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Anthocyanin production in calyx and callus of Roselle (*Hibiscus sabdariffa* L.) and its impact on antioxidant activity

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Abstract
Objective: Identifiaction of anthocyanin froù calyx and callus of *Hibiscus sabdariffa* and comparison of their antioxidant properties. Methods: HPLC fingerprints for different extracts were carried out using Agilent HPLC system. HPLC experiments were conducted using a reversed-phase C18 column. Antioxidant activity of anthocyanin was analyzed by using the 1,1-dipheny-2-picrylhydrazyl (DPPH).
Results: The HPLC studies using mobile phase system for each fraction showed various peaks in each extract. Six anthocyanins were determined in callus with the major compounds were cyanidin3-O-sambubioside and delphinidin 3-O-glucoside. In calyx, four anthocyanins were determined with the major compounds were delphinidin 3-O-sambubioside and cyanidin-3-O-sambubioside. Antioxidant activity is higher with anthocyanin from callus than that of callus. Conclusion: *Hibiscus sabdariffa* is a potential source of natural antioxidants. *In vitro* culture has increased the number of anthocyanins; this shows interest of *vitro* methods in improving dietary and pharmacological qualities of Hibiscus anthocyanin.

Keywords: Anthocyanin, Antioxidant activity, Callus, Calyx, *Hibiscus sabdariffa*, HPLC.

1. Introduction
Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals. Free radicals are types of reactive oxygen species (ROS), which include all highly reactive, oxygen-containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, singlet oxygen, nitric oxide radical, hypochlorite radical and various lipid peroxides [9, 17]. At high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage important cellular components especially proteins, nucleic acids and polyunsatured fatty acids in cell membranes and plasma lipoproteins [22]. Free radicals and reactive oxygen species have received a lot of attention especially in experimental or clinical medicine and biology because of their role in the aetiology of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, cancer,rheumatic and neurodegenerative desorders [14, 20, 30]. The damaging effects of reactive oxygen species on cells has been shown to be abrogated by plants with antioxidant compounds [2, 14, 38]. Plants are endowed with antioxidant and free radical scavenging molecules including vitamins, terpenoids, phenolic acids, tannins, flavonoids, coumarins, and other secondary metabolites [25, 35, 38]. The search for compounds, that can protect the human body from oxidative damage and retard the progress of many chronic diseases, has therefore greatly focused on plant sources as they produce significant amount of anti-oxidants and represent a potential source of new compounds with antioxidant activity.
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 [8, 31, 44]. 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, 38]. Roselle calyx extract is a good source of anthocyanins [3]. 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 [1, 26]. 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 [29, 41]. Some fractions of Roselle anthocyanins could present significant potential benefits for human health [4, 10, 18].

Anthocyanins are a group of plant pigments that are widely distributed in nature, which are responsible for the attractive colors of many flowers, fruits, grains and related products derived from them [49]. Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, and they are found in the form of polyhydroxylated and or methoxylated heterosides which derive from the flavylium ion or 2-phenylbenzopyrilium in nature [50]. Six anthocyanidins are widespread in fruits and vegetables, which are cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin [13]. Anthocyanins are valuable as kinds of important quality indicators in foods and chemotaxonomic indicators in plants. Recent research has shown that anthocyanins have numerous health beneficial properties, which include antioxidant [23, 48], anticarcinogenic [28], antimicrobial [44], anti-inflammatory [33, 36], cardioprotective [36, 39] and hepatoprotective properties [37, 40].

The aim of the present study was to evaluate the free radical scavenging properties of anthocyanins from calyx and callus of *Hibiscus sabdariffa*. In this study, the *Hibiscus* anthocyanins producing by calyx and callus were identified by HPLC method, compared as well as their antioxidant activity.

2. Materials and methods

2.1. Chemicals

All chemicals used were at least analytical grade. 1,1-diphenyl-1-picrylhydrazyl (DPPH), methanol, trifluoroacetic acid and anthocyanins standards (cyanidin, delphinidin, malvidin, peonidin, petunidin, cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, malvidin3-O-glucoside, peonidin 3-O-glucoside and petunidin3-O-glucoside) were purchased from Sigma-Aldrich (Steinheim, Germany). Ascorbic acid and trolox were obtained from Merck (Darmstadt, Germany).

2.2. Plant material

The dried calyces of Roselle were used as source of plant materiel investigated in the present study. This materiel was obtained according to the method described by Obouayeba *et al*. [38].

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*. [1].

2.3. Preparation of the extracts

2.3.1. Preparation of *Hibiscus* anthocyanins from calyx

The extract was prepared according to the method of Kouakou *et al*. [27]. One hundred grams (100 g) calyces of *Hibiscus sabdariffa* were extracted from 200 mL of acidified methanol with trifluoroacetic acid 0.1 % (v/v) for 24 hrs at 4 °C. The macerate was filtered successively on cotton wool and Whatman paper. After low-pressure vacuum evaporation of the methanol in BÜCHI Rotavapor R-114 at 38 °C, we obtained a dry extract. Two hundred milliliters (200 mL) of distilled water were added to the dry extract and the aqueous solution was submitted to a filtration on gel XAD-7, in order to eliminate sugars and chlorophyll pigments. One hundred milliliters (100 mL) of acidified methanol with trifluoroacetic acid 0.1 % (v/v) were poured over the gel XAD-7 and the methanolic filtrate obtained was resubmitted to low-pressure vacuum evaporation in BÜCHI Rotavapor R-114 at 38 °C. The dry extract obtained was dissolved in 100 mL of distilled water. The aqueous solution was lyophilized with the freeze dryer CHRIST ALPHA 1-2. The dried extract obtained represented the *Hibiscus* anthocyanins from calyx were previously determined by Obouayeba *et al*. [38].

2.3.2. Preparation of *Hibiscus* anthocyanins from callus

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.

2.4. Determination of *Hibiscus* anthocyanins from calyx and callus by HPLC analysis

The high performance liquid chromatography (HPLC) analysis was conducted using the method described by Drust and Wrolstad (2001) [15]. 10 mg of freeze-dried extracts from calyx and callus were dissolved with 5 mL of methanol during overnight at 4°C in 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 $mL.min^{-1}$. 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.

2.5. Radical scavenging activity

The free radical scavenging activity of the anthocyanins was analyzed by using the 1,1-dipheny-2-picrylhydrazyl (DPPH) assay [16, 38]. 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 ml of this solution are added to 0.5 ml of the anthocyanin solution in methanol (1.0 $mg.mL^{-1}$). 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 (%) $=[(OD_{control} - OD_{sample})/OD_{control}]$ x100, 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.

2.6. Statistical analysis

Data 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).

3. Results and Discussion

3.1. Characterization of *Hibiscus* anthocyanins from calyx and callus

Hibiscus anthocyanins were identified by their retention times, which were compared the standards being their characteristic wavelengths. The chromatographics of calyx and callus anthocyanins monitored at 521 nm is given in Figure 1.

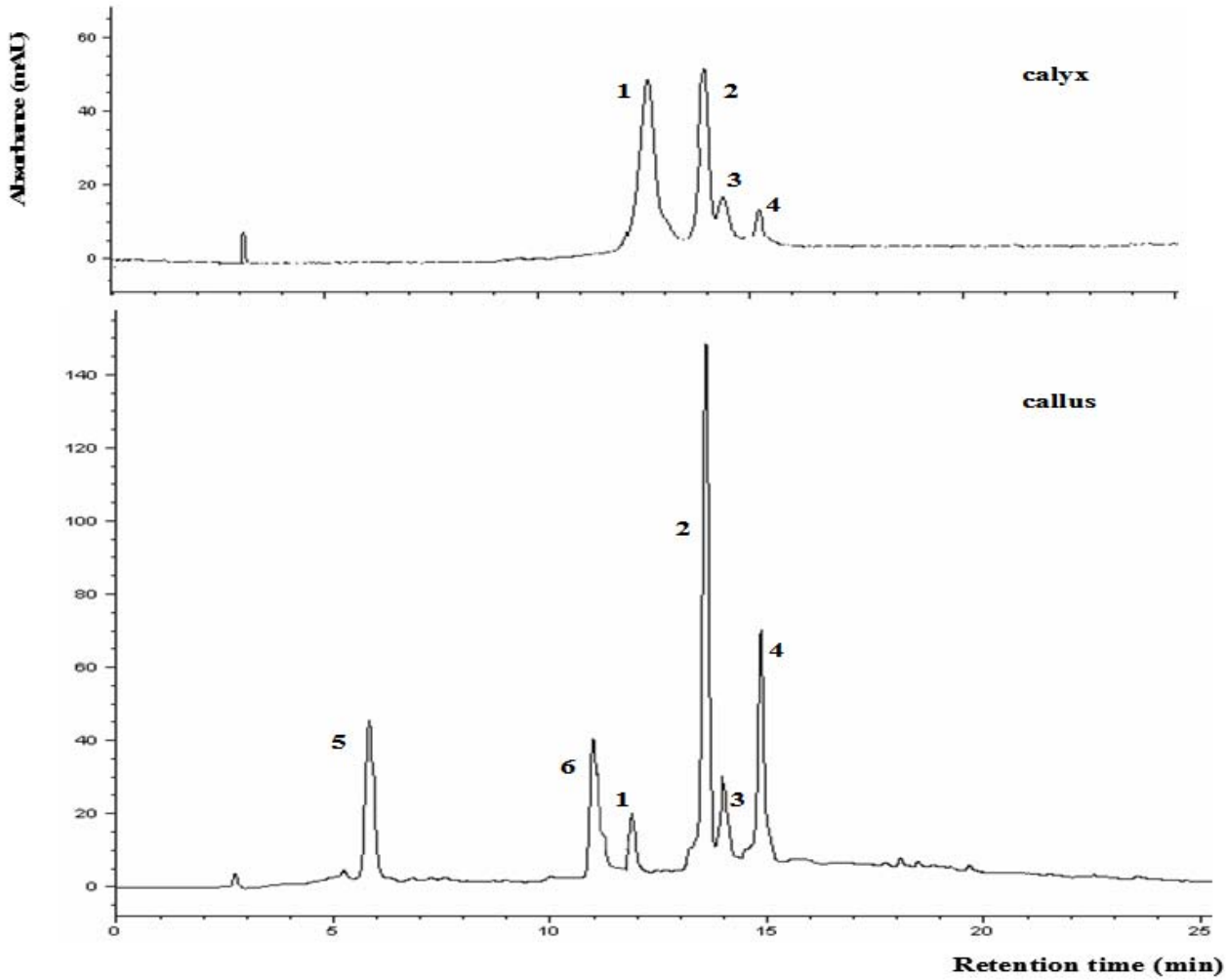

Fig 1: HPLC chromatogram of anthocyanins from calyx and callus extracts of *Hibiscus sabdariffa* monitored at 521 nm.

Peaks were identified by comparison with reference standards when available or by HNMR data (retention time).

1. delphinidin 3-O-sambubioside (12.056 min); 2. cyanidin 3-O-sambubioside (14.115 min); 3. cyanidin 3-O-glucoside (14.771 min); 4. delphinidin 3-O-glucoside (15.089 min); 5. malvidin-3-O-glucoside (6.125 min);6. petunidin-3-O-glucoside (12.569 min).

Six anthocyanins were detected in callus of *hibiscus sabdariffa* while four anthocyanins were detected in calyx of this plant. However all the anthocyanins present in the calyx is also found in callus. In these chromatograms the peaks 3, 4, 5 and 6 were identified as -3-O-glucosides: cyanidin- (3), delphinidin- (4), malvidin- (5), delphinidin- (2), petunidin-3-O-glucoside (6); the peaks 1 and 2 were identified as -3-O-sambubioside: the delphinidin- (1) and cyanidin-3-O-sambubioside (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 [41, 47]. The absence of anthocyanins as the Mv and Pt-3-O-glucoside in the calyx of *Hibiscus sabdariffa* shows that *in vitro* culture conditions enabled the progress of chemical reactions mentioned above, thus highlighting optimization production of anthocyanins in the callus.

During the metabolism, there would be aglycosylation under the action of 3-O-glucosyltarnsferase to transform Mv-, Dp-, Pt- and Cy- in their monoglucoside form [5, 51] under the action of 3-O-diglucosyltranferase,Cy-andDp-mainlyto give diglucoside form sambubioside [24]. The presence of anthocyanin compounds such as cyanidin 3-O-sambubioside, delphinidin 3-O-sambubioside, cyanidin 3-O-glucoside,delphinidin 3-O-glucoside,malvidin 3-O-glucoside and petunidin 3-O-glucoside in callus or calyx as been reported by several authors [1, 21, 38]. All *Hibiscus* anthocyanins isolated in callus or calyx have pharmacological properties reported by several authors [6, 7,19, 43, 48]. This clearly shows the use of this plant as herbal medicine. The results of quantitative phytochemical screening presented in Table 1 shows the content of various anthocyanin compounds in calyx and callus.Anthocyanin production in callus with the sum of the amounts of anthocyanins (42.99 mg.g^{-1} dm) is three times greater than the calyx (131.13 mg.g^{-1} dm). These results show that the distribution of the anthocyanins in roselle may depend on the conditions and growing techniques. Indeed, the number of anthocyanins extracted in callus was significantly greater than that of calyx [26, 38]. generally, the anthocyanin compoudspresent in calyx and callus are most abundant in the latter. For exemple the content of cyanidin 3-O-sambubioside (53.93 mg.g^{-1} dm), cyanidin 3-O-glucoside (08.01 mg.g^{-1} dm) anddelphinidin 3-O-glucoside (27.04 mg.g^{-1} dm)in callus were statistically superior to those of cyanidin 3-O-sambubioside (17.11 mg.g^{-1} dm), cyanidin 3-O-glucoside (02.40 mg.g^{-1} dm) anddelphinidin 3-O-glucoside (02.20 mg.g^{-1} dm)in calyx.Our results show that in vitro culture enables the production optimization of *Hibiscus* anthocyanins. These results are in accordance with those obtained by Tarrahi and Rezanejad [43] and Maharik *et al*. [32].

The findings showed that Cy-3sam (53.93 mg.g^{-1} dm), Dp -3sam (21.28 mg.g^{-1} dm) were the most abundant anthocyanins respectively in callus and calyx Roselle. otherwise, it is wise to announce that several studies reported thatthese anthocyanins are the majors compouds in Roselle calyx and callus [6, 11, 21, 16, 37, 48].

Table 1: Quantitative data of various *Hibiscus* anthocyanins isolated in calyx and callus.

Name of anthocyanin	Anthocyanins content (mg.g^{-1} dm)	
	Calyx	Callus
Cyanidin-3-O-glucoside	02.40 ± 0.02^{c}	08.01 ± 0.04^{d}
Delphinidin-3-O-glucoside	02.20 ± 0.01^{c}	27.04 ± 0.07^{b}
Cyanidin-3-O-sambubioside	17.11 ± 0.10^{b}	53.93 ± 0.20^{a}
Delphinidin-3-O-sambubioside	21.28 ± 0.05^{a}	07.07 ± 0.05^{d}
Malvidin-3-O-glucoside	nd	18.19 ± 0.10^{c}
Petunidin-3-O-glucoside	nd	16.89 ± 0.02^{c}
ACH	42.99 ± 0.70	131.13 ± 1.50

Same letters within a column and on a line indicate no significant difference (P<0.05). Mean of three replicates ± standard deviation; 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%). ACH, Anthocyanins characterized by HPLC (sum of the amounts of anthocyanins); nd, not detected.

3.2. Antiradical Activity of *Hibiscus* anthocyanins from calyx and callus

In this study, the antioxidant activity of *Hibiscus* anthocyanins from calyx and callus were investigated using the DPPH scavenging assay by determining the total antioxidant capacity of these compounds. All these have proven the effectiveness of *Hibiscus* anthocyanins from calyx and callus compared with the reference standard antioxidant ascorbic acid. The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 517 nm and also for the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Comparison of the antioxidant activity of the extract and ascorbic acid is shown in Figure 2.

The concentration required to inhibit 50 % radical-scavenging effect (IC_{50}) was determined from the results of a series of concentrations tested. A lower IC_{50} value corresponds to a larger scavenging activity. The IC_{50} values of *Hibiscus* anthocyanins from callus was 0.18 mg.mL^{-1} while that to those of *Hibiscus* anthocyanins from calyx (0.26 mg.mL^{-1}) and ascorbic acid, a well-known antioxidant compound used as the reference control in this study, its IC_{50} value was 0.32 mg.mL^{-1}. These results imply that all purified anthocyanin show a higher DPPH• radical scavenging activity.

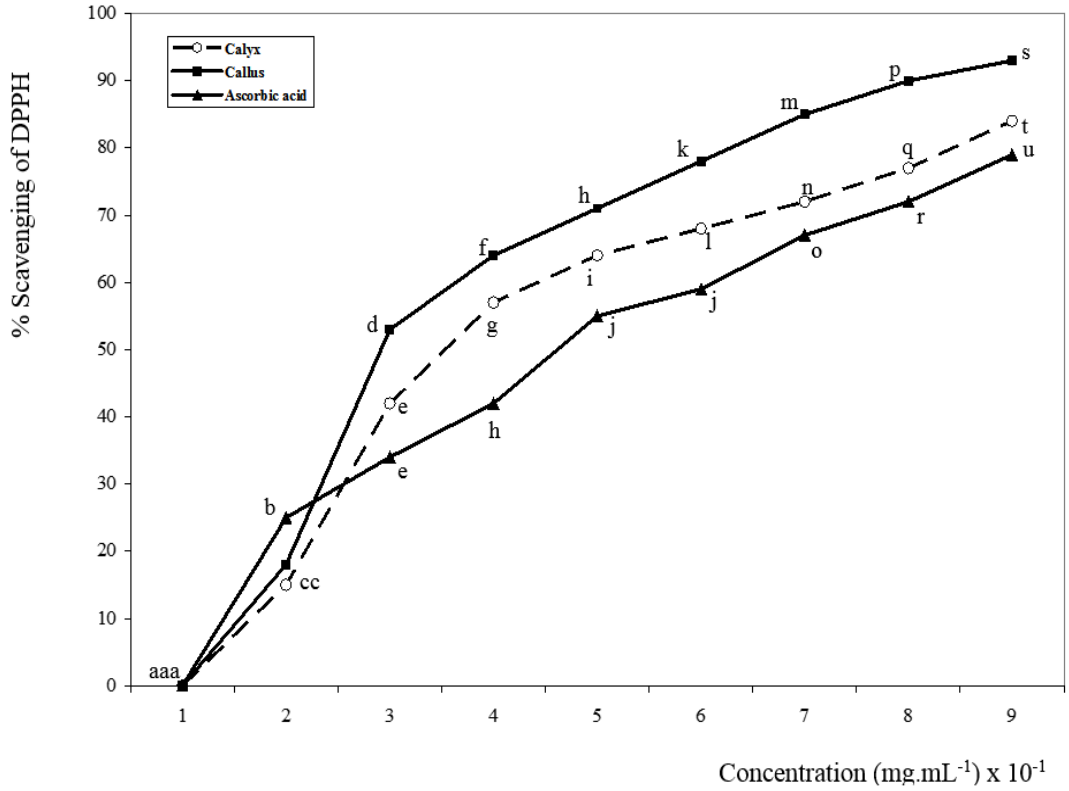

Fig 2: Free radical scavenging activity of *Hibiscus* anthocyanins from calyx and callus measured by DPPH assay

Indeed, 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.* [42] and Fauconneau *et al*. [16] 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 *o*-dihydroxide function on the B-cycle [12]. 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 [34, 41]. 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 calyx and callus anthocyanins of Roselle have good or very good free radical-scavenging activities.

4. Conclusion

Hibiscus sabdariffa is a medicinal and food plant rich in anthocyanins compounds of interest responsible for its pharmacological properties.This study which aimed to evaluatethe radical scavenging activity of the *Hibiscus* anthocyanins producing by calyx and callus.In Côte d'Ivoire, the juice of flowers of *H. sabdariffa* commonly known as Bissap is used in the preparation of local nonalcoholic cold beverage and as a hot drink is popular. These results show that the consumption of Bissap could help strengthen the antioxidant capacity of the organism. The use of *H. sabdariffa* calyces as natural antioxidants, natural colorants, and an ingredient of functional foods seems to be promising.

5. Conflict of Interest

We declare that we have no conflict of interest.

6. Acknowledgement

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