

## The In Vitro Antioxidant Properties of Hibiscus Anthocyanins Rich Extract (HAE)

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**Abstract:** This study investigate the antioxidant properties of Hibiscus anthocyanins rich extract (HAE) prepared from *Hibiscus sabdariffa*. The extract significantly inhibited superoxide production by xanthine – xanthine oxidase by 41.18% at 5% (v/v) of the reaction volume. In addition, the antioxidant investigation showed that HAE has potent scavenging ability against DPPH and ABTS radicals and these radicals scavenging abilities were found to be dose dependent. HAE was found to contain high level of total phenolic content ( $56.6 \pm 3.7$  mg/g in GAE/g dried weight). The anthocyanins components of HAE were identified through several chromatographic analyses. The results indicate that delphinidin-3-sambubioside, delphinidin-3-monoglucoside, cyanidin-3-sambubioside and cyanidin-3-monoglucoside as the major anthocyanins and delphinidin and cyanidin as major anthocyanidin. These studies demonstrated that *Hibiscus sabdariffa* is an efficient antioxidant plant *in vitro* and may prevent or reduce the development and progression of free radical mediated diseases. Antioxidant ability displayed by *Hibiscus sabdariffa* may in part be due to the presence of different phenolic compounds in the plant.

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### 1. Introduction

Plants extracts are rich source of important vitamins namely vitamin E, Vitamin C and  $\beta$ -carotene, flavonoids and other polyphenols which have been discussed as potential antioxidant prophylactic agents for both health and diseases management (Aruoma, 2003). Studies suggest that plant polyphenols such as the flavonoids are potent antioxidant compounds both *in vitro* and *in vivo*. They have been shown to scavenge free radicals, chelate redox-active metal ions and inactivate other pro-oxidants (Schroeter, 2002; Fraga, 2007). Their consumption have been shown to help reduce the risk and prevent against cardiovascular diseases, cancer and neuronal diseases (Damianaki et al., 2000; Mennen et al., 2004). These pharmacological properties of flavonoids which are mostly related to their antioxidant potential have been postulated to be due to their structural features (Schroeter et al., 2002; Valko et al., 2006; Fraga, 2007). This enables them to scavenge reactive radical species, interfere with the oxidation of macromolecules and inhibit key endogenous free radicals generating enzymes such as NAD(P)H-oxidase, xanthine oxidase, tyrosine kinase by their hydrogen (electron) donating ability and their hydroxylation/methylation patterns (Terao, 1999; Engler and Engler, 2006; Stratil et al., 2006).

*Hibiscus sabdariffa* is a rich source of phenolic compounds (Ali et al., 2005). Numerous phenolic compounds have been identified in the plant extracts (Salah et al., 2002; Lin, 2005). *Hibiscus sabdariffa* is also rich in anthocyanins which is responsible for its

deep red colouration. Anthocyanins are polyphenolic compounds responsible for cyanic colours ranging from salmon pink through red and violet to dark blue of most flowers, fruits, leaves and stems. They comprise the largest group of the water-soluble pigments in the plant kingdom (Strack and Wray, 1994). Anthocyanins have received increasing attention over the last fifteen years due to their potential health effects, and they are nowadays regarded as important nutraceuticals. This is mainly due to their possible antioxidant effects, and they have been given a potential therapeutic role related to cardiovascular diseases, cancer treatment, inhibition of certain types of virus including the human immunodeficiency virus type 1 (HIV-1), and improvement of visual acuity (Cecchini et al., 2005; Cooke et al., 2005; Jang et al., 2005; Talavera et al., 2006).

Due to use of improved analytical techniques, about 575 anthocyanins with complete structure have been reported (Andersen and Jordheim, 2006). Even though there are around 30 different anthocyanidins, approximately 90% of all anthocyanins are based on the six most common anthocyanidins; pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin, which only differ by the hydroxylation and methoxylation pattern on their B-rings. The anthocyanins differ with respect to glycosylation of hydroxyl groups, nature of glycosyl units, substitution pattern, and potential aliphatic and aromatic acylation (Andersen and Jordheim, 2006). *Hibiscus sabdariffa* pigment has been reported to be rich in delphinidin-3-

sambubioside, delphinidin-3-monoglucoside, cyanidin-3-sambubioside and cyanidin-3-monoglucoside (Shibata and Furukawa, 1969; Du and Francis, 1973; Hong and Wrostad, 1990; Wong et al., 2002; Sukwattanasinit et al., 2007), although only few studies have been able to link the antioxidant potential of *Hibiscus sabdariffa* to its anthocyanins content.

The aim of this study was to investigate the *in vitro* antioxidant potential and chemical constituents of Hibiscus anthocyanins rich extract (HAE). Evidence is presented to suggest that *Hibiscus sabdariffa* is an effective antioxidant plant *in vitro* and this antioxidant potential may be related to the high concentration of polyphenolic antioxidants in the plant.

## 2. Materials and Methods

### 2.1. Reagents

All chemical used were of analytical grade. Special reagents were Cytochrome C, Xanthine, Xanthine Oxidase (Grade III from bovine milk), ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline 6-sulfonic acid), Folin - ciocalteu's Phenol reagent, Trolox (6-hydroxy - 2, 5, 7, 8, - tetramethyl-chroman - 2-carboxylic acid (C-stock -solution = 2.5mmol/L), Gallic acid and all other chemicals were purchased from Sigma -Aldrich Company Limited, Dorsert, United Kingdom.

### 2.2. Plant Material

Flowers of *Hibiscus sabdariffa* (Malvaceae) were bought at a market in Nigeria. The identification and authentication of the plant was done by Dr A.J. Ogunkunle at Department of Pure and Applied Biology, Ladoké Akintola University of Technology, Ogbomoso, where a specimen was deposited in the herbarium. The dried flowers were further dried at room temperature and blended to a coarse powder.

### 2.3. Preparation of Hibiscus Anthocyanins Rich Extract (HAE)

HAE was prepared from the dried flower of *Hibiscus sabdariffa* L. as described by Chang, et al., (2006). The dried flower (20g) was mixed with 120ml methanol containing 1% HCl for 24 hrs at 4°C. The extract was filtered and the filtrate was applied to an amberlite Diaion HP-20 resin column for 24h, and then washed with distilled water containing 0.1% HCl solution and eluted with methanol. The filtrate was concentrated in a rotary evaporator and then lyophilized to obtain approximately 0.5g of HAE and stored at -20°C before use.

### 2.4. Preparation of a Diethyl Ether Extract of HAE

Due to intense colouring of HAE, it was necessary to prepare a less intense extracts, which did not interfere with some of the spectrophotometric assays. One part of HAE was gently mixed with two parts diethyl ether and centrifuge at 2000rpm for 10 minutes. The diethyl ether extract was removed and

dried under a stream of oxygen-free nitrogen gas. The residue was then re-suspended in phosphate buffered saline (PBS) pH 7.2 to its original volume.

### 2.5. Superoxide and xanthine oxidase activity

This was carried out as reported by Dillion et al., (2003). Superoxide production and xanthine oxidase activity were measured as cytochrome C reduction and uric acid production, respectively. Xanthine oxidase was prepared to a concentration of 107mU/ml in Phosphate Buffered Saline (PBS), pH 7.2 and Xanthine was prepared as a 1.6mM solution also in PBS. Superoxide ions were generated in a reaction volume of 1ml containing 160µM xanthine and 1.25mg cytochrome C. The reaction was initiated by the addition of 10.7mU xanthine oxidase, and superoxide ion production was monitored at 550nm (Edwards, et. al., 1987). In a series of separate experiments, xanthine oxidase activity was monitored as the production of uric acid at 284nm. Generation of superoxide ions was confirmed by the addition of 50U superoxide dismutase (SOD), which inhibited the reduction of cytochrome C without affecting xanthine oxidase activity. Extracts were added at 0 – 10% (v/v). Results for superoxide production are expressed as  $\Delta A_{550\text{nm}}/\text{minute}$  whilst, result for uric acid production are expressed as  $\Delta A_{284\text{nm}}/\text{minute}$ .

### 2.6. Trolox Equivalent Antioxidant Capacity (TEAC) with Manganese dioxide

The assay was performed as previously described by Schelesier et al., (2002). The ABTS radical cation was prepared by filtering a solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2µm syringe filter. This solution was diluted in 5mM PBS pH 7.4, adjusted to an absorbance of  $0.700 \pm 0.020$  at 734nm and preincubated at room temperature prior to use for 2 hours. 1 ml of ABTS<sup>•+</sup> solution and various concentrations of the extracts (diluted with water) were vortexed for 45 seconds in reaction tubes, and the absorbance (734nm) was taken exactly 2 minutes after initiation of mixing. PBS blanks were run in each assay. The antioxidant activity of the extracts was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\% \text{ antioxidant activity} = (A_{(\text{ABTS}^{\bullet+})} - A_{(\text{Extracts})}) / (A_{(\text{ABTS}^{\bullet+})}) \times 100.$$

where  $E$  is the extinction.

### 2.7. Trolox Equivalent Antioxidant Capacity with Potassium Persulfate

The assay was performed essentially as described by Re et al., (1999). ABTS radical cation was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and allowing the

mixture to stand in the dark at room temperature for 12–24 h before use. The ABTS<sup>•+</sup> solution was diluted with water for the hydrophilic assay and with ethanol for the lipophilic assay and adjusted to an absorbance of  $0.700 \pm 0.020$  at 734nm. For the photometric assay, 1ml of the ABTS<sup>•+</sup> solution and various concentrations of the extracts were mixed for 45 seconds and measured immediately after 1 minute at 734nm. The antioxidant activity of the extracts was calculated by determining the decrease in absorbance at different concentrations by using the following equation.

$$\% \text{ antioxidant activity} = ((A_{(\text{ABTS}^{\bullet+})} - A_{(\text{Extracts})}) / (A_{(\text{ABTS}^{\bullet+})}) \times 100.$$

## 2.8. DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging Activity

The assay was performed as previously described by Schelesier et al., (2002). The radical solution is prepared by dissolving 2.4 mg DPPH<sup>•</sup> in 100 ml methanol. For the photometric assay 1.95 ml DPPH<sup>•</sup> solution and 50  $\mu$ l antioxidant solution were mixed. At first, the absorbance of the disposable cuvette with 1.95 ml DPPH<sup>•</sup> was measured as blank, then the antioxidant solution was added and mixed. The reaction was measured at 5 min interval at 515 nm until  $\Delta A = 0.003 \text{ min}^{-1}$ . The anti-oxidative activity was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\% \text{ Inhibition activity} = ((A_{(\text{DPPH}^{\bullet})} - A_{(\text{Extracts})}) / (A_{(\text{DPPH}^{\bullet})}) \times 100$$

## 2.9. Determination of Total Phenolic Compounds in HAE

The content of total phenolic compounds in HAE was determined by Folin–Ciocalteu method as described by Miliuskas et al., (2004). Briefly, 1 ml aliquots of 0.024, 0.075, 0.0105 and 0.3 mg/ml ethanolic gallic acid solutions were mixed with 5ml Folin-ciocalteu reagent (diluted ten-fold) and 4ml (75g/L) sodium carbonate. The absorption was read after 30 min at 20°C at 765 nm and the calibration curve was drawn. One ml of HAE (1mg/ml) was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c \cdot V/m'$$

Where: C-total content of phenolic compounds, mg/g plant extract, in GAE; c-the concentration of gallic acid established from the calibration curve,

mg/ml; V- the volume of extract, ml; m'- the weight of pure plant methanolic extract, g.

## 2.10 TLC Separation of Anthocyanin in HAE

Individual anthocyanins in anthocyanins rich extract of *Hibiscus sabdariffa* were isolated by TLC using merck 60 silica gel plates (0.2mm). For detection of anthocyanins the plates were developed using a mixture containing n-butanol, acetic acid and water in the ratio of 40:10:20 (v/v/v). Diluted HAE (10 $\mu$ l), and 10 $\mu$ l standards were loaded onto silica gel 60 TLC plates and left to dry. The plate was allowed to run, dried and then visualized under visible light.

## 2.11. HPLC Separation of Anthocyanin in HAE

The separation and identification of anthocyanin was performed according to the procedure describe by De Ancos et al., (2000). The anthocyanins in HAE were separated by HPLC, a symmetry shield RP 18 column (80A, 5 $\mu$ ) and UV/Visible detector (monitored at 530nm). The mobile phase was composed of 4 per cent phosphoric acid (solvent A) and 100 per cent acetonitrile (solvent, B). The solvent program was isocratic elution with 6% B from 0 to 10min, linear gradient to 20% B from 10 to 50min and isocratic elution at 20% B from 50 to 60 minutes. The flow rate was set at 1ml/minute.

## 2.12. HPLC Separation of Anthocyanindin in HAE

To standardised the Hibiscus anthocyanins, the delphinidin and cyanidin contained in the HAE were determine by HPLC, using a symmetry shield RP 18 column (80A, 5 $\mu$ ) and UV/visible detector (monitored at 540nm). The mobile phase was composed of 4% phosphoric acid (solvent A) and 100% Acetonitrile (solvent B) run on a gradient of 10% B to 50% B for 15minutes and monitored at 540nm. 1ml of the sample was boiled with 1ml HCl at 95°C for 30minute to assist in the process of hydrolysis and 10 $\mu$ l of this were injected into chromatography. The flow rate was set at 1ml/min.

## Statistical analysis

Results are expressed as means  $\pm$  SEM. Statistical analyses were performed using Student's t test; a  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Superoxide Scavenging Ability of HAE

Superoxide production by xanthine – xanthine oxidase gave a reaction rate of  $0.085 \pm 0.002 \Delta A_{550} \text{ nm/min}$  while xanthine oxidase activity gave a reaction rate of  $0.106 \pm 0.001 \Delta A_{284} \text{ nm/minutes}$ . At 5% (v/v) of the reaction volume, diethyl ether extract of HAE significantly inhibited superoxide production i.e the reduction of cytochrome C by 41.18% (Table 1). Superoxide production was inversely related to the concentrations of diethyl ether extract of HAE. The extract significantly affects uric acid production indicating that the components of HAE inhibit xanthine oxidase.

**Table 1** The effect of SOD, diethyl ether extracts of HAE on superoxide and uric acid production by xanthine-xanthine oxidase.

Extract/Agent	Superoxide Production ( $\Delta A_{550\text{nm}/\text{min}}$ )	Uric Acid Production ( $\Delta A_{295\text{nm}/\text{min}}$ )
None <sup>1</sup>	0.085 $\pm$ 0.002	0.106 $\pm$ 0.001
50U SOD	0.001 $\pm$ 0.001*	0.099 $\pm$ 0.002
5% diethyl ether extract of HAE	0.050 $\pm$ 0.001*	N.D

Values are means of three experiments  $\pm$  SEM. Significant differences ( $p < 0.05$ ) from control<sup>1</sup> (xanthine-xanthine oxidase only) are indicated by \*

### 3.2. ABTS Radical Cation Scavenging Ability of HAE

In the three versions of TEAC assay, TEAC II and TEAC III (hydrophilic and lipophilic version) the inhibition of ABTS<sup>•+</sup> radical cation was directly related to the concentration of the extracts and the TEAC value of Trolox is 1.00. HAE showed comparable antioxidant activity in TEAC II and TEAC III hydrophilic version but TEAC values analysed in TEAC III (lipophilic version) were much lower when compared with the other two TEAC assays (Table 2).

**Table 2** Trolox equivalent antioxidant capacities (TEAC) (Mmol/L) of Trolox, Gallic acid and HAE

Assay/antioxidant	Trolox	Gallic	HAE
TEAC II	1	3.96 $\pm$ 0.31	0.85 $\pm$ 0.07
TEAC III hydrophilic	1	3.34 $\pm$ 0.20	0.74 $\pm$ 0.04
TEAC III lipophilic	1	3.99 $\pm$ 0.16	0.67 $\pm$ 0.05

### 3.3. Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of HPE

The Hibiscus anthocyanin rich extract (HAE) demonstrated a concentration dependent scavenging activity by quenching DPPH radicals (data not shown) and was compared with gallic acid, as a positive control. The IC<sub>50</sub> values (defined as the concentration of test compound required to produce 50% inhibition) for DPPH scavenging by HAE and gallic acid were 260.7  $\pm$  6.4  $\mu\text{g}/\text{dL}$  and 16.33  $\mu\text{g}/\text{dL}$  respectively (Table 3).

**Table 3** Total phenolic content and DPPH radical scavenging value of HAE

Sample	Total Phenol <sup>a</sup>	DPPH Scavenging activity (IC <sub>50</sub> ) <sup>b</sup>
HAE	56.6 $\pm$ 3.7	260.7 $\pm$ 6.4
Gallic	–	16.3 $\pm$ 1.5

Each value represents the mean  $\pm$  SEM. ( $n=3$ ).

a Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.

b Expressed as  $\mu\text{g}/\text{mL}$

### 3.4. The Phenolic Content of HAE

The phenolic content of the three extracts of *Hibiscus sabdariffa* (HAE) were determined using a colorimetric assay namely Folin-Ciocalteu assay, and

by constructing a standard curve using Gallic acid as the standard. The total amount of phenolic compounds present in HAE was found to be 56.6  $\pm$  3.7 mg/g (Table 3)

### 3.5 Analysis of Anthocyanins in HAE by TLC

Thin layer chromatography (TLC) analysis was performed to compare the nature of anthocyanins present in HAE with two standard anthocyanidins (delphinidin and cyanidin). For comparative TLC analysis Silica gel 60 plates and an organic solvent system (n-butanol, acetic acid and water (40:10:20 v/v/v)) were employed. The plates were visualized with visible light. Under this system five well resolved bands were detected (Table 4).

**Table 4.** TLC separation of anthocyanins in anthocyanin rich extract (HAE). Values are the means of three experiments  $\pm$  SEM

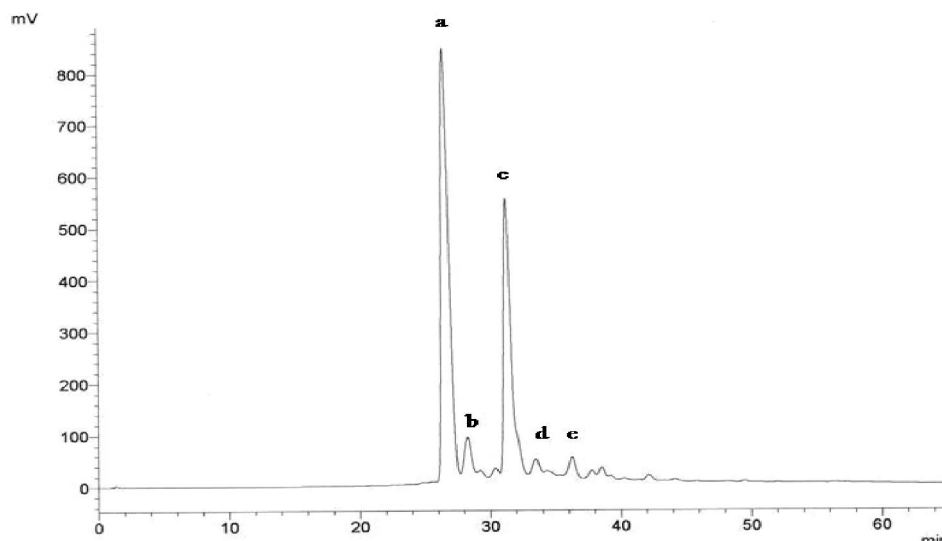
Standard/Sample	Standard Name/Band	Visible Light Rf value
Standards	Delphinidin Chloride	0.25
	Cyanidin Chloride	0.49
HAE	Band 1	0.19
	Band 2	0.25
	Band 3	0.32
	Band 4	0.51
	Band 5	0.71

### 3.6. Analysis of Anthocyanins in HAE by HPLC

Separation of anthocyanins in HAE was achieved using high pressure liquid chromatography (HPLC). The acetonitrile/water gradient system used allowed separation of anthocyanins compounds present in HAE by polarity with the most polar compounds eluted first. Five peaks were obtained (two major and three minor). The chromatogram (Figure 1), compared well with the results of previous workers, who separated and identified four anthocyanins in *Hibiscus sabdariffa* using HPLC (Hong and Wrolstad, 1990; Wong, et. al., 2002). In this study anthocyanins a-d (Figure 1) were identified by matching their retention times to those of the anthocyanins present in an authentic sample of

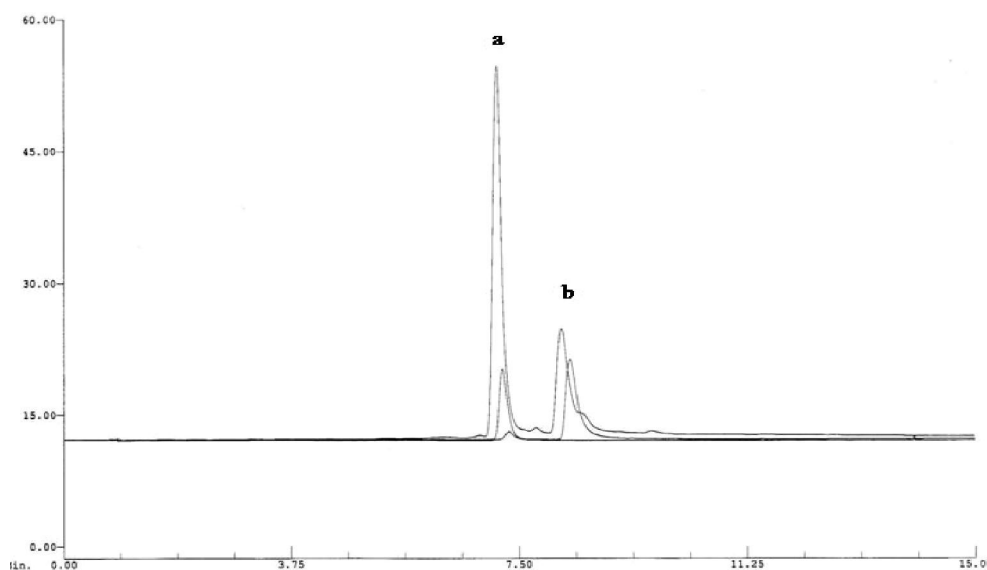
*Hibiscus sabdariffa*, as described by Hong and Wrolstad. The anthocyanins were expressed as percentage based on total peak area. The relative percentages distributed by delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin-3-sambubioside and cyanidin-3-glucoside in HAE were 56.85%, 4.50%, 36.60% and 1.00% respectively (Figure 1 & 3). Results of this study showed that delphinidin-3-sambubioside and cyanidin-3-sambubioside were the major anthocyanins present

and this corresponded to those reported previously by Hong and Wrolstad, (1990). In a separate study, HPLC analysis on Acid-hydrolysed HAE showed two peaks with retention time of 7.1 minutes and 8.18 minutes. These two peaks corresponded to peaks obtained with delphinidin and cyanidin standards (Figure 2 & 3). This showed that delphinidin and cyanidin are the major anthocyanins in *Hibiscus sabdariffa*.



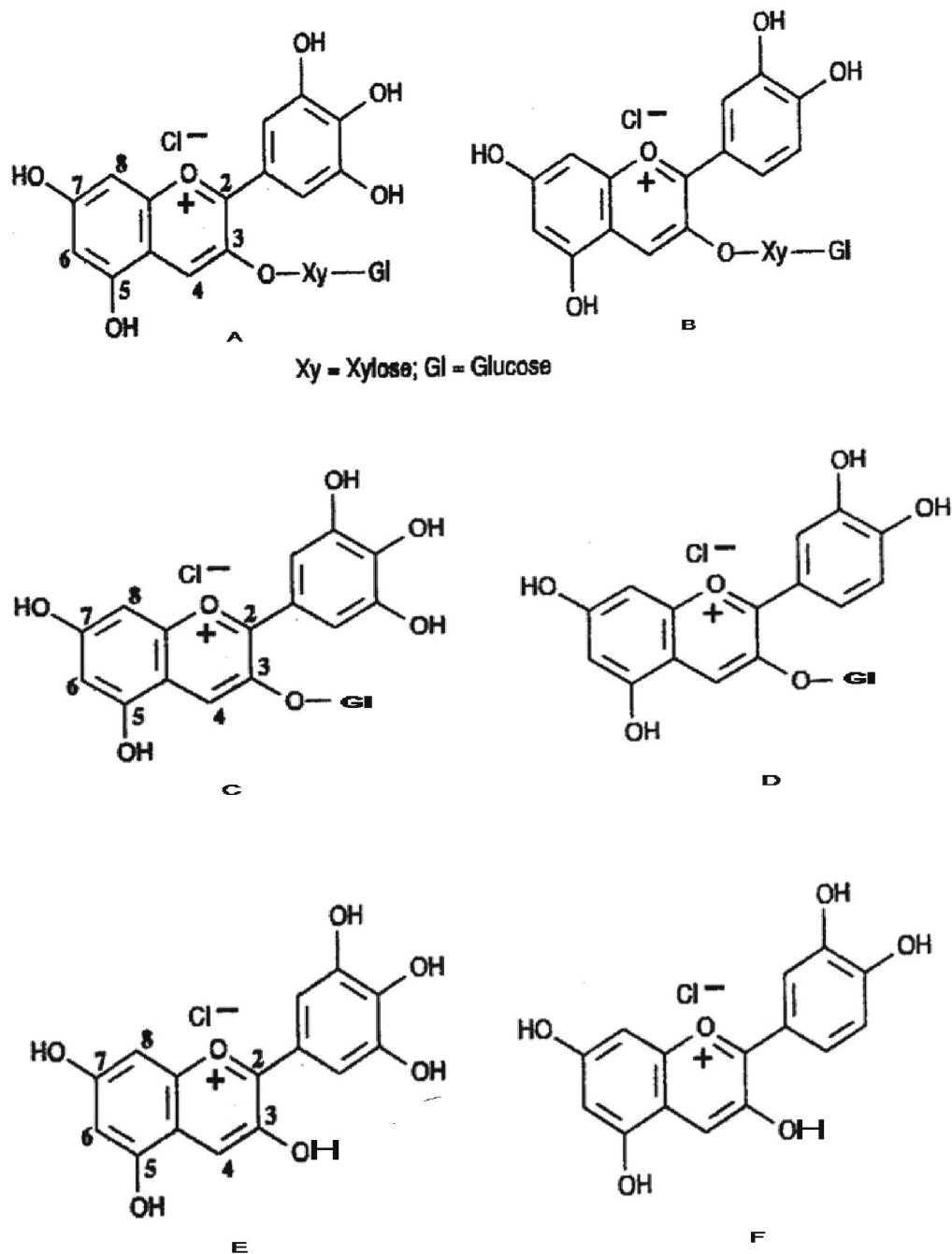
**Figure 1 A**  
areas calc  
delphinidin  
unknown (1  
experiment

lative peak  
56.8%); b.  
(1.0%); e.  
37.6%. All



**Figure 2 A** typical HPLC chromatogram of acid hydrolysed HAE. Peak (a) co-elute with delphinidin at 7.11 minutes and Peak (b) co-elute with cyanidin at 8.18 minutes. All experiments were repeated three times.





**Figure 3** Chemical structures of anthocyanin and anthocyanidin identified in HAE. (A) delphinidin-3-sambubioside (B) cyanidin-3-sambubioside (C) delphinidin-3-monoglucoside (D) cyanidin-3-monoglucoside (E) delphinidin (F) cyanidin.

#### 4. Discussions

*Hibiscus sabdariffa* has been demonstrated to be rich in polyphenols such as flavonoids, phenolic acids and anthocyanins (Ali, et. al., 2005; Salah, et. al., 2002; Lin, et. al., 2005). Studies suggest that plant polyphenols such as the flavonoids and anthocyanins

are potent antioxidant compounds both *in vitro* and *in vivo* (Fraga, 2007). Therefore it would not be surprising if the antioxidant properties associated with *Hibiscus sabdariffa* is related to the flavonoids and anthocyanins components of the plant.

This study investigate the antioxidant potential of Hibiscus anthocyanins rich extract (HAE). The result suggest that diethyl ether extract of HAE did not scavenge superoxide radical but rather inhibited xanthine oxidase in the production of the radical. This result therefore supports the works of Tseng et al., (1997) that *Hibiscus sabdariffa* and its constituents have inhibitory effect on xanthine oxidase activity. In addition, this study demonstrates that, the extract has ABTS<sup>•+</sup> and DPPH radicals cation scavenging ability. *Hibiscus sabdariffa* is a rich source of a plant polyphenols such as flavonoids and phenolics acids (Salah, et. al., 2002; Lin, et. al., 2005; Jamilah, 2014). The results presented in this study suggest the same is true for HAE. Using Folin-Ciocalteu assay HAE was shown to have high phenolic content (Table 3).

The pigment of *Hibiscus sabdariffa* is rich in anthocyanins (Hong and Wrolstad, 1990; Wong, et. al., 2002; Du and Francis, 1973). The anthocyanins composition of HAE was determined in this study. The results obtained through various methods of analysis suggest the presence of delphinidin-3-sambubioside, delphinidin-3-monoglucoside, cyanidin-3-sambubioside and cyanidin-3-monoglucoside as the major anthocyanins and delphinidin and cyanidin as major anthocyanidin in HAE, these results support earlier findings (Hong and Wrolstad, 1990). Anthocyanins have been reported to exert multiple biological effects, such as, anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities (García-Alonso, et. al., 2004; Weisel, et. al., 2006). Also, high antioxidant activity of anthocyanins has been demonstrated in many studies (Weisel, et. al., 2006). As observed in this study, a substantial proportion of the total phenolics in *Hibiscus sabdariffa* is represented by monomeric anthocyanins, and since anthocyanins are known to exhibit antioxidant activities, this group of compounds would therefore play a significant role in antioxidant effect demonstrated by *Hibiscus sabdariffa*, although it is, not yet clear, whether the protective potential of *Hibiscus sabdariffa* *in vivo* can be ascribed to this class of compounds alone.

It is however important to note that the extent of the anthocyanins and other polyphenolic compounds, antioxidant potential in humans, and other observed positive health effects studied *in vitro*, are of course *in vivo* dependent on the absorption, metabolism, distribution, and excretion of these compounds within the body after ingestion (Rice-Evans and Parker, 2003). In addition, since plants also contain other antioxidant micronutrients such as vitamin C,  $\beta$ -carotene and lycopene, it has to be kept in mind that their antioxidant efficacy may not be attributed alone to the presence of these phenolic compounds (Bonorden and Pariza, 1994).

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