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Evaluation of the *In Vitro* Antioxidant Properties of *Hibiscus Sabdariffa* L. Anthocyanins

Orororo, O.C*[1], Ogbuta, A.A[2] and Asagba, S.O[3],
[1]Department of Chemical Sciences, Edwin Clark University, Kiagbodo, Delta State
[2]Department of Chemical Sciences, University of Africa, Toru-Orua, Bayelsa State
[3]Department of Biochemistry, Delta State University, Abraka, Nigeria.
*Corresponding author's email: osuvwec@yahoo.com

Abstract
Given the huge health benefits of anthocyanins and their application in various industrial processes, there has been an upsurge in researches related to the isolation, purification and characterization of anthocyanins. Therefore, this study was designed to evaluate the in vitro antioxidant activities of anthocyanins extracted from Hibiscus sabdariffa L and compare it with the aqueous extract. Hibiscus sabdariffa aqueous extract as well as the anthocyanins were obtained using standard protocols and were subjected to different in vitro antioxidant capacity and free radical scavenging and metal chelating assays. The ability of H. sabdariffa anthocyanins to trap free radicals produced by stable DPPH' free radical (12.78±2.0) was higher than that of the aqueous extract. Similarly, H. sabdariffa anthocyanins recorded a higher total antioxidant activity (74.53±2.8%) than the aqueous extract (73.6±1.9%). On the ability of the extracts to chelate metal ions (Fe^{2+}), H. sabdariffa anthocyanin extract had the higher chelating activity (36.38±2.1%) than the aqueous extract and EDTA (34.36±2.3%), which was used as a standard. In addition, Hydrogen peroxide scavenging activity recorded was higher in H. sabdariffa anthocyanins (59.53 ±2.3%) than in the aqueous extract (54.53±1.5%). The in vitro assays conducted in this study shows that H. sabdariffa anthocyanins are better antioxidant and scavenger of free radicals than crude aqueous extract, which is also a source of antioxidants. The results presented in this study supports the fact that anthocyanins have a high chemical (DPPH, FRAP, Hydrogen peroxide scavenging and metal chelating) antioxidant activity.

Keywords: *Hibiscus sabdariffa, Anthocyanins, Free radicals*

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1. Introduction

Anthocyanins are members of the flavonoid group of phytochemicals, which are found in many fruits and vegetables and are responsible for their colours (blue, purple, red, orange etc) (Horbowicz *et al*., 2008; Ajiboye *et al*., 2011). According to Mazza (2007), anthocyanins are distinguished from other flavonoids by their ability and capacity to form flavylium cations, possessing anti-inflammatory, antioxidative and metal ion chelating properties (Oszmiañski, 2001; Wawer, 2001; Ajiboye *et al*., 2011).

According to Prior and Wu (2006), anthocyanins are usually found as glycosides of their respective aglycone anthocyanidin chromophores with the sugar moiety generally fixed at the 3-position on the C-ring or the 5-position on the A-ring. Jaganath and Crozier (2010) noted that there are about 17 anthocyanidins found in nature, with cyanidin being the most popular and only six (cyaniding, petunidin, delphinidin, pelargonidin, malvidin and peonidin) being of great benefit in human diet (Fig. 1). The anthocyanins obtained from different sources are also of great variance (Ha and Le, 2022). Thus, there are many different types of anthocyanins and according to researchers, this is due to a number of reasons such as the quantity of hydroxyl and methoxy groups on the basic anthocyanidin skeleton as well as their positions of attachment; identity of attached sugar moieties and their points of attachment, the type of acylating agent and the extent to which attached sugars are acylated (Jaganath and Crozier, 2010).

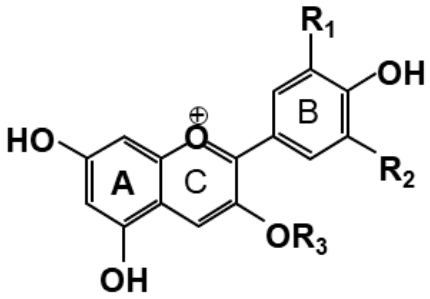

Fig. 1: Anthocyanins structure
R_3 = sugar, and anthocyanidins; R_3 = H. Such that, **Delphinidin** (R_1=OH; R_2=OH; R_3=H); **Petunidin** (R_1=OH; R_2=OCH_3; R_3=H); **Malvidin** (R_1=OCH_3; R_2=OCH3; R_3=H); **Cyanidin** (R_1=OH; R_2=H; R_3=H); **Pelargoridin** (R_1=H; R_2=H; R_3=H) (Tena *et al*., 2020).

Hibiscus sabdariffa Linn, also called roselle or red sorrel, is a an herbaceous sub shrub cultivated widely in West and Central Africa, whose calyces are commonly consumed as a cold or a hot beverage all over the world and its extracts are used in folk medicine to treat cases of high blood pressure, fever, liver diseases and a host of other ailments (Wang, 2000). Roselle flowers and calyces contain significant quantities of vitamins, minerals, polyphenols, organic acids, flavonoids and anthocyanins with varied health benefits (Sindi *et al*., 2014; Da-Costa-Rocha *et al*., 2014; Lin *et al*., 2015).

Anthocyanins have been implicated in the high antioxidant properties and positive physiological effects observed in the extract of *H. sabdariffa* (Formagio *et al*., 2015; Al-Groom and Al-Kubaisy, 2016; Tahir *et al*., 2016; Orororo *et al*., 2018a; Orororo *et al*., 2018b). Thus, the fact that anthocyanin pigments are potent medicinal agents has been long accepted in folk medicine throughout the world and they have been implicated in many disease states to have great health benefits. However, though the beneficial nature and uses of anthocyanins has been shown, the precise and measurable biochemical qualities and properties of purified anthocyanins *in vitro*, *in vivo* and in clinical research trials is relatively novel (Tsuda *et al*., 2003). Thus, given the huge health benefits of anthocyanins and their application in various industrial processes, there has been an upsurge in researches related to the isolation and purification of anthocyanins (Sari *et al*., 2009). However, studies describing the chemical properties, structures and *in vitro* antioxidant properties of purified anthocyanins in general are not popular. In addition, although some studies have been done on the antioxidant properties of *H. sabdariffa* extract *in vitro* (Fernández-Arroyo *et al*., 2011; Mohd-Esa *et al*., 2010; Owoade *et al*., 2015), studies on extracted and purified *H. sabdariffa* anthocyanins are limited. Therefore, this study was designed to evaluate the *in vitro* antioxidant activities of anthocyanins extracted from *Hibiscus sabdariffa* L.

2. Materials and methods

2.1 Chemicals

Quality chemicals and regents of analytical standard were utilized in carrying out this study. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, citric acid,sodium hydroxide,sodium carbonate (Na_2CO_3), hydrochloric acid (HCl), methanol, ethanol, and ascorbic acid which were obtained from Sigma Chemicals Co. (St. Louis, USA).

2.2 Plant material

Fresh calyces of *H. sabdariffa* L.were obtained from Warri main market, Warri South L.G.A., Delta State, Nigeria. Thereafter, they were dried under continuous air-flow maintained at room temperature until constant weight.

2.3 Preparation of aqueous extract

Aqueous extract of *H. sabdariffa calyces* was prepared as described by Iyare and Adegoke (2008). Dried *Hibiscus sabdariffa* calyx were boiled in distilled water for 15min.The boiled sample was allowed to cool and then filtered and the filtrate was evaporated to dryness at 400C in an oven to produce a dark red residue.

2.4 Extraction of *H. sabdariffa* anthocyanins

Anthocyanins were extracted from *H. sabdariffa* calycesaccording to the method of and Wrolstad (1990a) as reported in our previous study (Orororo *et al*., 2018a). The extraction began by pulverization of one (1) kg of *H. sabdariffa* calyces. The pulverized calyces were then extracted using ten litres of 0.1% trifluoroacetic acid (TFA) for a period of twelve hours at 40°C. Thereafter, the extract was filtered with Whatman No. 1 filter paper and the filtrate was applied to Sepabeads SP-207 resin column for fractionation of the different compounds in the extract. The resin bed became red as it absorbed anthocyanins while sugars, acids and other water-soluble compounds were washed off with three litres of water. Anthocyanin pigments were thereafter eluted with 50% ethanol solution containing 0.1% TFA. The resulting eluate was dried at 40°C under vacuum to obtain a concentrated eluate, used for assays.

2.5 Determination of antioxidant properties of the extract

2.5.1 2,2 diphenyl-1-picrylhydrazyl (DPPH) Free Radical scavenging assay

DPPH scavenging property of the anthocyanin extract was determined by the method of Brand *et al*. (1995) as reported by Lim and Quah (2007) with slight modification. 2,2-diphenyl-1-picrylhydrazyl was dissolved in methanol at a final concentration of 0.101 mmol/L to form a purple-colored methanol solution. Thereafter 0.1 ml of the methanolic solution of *H. sabdarriffa* crude and anthocyanin extracts were added to 3.9 ml DPPH solution. The resulting solutions were incubated for 1 hour at room temperature, and then absorbance was measured against a blank at 510 nm with ascorbic acid solution serving as positive control.

% DPPH radical scavenging rate was calculated by the formula in Equation (1):

$$Radical\ scavenging\ rate\ (\%) = \frac{A\ blank - A\ sample}{A\ blank} x\ 100 \quad (1)$$

where *A* blank = the absorbance of the control reaction, and *A* sample = the absorbance of the test compounds. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined (IC_{50}) for each sample. IC_{50} value is the inhibitory concentration of sample that could scavenge half (that is 50%) of the DPPH radicals.

2.5.2 Ferric reducing antioxidant power (FRAP) assay

The ability of crude and anthocyanin extracts of *H. sabdarriffa* to reduce the ferricyanide complex to the ferrous form was determined by the method proposed by Oyaizu (1986) with some modification as reported by Amin and Razieh (2007). First, the FRAP reagent was prepared by mixing 2.5 ml of 10 mmol/L tripyridyltriazine (TPTZ) solution in 2.5 ml of 40 mmol/L HCl; 2.5 ml of 20 mmol/L $FeCl_3$ and 25 ml of 0.3 mol/L acetate buffer, pH 3.6. Thereafter, the extracts were diluted in methanol at three different concentrations (0.5 ml, 1 ml and 1.5 ml). Then 0.3 ml of the extract solution was mixed with 2.7 ml of FRAP reagent and the absorption of the resulting mixture was measured at 593 nm. A mixture of 0.3 ml methanol and 2.7 ml TPTZ served as reagent Blank while the calibration curve was obtained by using aqueous solutions of known Fe (II) concentration (0 to 1000 µmol/L, $FeSO_4$). The concentration of extract having a ferric reducing ability equivalent to that of 1 µmol $FeSO_4$ was used as FRAP parameter and the reducing power of sample was expressed as equivalent concentration of $FeSO_4$.

2.5.3 Total antioxidant activity

Total antioxidant activity of *H. sabdarriffa* crude and anthocyanin extracts was determined by the method of Orak (2006) as reported by Al-Fartosy and Abdulwahid (2015). Linoleic acid solution (5.0 ml) 6 mg/mL in 99% methanol was prepared. To this, 1 ml of the extract was added and the resulting mixture was incubated for 10 min at 37˚C. Thereafter, 0.1 ml of this solution was added to 4.7 ml of 75% ethanol, 0.1 ml of 0.1 M ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in 3.5% HCl solution. The reaction was then allowed to stand in the dark for 5 min at 30˚C. Thereafter, the absorbance was read at 500 nm. The percentage of lipid peroxidation, which indicates the total antioxidant activity, was calculated according to the formular in Equation (2):

$$\%\ of\ inhibition = \frac{A\ blank - A\ sample}{A\ blank} x\ 100 \quad (2)$$

where A_{blank} was the absorbance of control (blank, without extract) and A_{sample} was the absorbance in the presence of the sample.

2.5.4 Hydrogen Peroxide Scavenging Activity

The Hydrogen peroxide scavenging activity of *H. sabdarriffa* crude and anthocyanin extracts was determined using the method described by Akinpelu *et al*. (2010) as applied by Al-Fartosy and Abdulwahid (2015). H_2O_2 solution (4 mM) was first prepared in 0.1 M (pH 7.4) phosphate buffer. To 0.6 ml of this solution was added 0.1 ml of sample. The resulting solution was then incubated for 10 min and thereafter its absorbance was read at 230 nm against blank solution (H_2O_2 without sample). The hydrogen peroxide scavenging activity was calculated according to Equation 2.

2.5.5 Metal chelating activity

The method of Ponmozhi *et al*. (2011) with slight modification as reported by Al-Fartosy and Abdulwahid (2015) was used in determining the metal chelating activity of *H. sabdarriffa* crude and anthocyanin extract as compared with Ethylenediamine tetraacetic acid (EDTA), as standard. For the assay, 100 µl of standard and sample were added to a solution of 100 µL Ferric chloride (1 mM). Then the reaction was initiated by adding of 250 µL of 1 mM ferrozine. This mixture was then quantified to 1.3 mL with methanol, was shaken vigorously and left to stand for 10 min at room temperature. Absorbance was read at 562 nm after the mixture had reached equilibrium with

lower absorbance at 562 nm indicating stronger chelating effect. Equation (2) was used to calculate the percentage inhibition of ferrous-ferrozine complex formation.

2.6 Statistical analyses

The data obtained in the study are given in the form of mean ± standard deviation of triplicate determination. Fisher's LSD test in addition to the one-way analysis of variance (ANOVA) was used in evaluating the data using Statistical software SPSS 21.

3. Results

The *In vitro*Antioxidant properties of *Hibiscus sabdariffa* crudeaqueous extract and *Hibiscus sabdariffa* anthocyaninsis presented in Fig. 2 and Table 1. The ability of *H. sabdariffa* anthocyaninsto trap free radicals produced by stable DPPH' free radical (12.78±2.0) was higher than that of the aqueous extract. IC_{50} (Half maximal Inhibitory Concentration) value is the concentration of the sample that can scavenge 50% of DPPH free radical. It is inversely proportional to the free radical scavenging activity/ antioxidant property of the sample. Similarly, *H. sabdariffa* anthocyanins recorded a higher total antioxidant activity (74.53±2.8%) than the aqueous extract (73.6±1.9%). On the ability of the extracts to chelate metal ions (Fe2+), *H. sabdariffa* anthocyanins had a higher chelating activity (36.38±2.1%) than the aqueous extract and even EDTA (34.36±2.3%), which was used as a standard. In addition, Hydrogen peroxide scavenging activity recorded was higher in *H. sabdariffa* anthocyanins (59.53±2.3%) than in the aqueous extract (54.53±1.5%). The results suggest that *H. sabdariffa* anthocyanins have higher antioxidant potentials than the aqueous extract in vitro.

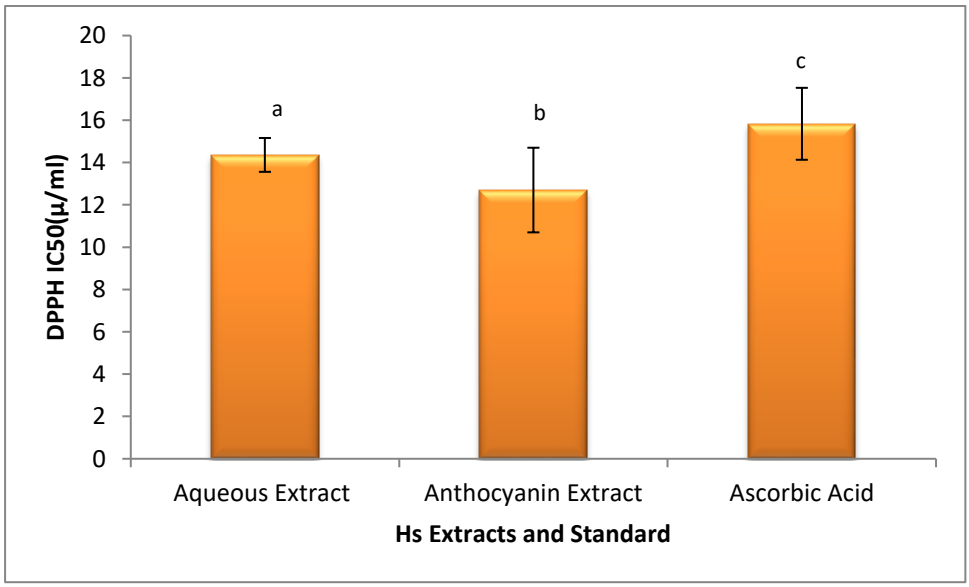

Fig. 2: (DPPH) Free Radical scavenging activity of*H. sabdariffa*L. Anthocyanins

Table 1: *In vitro*Antioxidant properties of *Hibiscus sabdariffa* crudeaqueous extract and anthocyanin extract

Properties	Aqueous Extract	Anthocyanin Extract	Control(s) Standards
FRAP (%)	32.53±2.2a	36.35±1.2b	-
Total Antioxidant Activity (%)	73.6±1.9a	74.53±2.8a	-
Hydrogen Peroxide Scavenging activity (%)	54.53±1.5a	59.53±2.3b	Ascorbic acid, 50.33±1.3c
Metal Chelating Activity (%)	26.47±2.8a	36.38±2.1b	EDTA, 34.36±2.3c

All values are means of triplicate determinations. Results are presented as Mean±standard deviation (SD). Values with different superscripts within same row differ significantly.

4. Discussion

The antioxidant properties of anthocyanins from *H. sabdariffa* were evaluated using different *in vitro* assays due to the accepted fact that in assessing the antioxidant prowess of natural compounds, single assays are usually inadequate (Rahman *et al*., 2015). The result showed that *H. sabdariffa* anthocyanin can mitigate free radical chain reactions. The ability of anthocyanin extract of *H. sabdariffa* to trap free radicals produced by stable DPPH• free radical was higher than that of the aqueous extract and that of ascorbic acid used as standard antioxidant. Hydrogen peroxide scavenging activity recorded was also higher in the anthocyanin extract than in the aqueous extract. This result agrees with earlier works which show that anthocyanins are potent in quenching oxygen radicals (Hale *et al*., 2002; Nuri *et al*., 2010; Chiunghui *et al*., 2010; Francilene *et al*., 2011). This radical scavenging activity of anthocyanins is related to the presence of their 3',4'-dihydroxy groups with which they relate with free radicals resulting in the formation of stable anthocyanin-metal complexes (Sarma *et al*., 1997). This in turn depends on the basic structural arrangement of the anthocyanin moiety as it determines the speed by which a hydrogen atom from a hydroxyl group can be donated to a free radical in addition to the capacity of the anthocyanin to support an unpaired electron (Kay et al., 2004). The implication of all this is that anthocyanins and other polyphenolics in the HS aqueous and anthocyanin extracts functioned as good electron and hydrogen atoms donor and as such were able to end radical chain reaction by helping to convert free radicals to more stable products. According to Qin and Xiaojun (2013), the ability of anthocyanins to scavenge free radicals, as witnessed in this study, is largely due to the presence of phenolic hydroxyl groups in their molecular structure. It has been reported that these phenolic hydroxyl groups are able to effectively stop peroxidation by making hydrogen atoms available to bond with the free radicals thereby cutting off the oxidation chain reaction (Owoade *et al*., 2015). On the ability of the extracts to chelate metal ions (Fe2+), the anthocyanin extract had the higher chelating activity than the aqueous extract. This may be due to the presence of more anthocyanin molecules in the anthocyanin extract compared to the aqueous extract. This result is supported by Al-Fartosy *et al*., (2015) who also reported that the chelating power of anthocyanin extracts increase with increase in its content of anthocyanin compounds which are responsible for metal chelating.

5. Conclusion

The *in vitro* assays conducted in this study shows that *H. sabdariffa* anthocyanins are better antioxidant and scavenger of free radicals than crudeaqueous extract, which is also a source of antioxidants. The results presented in this study supports the fact that anthocyaninshave is a high chemical (DPPH, FRAP, Hydrogen peroxide scavenging and metal chelating) antioxidantactivity.

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