ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Free Radical Scavenging Properties and Antioxidant Activities of Some Anthocyanins Purified from Roselle (*Hibiscus sabdariffa* L.) Callus Using *In-Vitro* Tests.

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ABSTRACT

The present study evaluated the anti-oxidative activity of Hibiscus callus anthocyanins (HCA) by measuring their effects on lipid peroxidation inhibition capacity. HCA have been tested in vitro examining their TBARS and the antiradical activity was estimated by the ability of HCA to scavenge the DPPH•. The anti-oxidative activity of HCA was defined by TBARS assay (IC_{50}) in the $Fe(2^+)$ and $Cu(2^+)$-mediated oxidize lipids. Six anthocyanins were determined in Roselle callus. The most prominent anthocyanin was Cya-3sam followed by Dp-3sam, Mv-3gluc and Dp-3gluc. Cya-3gluc and Pt-3gluc were the lowest anthocyanins. HCA showed strong potential in inhibiting lipid peroxidation. A high correlation between the number of glycoside, the number of hydroxyl and, radical scavenging and antioxidant activities of HCA was found. Thus, HCA classified as antioxidants are able to inhibit lipid peroxidation by scavenging free radicals: Dp-3sam > Cya-3sam > Dp-3gluc > Cya-3gluc. It can be concluded that HCA could be a potential source of antioxidant compounds.
Keywords: anthocyanin, *Hibiscus sabdariffa* L, antioxidant, callus, radical scavenger.

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ISSN: 0975-8585

INTRODUCTION

Chemical or biological attack may cause the peroxidation of cell membrane lipids and the release of toxic substances, such as free radicals [1; 2]. Studies concerning the relation between the morbidity due to cancer and heart diseases, and the consumption of fruits and vegetables indicated that polyphenols in large amount in fruits and vegetables have a significant impact to reduce the effects of these diseases [3; 4]. Vegetables, including Roselle, are the most important sources of phenolic compounds in our diets. Especially phenol acids, anthocyanins, flavonols, flavanols, benzoic and cinnanmic acid derivatives are frequently present [5; 6]. Many of these compounds exhibit a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory, cardioprotective, hepatoprotective and vasodilatory actions [7; 8]. Anthocyanin extracts of Roselle inhibit human low-density lipoprotein (LDL) and liposome oxidation [9-12]. Roselle has also shown a remarkably high scavenging activity toward chemically generated active oxygen species [6; 13]. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers [14; 15]. The antioxidant properties of Roselle are documented in the literature and Roselle flowers are ranked to have one of the highest antioxidant activities among many other vegetables [12; 16; 17]. Roselle anthocyanins have been shown to have free radical-scavenging properties against superoxide radical ($O_2^{-)}$, hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$), and they can also inhibit lipid peroxidation, as well as protein and lipid oxidation in liposomes [18]. Other phytochemicals found in Roselle (flavonols, flavanols, benzoic and cinnanmic acid derivatives) have attracted a great deal of attention because of their biological activity [13; 15; 19-21]. Anthocyanins are representative of plant pigments widely distributed in colored fruits and flowers. They are the more important plant pigments visible to the human eye. The differences between individual anthocyanin relate to the number of hydroxyl groups, the nature and number of sugar attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule. The most significant function of anthocyanin is their ability to impact color to the plants or plant products in which they occur. They play a definite role in the attraction of animals for pollinisation and seed dispersal, and hence they are of considerable value in the co-evolution of these plant-animal interactions [22]. Because anthocyanins are widely consumed, finding out additional biological activities related to these compounds would be of great interest [23].

Roselle (*Hibiscus sabdariffa*) is an edible plant used in various applications including foods production. The most used parts are the fleshy red calyces used for making wine, juice, jam, syrup, pudding, cakes, ice cream or herbal tea. Roselle flowers and calyces are also known for their antiseptic, diuretic, antioxidant and antimutagenic properties [24]. Roselle is an important source of vitamins, minerals and bioactive compounds such as organic acids, phytosterols and polyphenols, and some of them, with antioxidant properties. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside and cyanidin-3-sambubioside [5; 6]. Roselle calyx extract is a good source of anthocyanins [25]. But, they have some restrictions related to their seasonality, large plantation requirements, extended period of time to produce the source, storage conditions of flowers and the excessive use of flowers as they fruiting conditions i.e. the problem of the existence of the species, etc. Thus, culture of Roselle cells lines derived from calli seems to be an important alternative for producing anthocyanins [26; 27]. Additionally, there has been an increasing interest in exploring new antioxidants of natural origins because of the potential toxicity of synthetic antioxidants and consumers' preference [11; 16]. Some fractions of Roselle anthocyanins could present significant potential benefits for human health [16; 28].

The aim of the present study was to evaluate the free radical scavenging properties and antioxidant activities of anthocyanins from callus. In this work, anthocyanin high producing callus line was collected and an HPLC method for anthocyanins separation was established. Then, a rapid colorimetric method for the measurement of free radical scavenging capacity was applied and a lipid peroxidation was used to evaluate the ability of anthocyanins to inhibit oxidation (antioxidant effect) in order to prevent cell-walls damage.

MATERIALS AND METHODS

Plant material

The callus lines of Roselle, highly rich in anthocyanins (red callus), were used as plant material. They were obtained according to the method described by **Abeda *et al*.** [26].

Methods

Extraction of anthocyanins and sample preparation

Approximately 50 mg of freeze-dried callus was mixed in 5 mL of methanol acidified with trifluoroacetic acid 0.1% (v/v) and anthocyanins were extracted overnight at 4°C with a blender. The supernatant was concentrated by evaporation of solvent using the SpeedVac Automatic evaporation system (Savant System, Holbrook, NY). Freeze-dried extract was then dissolved in a water/methanol mixture and filtered through a Millipore membrane with 0.45 µm porosity. The filtrate was twice diluted with purified distilled water.

Anthocyanin determination by HPLC

The high performance liquid chromatography (HPLC) analysis was conducted using the method described by Drust and Wrolstad [29]. Callus were harvested and freeze-dried. 10 mg of dry biomass were extracted by 5 mL methanol during overnight at 4 °C with a blender. Samples were centrifuged at 3000 rpm for 10 min, supernatant was collected and filtered through a Millipore membrane (0.45 µm). The filtrate was twice diluted with purified distilled water. The analyses were performed on a HPLC (Agilent), model-LC 1100 series, equipped with a degasser, an autosampler automatic injector, a high pressure pump and a UV/Visible detector at multiple wavelengths wave, and running on Windows XP Workstation. HPLC experiments were conducted using a reversed-phase C18 column (Prontosil, 250 x 4.0 mm, 5 µm, Bischoff). The mobile phase used was a binary gradient eluent (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile). Acetonitrile used was of HPLC grade (Sigma/Aldrich) and was degassed in an ultrasonic bath before using. The water was distilled using a Milli-Q system (Millipore). The elution program was 5-20 %B (0-5 min), 20-35 %B (5-10 min), 35-100 %B (10-25 min) and 100 %B (25-40 min) with a flow rate of 0.8 mL/min. The chromatograms were monitored at 521 nm. The anthocyanins identification and peak assignments are based on their retention times, UV-VIS spectra comparing with standards and published data. The anthocyanin quantification was performed using cyanidin 3-O-galactoside.

Radical scavenging activity

The free radical scavenging activity of the anthocyanin was analyzed by using the 1,1-dipheny-2-picrylhydrazyl (DPPH) assay [6; 30]. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 515 nm. In this method, a 0.1 mM solution of DPPH in methanol is prepared (6 mg DPPH/100 mL methanol), and 2.0 mL of this solution are added to 0.5 mL of the anthocyanin solution in methanol (1.0 mg/mL). The mixture was left to stand at room temperature for 30 min in the dark before absorbance measurement at 515 nm to assess the stability of the coloured reactive action. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the anthocyanin. The antioxidant activity of the anthocyanin was estimated by the ability to scavenging the DPPH radical. Three analytical replicates were carried out on each isolated anthocyanin. Ascorbic acid and trolox were used as standard references and dissolved in methanol to bring the stock solution to the same concentration (100 µM). Control sample was prepared, which contained the same volume without any extract and methanol was used as the blank. The scavenging or inhibition percentage was calculated according to the following equation:

Scavenging (%) = [($OD_{control}$ - OD_{sample}) / $OD_{control}$] x 100, where OD is optical density.

The actual decrease in absorption induced by the tested compounds was compared with the positive control. Measurement was performed at least in triplicate. Inhibition of coloration was expressed as a percentage, and the effective concentration 50 % (EC_{50}) was obtained from the inhibition curve.

Determination of the lipid peroxidation inhibition capacity

The determination of antioxidant activity of each anthocyanin extracted from Roselle callus was done using a microtechnique based on non-enzymatic peroxidation of microsomes and human low density lipoprotein (LDL) obtained from the plasma of healthy donors (blood comes from blood transfusion center).

These tests measure by spectrophotometry the thiobarbituric acid reactive substances (TBARS) concentration produced during the lipid peroxidation. Indeed, in acid and hot medium, substances derived from the lipid peroxidation, such as malondialdehyde (MDA), react with thiobarbituric acid (TBA) to form a pink colored complex and absorbing at 532 nm. The antioxidant activity is equivalent to the lipid peroxidation inhibition capacity. Ascorbic acid and trolox, the well-known antioxidants, were used as references. The relative antioxidant activity (AA) or Inhibition of lipid peroxidation by anthocyanin was calculated using the following equation:

$AA\ (\%) = 100 - [(OD_{sample} - OD_{negative\ control}) / OD_{positive\ control} - OD_{negative\ control}] \times 100,$ where OD is optical density.

Thus concentration of anthocyanin able to inhibit completely the lipid peroxidation will have a 100 % antioxidant capacity. Then, data were reported to the quantity of dry matter of each sample. Inhibition of lipid peroxidation was expressed as a percentage, and EC_{50} was obtained from the inhibition curve.

Test on microsomes

Rat liver microsomes were prepared by homogenization with five volume of ice-cold KCl 0.15. Microsomal fractions were isolated in Tris-HCl 0.05M/KCl 0.15 M (pH 7.4), by removal of the nuclear fraction at 5000 rpm for 15 min, removal of the mitochondrial fraction at 10 000 rpm for 15 min and sedimentation at 20 000 rpm for 30 min. Fractions were washed twice in buffer by centrifugation, with subsequent sedimentation at 20 000 rpm for 15 min and pellets were constituted microsomal fractions. Two (2) mL of trichloroacetic acid (TCA) 20% was added to 0.5 mL of microsomal fractions in order to precipitate the serum proteins. After centrifugation at 5000 rpm for 10 min, 3.0 mL of Tris-HCl 0.05 M/KCl 0.20 M (pH 7.4v) were added to pellets. The mixture was pre-incubated water bath for 1 h with each isolated anthocyanin.

The inhibitory effect on lipid peroxidation was assessed by measuring the TBARS, mainly constituted by malonaledialdehyde (MDA). For tests, microsome fractions were diluted with Tris-HCl 0.05 M (pH 7.4) containing KCl 0.20 M. The mixture was then pre-incubated in a shaking water-bath at 37 °C for 10 min with different concentrations of each anthocyanin isolated. Lipid peroxidation was initiated with 10 µM $FeCl_2$ (200 µM). After incubating the samples for 30 min, 1 mL of trichloroacetic acid (TCA) at 20 % was added to stop the lipid peroxidation reaction. The mixture was centrifuged and 1 mL of thiobarbituric acid (TBA) at 0.50% was added in the supernatant followed by 15 min incubation in boiling water. The absorbance was measured at 532 nm for TBARS determination [31] against a blank without microsome fraction. Measurement was performed at least in triplicate. The negative control was represented by the reaction mixture in presence of $FeCl_2$ without the sample. The positive control contained only the reaction mixture without $FeCl_2$.

Tests on low density lipoproteins (LDL)

Blood (20 mL) was centrifuged at 5000 rpm at 4 °C during 20 min. The supernatant (plasma) was distributed in 5 mL tubes. Ultra-centrifugation at 40 000 rpm for 24 hours at 4 °C was performed and very-low density lipoproteins (VLDL) which are in surface of supernatant were removed. The volume in the tube was then adjusted with saline to 5 mL and 300 mg of KBr (potassium bromide) was added in each tube. The mixture was again ultra-centrifuged as above and plasma LDL were collected in surface of supernatant and dialyzed against a buffer NaCl 160 mM/NaH_2PO_4, $2H_2O$ 10 mM, to remove KBr. Two (2) mL of trichloroacetic acid (TCA) 20 % was added to 0.5 mL of LDL fraction in order to precipitate the serum proteins. After centrifugation at 5000 rpm for 10 min, 3 mL of NaCl/NaH_2PO_4 were added to pellets. Prior to testing, lipoproteins were diluted 10 times with above buffer. Lipoproteins were pre-incubated in the presence of different concentrations of each anthocyanin isolated at 37 °C for 1 h in a shaking water-bath. After incubating the samples for 30 min, 1 mL of TCA (20 %) was added to stop the lipid peroxidation reaction. The mixture was centrifuged and 1 mL of TBA (0.70 %) was added in the supernatant followed by 15 min incubation in boiling water. The absorbance was measured at 532 nm for TBARS determination [31] against a blank without LDL fraction. Measurement was performed at least in triplicate. The negative control was represented by the reaction mixture in presence of $CuSO_4$ without the sample. The positive control contained only the reaction mixture without $CuSO_4$.

ISSN: 0975-8585

Statistical analysis

The data's are expressed as mean ± standard deviation (SD) from three parallel measurements. In order to determine the significant differences between values, analysis of variance (ANOVA) and Newman-Keuls test were performed. Significance of means difference was defined at the 5% level ($p < 0.05$). Kruskal-Wallis's test was used to determine significant differences ($P < 0.05$) between the inhibition percentages. All statistical analysis was carried out using Statistica software (release 7.5).

RESULT AND DISCUSSION

Characterization of anthocyanins

Anthocyanins were identified by their retention times, which were compared the standards being their characteristic wavelengths. The chromatographic of callus anthocyanins monitored at 521 nm is given in figure 1.

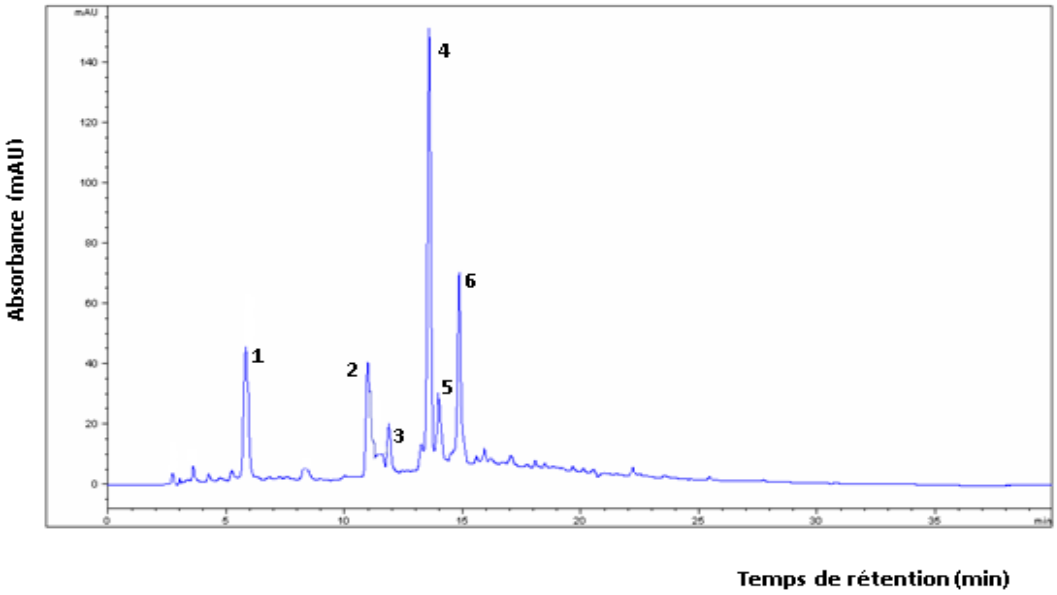

Figure 1: HPLC chromatogram of anthocyanins from callus extracts of *Hibiscus sabdariffa* monitored at 521 nm.

Peaks were identified by comparison with reference standards when available or by HNMR data (retention time). 1. malvidin-3-O-glucoside (5.650 min); 2. delphinidin-3-O-glucoside (10.812 min); 3. petunidin-3-O-glucoside (11.704 min); 4. cyanidin-3-O-sambubioside (13.561 min); 5. cyanidin-3-O-glucoside (13.978 min); 6. delphinidin-3-O-sambubioside (14.866 min).

Six anthocyanins were detected in callus of *Hibiscus sabdariffa*. In this chromatogram the peaks 1, 2, 3 and 5 were identified as -3-O-glucosides: malvidin- (1), delphinidin- (2), petunidin- (3) and cyanidin-3-O-glucoside (4); the peaks 4 and 6 were identified as -3-O-sambubioside: the cyanidin- (4) and delphinidin-3-O-sambubioside (6). Their chemical structures are showed in figure 2. In *Hibiscus sabdariffa* callus there are only malvidin (Mv-), delphinidin (Dp-), petunidin (Pt-) and cyanidin (Cy)-3-O-glucosides, along with the corresponding sambubioside derivatives of Cy- and Dp-. Cyanidin is the precursor pigment of the other anthocyanidins, and it can be transformed into delphinidin by the action of a 3'-O-hydroxylase. A 3'-5'-O-methyltransferase transforms Delphinidin into petunidin, and petunidin into malvidin [11; 32].

During the metabolism, there would be a glycosylation under the action of 3-O-glucosyltarnsferase to transform Mv-, Dp-, Pt- and Cy- in their monoglucoside form [33] under the action of 3-O-diglucosyltranferase, Cy- and Dp- mainly to give diglucoside form (sambubioside) [34].

ISSN: 0975-8585

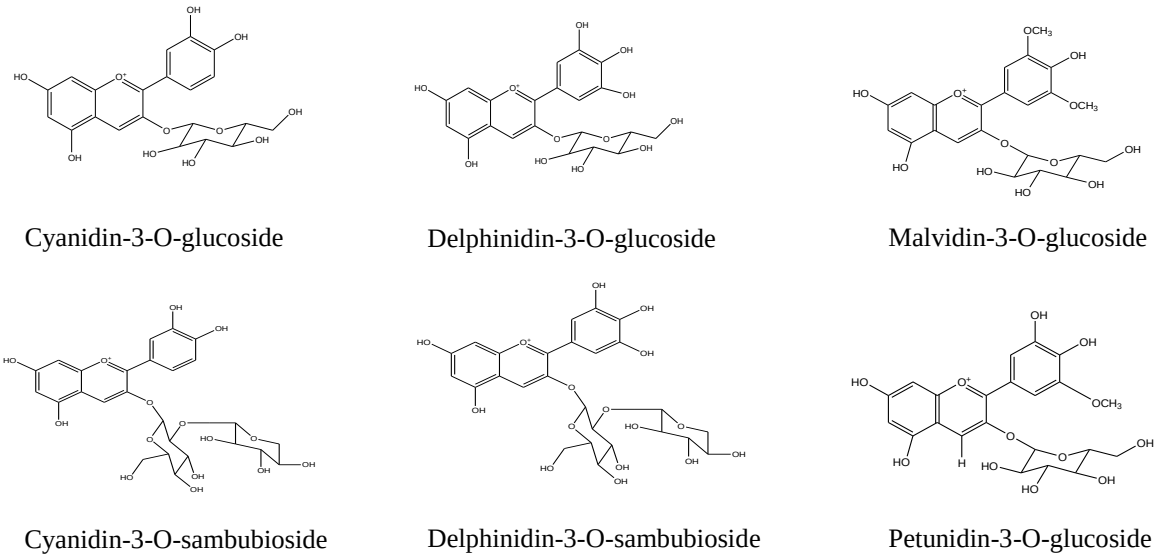

Figure 2: Structure of different anthocyanins detected in callus extracts of *Hibiscus sabdariffa*

In addition, several studies showed the presence of four anthocyanins namely cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-sambubioside and delphinidin-3-O-sambubioside in the flowers of Roselle [6; 9]. Quantitatively, cyanidin-3-O-sambubioside (53.93 mg/g DM) was predominant in the investigated callus. Delphinidin-3-O-sambubioside (17.07 mg/g DM), malvidin-3-O-glucoside (15.19 mg/g DM) and delphinidin-3-O-glucoside (10.04 mg/g DM) were intermediate anthocyanins followed by cyanidin-3-O-glucoside (5.01 mg/g DM) and petunidin-3-O-glucoside (3.89 mg/g DM) which were minor anthocyanins. The quantification of each is showed in table 1.

The distribution of the anthocyanins in Roselle can depend on the culture technique: Indeed, the number of anthocyanins extracted in callus was significantly greater than that of calyces [6; 27]. Malvidin-3-O-glucoside (Mv-3gluc) represented 14.45%; Delphinidin-3-O-glucoside (Dp-3gluc) 10.04%; Petunidin-3-O-glucoside (Pt-3gluc) 3.89%; Cyanidin-3-O-glucoside (Cy-3gluc) 5.01%. The most prominent anthocyanin was Cyanidin-3-O-sambubioside (Cy-3sam), which accounted for 53.93% of total content, followed by Delphinidin-3-O-sambubioside (Dp-3sam), 17.07% in all investigated Roselle callus.

Table 1: Distribution of individual anthocyanins detected in callus extracts of *Hibiscus sabdariffa*

N° peak	Name of anthocyanin	Content (mg/g DM)	Percentage (%)
1	Malvidin-3-O-glucoside	15.19 ± 0.04^{a}	14.45 ± 0.08^{a}
2	Delphinidin-3-O- glucoside	10.04 ± 0.05^{b}	09.55 ± 0. 10^{b}
3	Petunidin-3-O-glucoside	03.89 ± 0.01^{c}	03.70 ± 0.03^{c}
4	Cyanidin-3-O-sambubioside	53.93 ± 0.05^{d}	51.30 ± 0.50^{d}
5	Cyanidin-3-O-glucoside	05.01 ± 0.02^{c}	04.76 ± 0.09^{c}
6	Delphinidin-3-O-sambubioside	17.07 ± 0.08^{a}	16.24 ± 0.10^{a}
Sum of monoglucoside		34.13 ± 0.08^{e}	32.46 ± 0.08^{e}
Sum of diglucoside		71.00 ± 0.08^{f}	67.54 ± 0.08^{f}
Sum of methoxylated forms		19.08 ± 0.08^{a}	18.15 ± 0.08^{a}
Sum of non methoxylated forms		86.05 ± 0.08^{g}	81.85 ± 0.08^{f}
Sum of purified anthocyanins		100.13 ± 0.08^{h}	100.00 ± 0.08^{h}

Analysis performed in triplicate ; values are means of triplicate determination (n = 3) ± standard deviation (SD); DM, dried matter; Peaks were identified by comparison with reference standards when available or by ^{1}H-NMR data; in a column, values followed of a same letter are not statistically different (Newman-Keuls test at 5%).

The calli of Roselle has a percentage of methoxylated (Mv-3gluc and Pt-3gluc, sum 18.15%) lower than non-methoxylated (Cy-3gluc, Dp-3gluc, Cy-3sam and Dp-3sam, sum 81.85%) anthocyanin forms. With regard to glycosylation, the distribution of diglucoside anthocyanins form, mean 67.54% (71.00 mg/g DM) were the most abundant fraction in callus followed by monoglucoside anthocyanins was 32.46% (34.13 mg/g DM). The findings showed that Cy-3sam (51.30%) was the most abundant anthocyanin in callus of Roselle. By contrast, Pt-3gluc was the least abundant anthocyanin pigment, as demonstrated. Another point worth mentioning is that the order of abundances based on percentage value of distribution for each anthocyanin was the following: Cy-3sam > Dp-3sam > Mv-3gluc > Dp-3gluc > Cy-3gluc > Pt-3gluc. Callogenesis has made it possible to induce malvidin-3-O-glucoside and petunidin-3-O-glucoside compared to the extract of Roselle's calyces [13-14; 35; 36]. Thus, the induction of the highly anthocyanins callus producing lines would increase the dietetic value of the Roselle. It is obvious that the content and the quality of anthocyanins in callus were different from that of explants which are directly taken plants [6; 37]. This study clearly revealed that the induction of anthocyanins biosynthesis from callus culture improves the gain of anthocyanins considerably. Otherwise, it is wise to announce that several studies reported the pharmacological properties of anthocyanins [7-9; 21; 30; 38].

Antiradical Activity

The ability of callus anthocyanins of Roselle to trap free radicals produced by stable DPPH• free radical was estimated. The decrease in absorbance at 517 nm is taken as a measure of the extent of radical-scavenging. All purified anthocyanin show a higher DPPH• radical scavenging activity. Considering 50% inhibition concentration (IC_{50}) of each anthocyanin (Table 2), we identified that delphinidin-3-O-sambubioside (2.80 µg/g), cyanidin 3-O-sambubioside (4.10 µg/g) and delphinidin-3-O-glucoside (6.20 µg/g) have greater than that of trolox (7.39 µg/g) and ascorbic acid (7.60 µg/g). Cyanidin-3-O-glucoside (7.40 µg/g) as almost the same anti-radical power than trolox (7.39 µg/g). Then, the malvidin-3-O-glucoside (10.40 µg/g) and petunidin 3-O-glucoside have lowest anti-radical capacity.

Table 2: IC_{50} value obtained with anthocyanins of callus Roselle on antiradical capacity

Compounds			DPPH (IC_{50}) µg/g DM
Delphinidin-3-O-sambubioside			02.80 ± 0.02a
Cyanidin-3-O-sambubioside			04.10 ± 0.04b
Delphinidin-3-O- glucoside			06.20 ± 0.03c
Cyanidin-3-O-glucoside			07.40 ± 0.08d
Malvidin-3-O-glucoside			10.60 ± 0.07e
Petunidin-3-O-glucoside			12.30 ± 0.04f
References		Ascorbic acid	07.60 ± 0.07d
		Trolox	07.39 ± 0.09d

Ascorbic acid: Vitamin C; Trolox: Analogue of vitamin E soluble in water; DM: dry matter; DPPH: 1,1-diphenyl-2-picryl-hydrayl; IC_{50}: 50 % of inhibition concentration.

The most active substances in this test are: delphinidin-3-O-sambubioside and cyanidin 3-O-sambubioside (4.10 µg/g), which are twice more active than the references (ascorbic and trolox). In comparison, the OH bonds in the B ring of anthocyanins increase the anti-radical capacity. The importance of the OH group was previously noted by Rice-Evans *et al.* [39] and Fauconneau *et al*. [30] who observed an increased activity (6 times) of astringin (stilbene with two OH on ring B) relative to the trans-piceid (stilbene having one –OH on the B ring). The scavenging properties of these substances is related to the hydroxyl group (OH) specifically one with an orthodihydroxide function on the B-cycle [40]**.** Generally, the number of sugar residues at the 3-position, the oxidation state of the C ring the hydroxylation and as well as methylation pattern are considered crucial factors for the expression of anti-radical effects [11; 12]. In our case, the methylated forms of anthocyanin seem to reduce the anti-radical activity while diglucoside forms increase the

anti-radical capacity of anthocyanins. It is also important to note that the purified callus anthocyanins of Roselle have good or very good free radical-scavenging activities, except for Mv-3gluc and Pt-3gluc which were pro-oxidant compounds. The classification of anthocyanins from their scavenging activity was presented as follows: Dp-3sam > Cya-3sam > Dp-3gluc. Cyanidin-3-O-glucoside possesses the same anti-radical capacity as the references (trolox and ascorbic acid). On the other hand, Mv-3gluc followed by Pt-3gluc has weaker anti-radical activity than the references. Thus, the six purified anthocyanins in Roselle callus can be classified according to their anti-radical capacity compared to references as follows:

DP-3sam > Cy-3sam > Dp-3gluc > Cya-3gluc >> Mv-3gluc > Pt-3gluc.

Antioxidant activity

Anthocyanin composition is an important quality parameter for because of the significance of these compounds in determining biological activities [15; 21; 23]**.** The determination of the antioxidant activity of each anthocyanin from Roselle callus extracts was done using a microtechnique based on the non-enzymatic peroxidation of rat liver microsome and Human low-density lipoprotein. Six different anthocyanins purified from Roselle callus were examined for their ability to inhibit lipid peroxidation induced either by Fe (II) ions in microsomes or Cu (II) ions in LDL. Microsomes are membrane fragments of the endoplasmic reticulum. Indeed, it is worth noting that particularly rich cell membrane polyunsaturated fatty acids are the main target of free radicals [9; 10]. LDL is the function for transporting cholesterol, free or esterified in the blood and through the body to furnish the cells. A defect of LDL uptake by cells increases the cholesterol level in the blood vessels. LDL deposit can then result in atherosclerosis. For this reason, LDL is often called bad cholesterol as opposed to HDL called good cholesterol. However, the endothelium of the vessel wall is impermeable to lipoproteins. That's why finding substances capable of inhibiting LDL lipid peroxidation is essential to improve the health of Human [41]. The antioxidant abilities of anthocyanins were compared with a water-soluble tocopherol derivative, trolox and ascorbic acid. The antioxidant efficiency of these compounds was evaluated by their ability to inhibit the free radicals generated during lipid peroxidation. The capacity of lipid peroxidation inhibition of each anthocyanin extracted from Roselle callus is presented in **Table 3**.

Table 3: IC_{50} value obtained with anthocyanins of callus Roselle on lipid peroxidation inhibition capacity

Compounds		Microsomes (IC_{50}) µg/g DM	LDL (IC_{50}) µg/g DM
Delphinidin-3-O-sambubioside		02.73 ± 0.04a	01.14 ± 0.03b
Cyanidin-3-O-sambubioside		03.45 ± 0.03c	01.96 ± 0.06d
Delphinidin-3-O-glucoside		03.91 ± 0.05e	02.35 ± 0.07d
Cyanidin-3-O-glucoside		04.49 ± 0.07f	03.31± 0.05e
Malvidin-3-O-glucoside		09.22 ± 0.05g	05.35 ± 0.04h
Petunidin-3-O-glucoside		12.38 ± 0.04b	06.29 ± 0.08a
References	Ascorbic acid	06.23 ± 0.03d	04.57± 0.01c
	Trolox	05.82 ± 0.04h	03.64 ± 0.07e

Ascorbic acid: Vitamin C; Trolox: Analogue of vitamin E soluble in water; LDL: low density lipoprotein; DM: dry matter; IC_{50}: 50 % of inhibition concentration; In a column, values followed by the same letters are not significantly different according to Newman-Keuls test (5 %); Values are means of triplicate determination (n = 3); ± standard deviation.

The results showed that the antioxidant activity or lipid peroxidation inhibition of sample varies with the type of anthocyanin. Except Mv- and Pt-3gluc, the tested anthocyanins exhibited higher antioxidant activities against Fe (II)-induced peroxidation than Cu (II). It was also found that diglucoside anthocyanins (Dp-3sam and Cya-3sam) appeared to be the most efficient at inhibiting the lipid peroxidation followed by monoglucoside anthocyanins (Dp-3gluc and Cya-3gluc). Methoxylated anthocyanins represented by malvidin- and petunidin-3-O-glucoside which have an IC_{50} higher than references did not inhibit lipid peroxidation. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers [14; 15]. Among the samples, the antioxidant activity of Dp-3sam, Cya-3sam, Dp-3gluc and Cya-3gluc was higher than those of references. Otherwise, Dp-3sam showed the

highest capacity of lipid peroxidation inhibition followed by Cya-3sam (microsomes, 02.73 µg/ml; LDL, 01.14 µg/ml and microsomes, 03.45 µg/ml; LDL, 01.96 µg/ml, respectively) when results were expressed in IC_{50}. Additional, Dp-3gluc and Cya-3gluc have intermediate activity on lipid peroxidation inhibition (microsomes, 03.91 µg/ml; LDL, 02.35 µg/ml and microsomes, 04.49 µg/ml; LDL, 03.31µg/ml, respectively). It is also interesting to note that Mv-3gluc and Pt-3gluc showed a low capacity of lipid peroxidation inhibition (microsomes, 09.22 µg/ml; LDL, 05.35 µg/ml and microsomes, 12.38 µg/ml; LDL, 06.29 µg/ml, respectively) by comparison with ascorbic acid (microsomes, 06.23 µg/ml; LDL, 04.57 µg/m) and trolox (microsomes, 05.82 µg/ml; LDL, 03.64 µg/ml). Mv-3gluc and Pt-3gluc have pro-oxidant properties. Regarding anthocyanin antioxidant activity, it would have a structure-activity relationship of molecules. By comparing the derived glycosides, the number of hydroxyl on the derivatives would have a role in the antioxidant capacity. Dp-3sam and Cya-3sam which have most OH in the B ring and a diglucoside seems to explain their ability to inhibit lipid peroxidation. The methylation on B ring inhibits lipid peroxidation capacity. These results are consistent with the work of some authors [18; 41]. Besides, Garcia-Viguera *et al.* [42] reported that anthocyanins act as antioxidant on human low density lipoprotein (LDL) and *in vitro* systems of lecithin-liposomes. They found that the inhibition of oxidation increases with the concentration of the antioxidant and the order of antioxidant activity may vary depending on the catalyst concentration. Thus, in systems with LDL, malvidin have an antioxidant activity when the oxidation is catalyzed by copper. However, they noted that the delphinidin and cyanidin have a higher antioxidant activity than malvidin. This result suggests that glycosylation on methylated forms of anthocyanins decrease or rather eliminates their capacity to inhibit lipid peroxidation. The classification of anthocyanins extracted from Roselle callus compared to their antioxidant activity arises as follows: Dp-3sam > Cya-3sam > Dp- 3gluc > Cya-3gluc >> Mv-3gluc > Pt-3gluc. Moreover, works carried out in rats have helped to highlight vasodilatory and hypotensive protective effects against cardiac ischemia and myocardial, fibrosis against flavonoids, mainly anthocyanins [43]. The ability of anthocyanins to prevent the oxidation of low density lipoprotein (LDL) could lead to a reduction of atherosclerosis i.e. atherosclerotic plaque formation in the vascular wall [44 ; 45]. Thus, consumption of anthocyanins extracted from callus of Roselle could reduce cardiovascular events.

CONCLUSION

The antioxidant properties of anthocyanins from Roselle are ranked one of the highest antioxidant activities among many other plants. Roselle anthocyanin extracted from callus has been shown to have free radical-scavenging properties and they can also inhibit lipid peroxidation. Regardless the activity, anthocyanins can be classified from the most active to the lowest as follows: Dp-3sam > Cya-3sam > Dp- 3gluc > Cya-3gluc. Mv-3gluc followed by Pt-3gluc are considered pro-oxidant. These data show that anthocyanins extracted from Roselle callus may have a protective effect against atherogenesis through their antioxidant capacity. Within sight of these various biological properties, Roselle anthocyanins consumption could be encouraged in order to improve the Human health. Then, the use of Roselle anthocyanins as nutritional supplements appears to be necessary. They could be encapsulated in gellules to the appropriate dose to cure some pathologies. Further investigations into the specificity and mechanism(s) of HCA are needed.

ACKNOWLEDGMENT

The authors express their gratitude to Centre National de Recherche Agronomique (Abidjan, Côte d'Ivoire) through the *in vitro* culture laboratory for the technical material provision and to Groupe d'Etude des Substances Végétales à Activités Biologiques of University Victor Segalen (Bordeaux, France) for HPLC analyze.

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