed to intercept the free radical chain of oxidations, and to contribute hydrogen, thereby forming stable free radicals which do not initiate or propagate further oxidation of lipids. The results in this study showed that HSC, D-3-S and C-3-S were able to quench DPPH radicals (Table 1). The scavenging effects decreased in the order of HSC > D-3-S > C-3-S. At 0.1mg/ml, the three extracts showed a scavenging effect of 66.78, 52.45 and 37.93% for HSC, D-3-S and C-3-S respectively. Although the extracts showed noticeable effect on scavenging free radicals, the effect was much lesser than BHA which scavenged 78.27% of the DPPH radicals at a concentration of 0.025mg/ml. These results demonstrated that HSC, D-3-S and C-3-S have effective activities as hydrogen donors and as primary antioxidants by reacting with the lipid radical. This may be responsible for the cause of suppression of autoxidation in the linoleic acid system.

TABLE 1 Scavenging effect (DPPH) radical

	Concentration of extracts (mg/ml)	**% of DPPH bleaching**
Control	0	—
HSC extract	0.025	28.74±0.28[a]
	0.050	45.32±0.94
	0.075	58.97±0.10
	0.100	66.78±0.95
C-3-S	0.100	37.93±1.26
D-3-S	0.100	52.45±0.22
BHA	0.025	75.27±1.82

[a] Values are mean ± standard deviation of three separate experiments. HSC= *Hibiscus sabdariffa* crude; C-3-S=cyanidin-3-sambubioside; D-3-S=delphinidin-3-sambubioside; BHA=butylated hydroxyanisole.
% of DPPH bleaching = (Absorbance of control group- Absorbance of the sample added group) / Absorbance of control X 100%)

Reducing Power

Previous reports have shown that the reducing power of extracts from plants were significantly correlated to the extent of antioxidative activity (Duh &Yen, 1997). The reducing power of HSC and the individual anthocyanins isolated from *Hibiscus sabdariffa* are summarized in Figure 5.

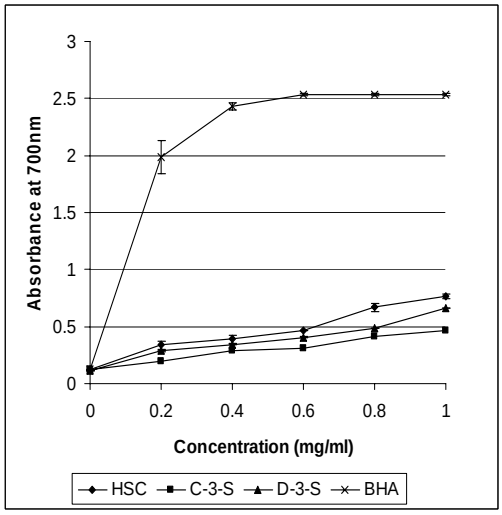


FIGURE 5 Reducing power of HSC, C-3-S, D-3-S and BHA extracts. Each value represents the mean of 3 separate experiments. Abbreviations as in TABLE 1.

The reducing power decreased in the order HSC>D-3-S >C-3-S. BHA was shown to have

a greater reducing power compared to HSC and the isolated pigments at all concentrations tested. HSC, D-3-S and C-3-S are electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions. Gordon (1990) reported that the antioxidative activity of reductones is believed to break radical chains by donating of a hydrogen atom.

Deoxyribose Assay (OH• Scavenging Activity)

It has been revealed that active oxygen species such as OH• are thought to be agents that cause oxidative damage. In this study, hydroxyl radicals (OH•) were generated by a mixture of Fe 3+ and H_2O_2 in the presence of slight molar excess of EDTA over the Fe3+ salt. Figure 6 shows the OH• scavenging activity of the extracts. All the three extracts can be said to be a chain-breaking inhibitor of the peroxidation process due to its ability to scavenge oxygen radicals. However, HSC was the most effective scavenger among the three extracts tested and the activity decreased in the order of HSC>D-3-S>C-3-S.

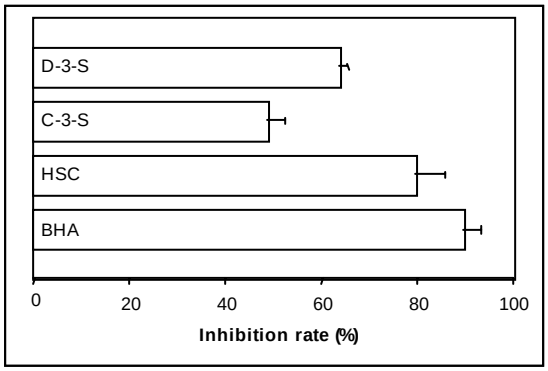


FIGURE 6 Hydroxyl radical-scavenging effects by the deoxyribose oxidation method. Each value represents the mean of 3 separate experiments Abbreviations as in TABLE 1.

Mechanism for the Antioxidant Effects of Anthocyanins from Hibiscus sabdariffa

Figure 4 shows the inhibitory effect of the anthocyanins, delphinidin-3-sambubioside (D-3-S) and cyanidin-3-sambubioside (C-3-S), on MDA formation in the linoleic acid oxidation system as measured by the TBA assay. D-3-S which has 3 hydroxyl groups in the B-ring showed higher inhibition activity than C-3-S which has two hydroxyl groups in the B-ring. The structural differences among D-3-S and C-3-S, lies only in the number of hydroxyl groups on the B-ring (Figure 3).Therefore the activity may be depended on the number of hydroxyl constituents of the B-ring.

Free radical-scavenging activities have been recognized as one of the mechanisms for the antioxidant effects of flavonoids in biological systems. Flavonoids are well known for the ability to scavenge peroxyl and alkoxyl radicals which are important intermediates in lipid peroxidation (Chimi *et al*., 1991).

Since anthocyanins comprise a class of flavonoids, similar mechanism may function in the antioxidant protection by anthocyanins in the inhibition on lipid peroxidation. The o-diphenol substitution in ring B of anthocynins and the conjugated double-bond system are related to their radical scavenging activity, due to hydrogen donation and subsequent radical stabilization (Gracia *et al*., 1997). The results of the DPPH radical and OH• scavenging effect showed that D-3-S was a greater inhibitor than C-3-S. Husain *et al*. (1987) indicated that flavonoids such as myricetin, quercetin and rhamnetin were scavengers of hydroxyl radical and that the scavenging effect increased with increasing number of hydroxyl groups substituted in the aromatic B-ring. This could explain the higher activity of D-3-S that has an additional hydroxyl group in the B-ring compared to C-3-S.

Hence, it is possible that the antioxidative property of the pigments, D-3-S and C-3-S isolated from *Hibiscus sabdariffa* could be due to free radical scavenging activity and these results suggest that the scavenging mechanism of the pigments is the same as that of the flavonoids. Further research is needed with more anthocyanins to clarify the antioxidant mechanisms of anthocyanins.

Acknowledgements

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