Original Research Article

Identification of Phenolic Compounds in *Hibiscus sabdariffa* Polyphenolic Rich Extract (HPE) by Chromatography Techniques

ABSTRACT

The study was designed to determine the phenolic constituents of *Hibiscus sabdariffa* polyphenolic rich extract (HPE), (a group of phenolic compounds occurring in the dried red calyx of *Hibiscus sabdariffa*). While colorimetric analysis revealed that HPE contain high level of total phenolic content (97.9 ± 1.31 mg/g in GAE/g dried weight) combination of results obtained through several chromatographic analyses indicates that phenolic compounds such as ferulic acid, chlorogenic acid, naringenin, rutin and quercetin may be present in HPE. These phenolic compounds identified in HPE are known for possessing one pharmacological properties or the other, therefore the presence of these antioxidants phenolic compounds in HPE along with several other phenolics not identified in this study would explain the medicinal principle behind the ethnomedical practices associated with *H. sabdariffa.*

Key Words: *Hibiscus sabdariffa*, Antioxidants, ethnomedical, phenolic compounds.

1. INTRODUCTION

There is compelling epidemiological evidence linking a greater consumption of diet rich in fruit and vegetables with decrease risk of cancer and cardiovascular disease associated with oxidative damage to biomolecules [1] [2]. These foods contain phytochemicals with inherent antioxidant properties, such as Vitamin E, Vitamin C, Carotenoids and phenolic compounds [3] [4]. Phenolic

compounds are in particular presently gaining acceptance as responsible for the health benefits offered by most fruit and vegetables [5]. Studies suggest that plant polyphenols such as the flavonoids are potent antioxidant compounds both *in vitro* and *in vivo*. Their consumption has been shown to help reduce the risk and prevent against cardiovascular diseases [6]. *H. sabdariffa* is a rich source of phenolic compounds [7]. Numerous phenolic compounds such as quercetin, luteolin, chlorogenic acid, protocatechuic acid, catechin, epigallocatechin, epigallocatechingallate and caffeic acid have been identified in the plant extracts [8] [9]. In addition, *H. sabdariffa* is particularly rich in anthocyanins which is responsible for the deep red colouration of the red variety.

There is increasing need to know the phytochemical profiles of antioxidants in different plants, however, conventional chromatographic techniques (i.e Paper Chromatography, Thin Layer Chromatography and Column Chromatography) in general lack the sensitivity and resolution that are often required for trace amount of antioxidant phytochemicals. Gas chromatography (GC) meets these requirements, but its use is somewhat limited due to the non-volatility of many antioxidants. High performance liquid chromatograpy (HPLC) is presently, perhaps the most popular and reliable system among all chromatographic separation techniques for the separation of antioxidant phytochemicals. Recently, a new hyphenated technique, liquid chromatography/mass spectrometry (LC/MS) has proved to be a powerful and reliable analytical approach for structural analysis of components in herbal extracts with high sensitivity and low consumption of samples [10] [11] [12]. Although, LC/MS is very expensive and consequently not widely used in routine laboratories, it is advantageous in that it provides high resolution and rapid compound identification without the need to isolate individual compounds [13].

The aim of the study was to determine the chemical constituents of *Hibiscus sabdariffa* polyphenolics rich extract (HPE) extracted from calyx of red variety of *H. sabdariffa*. Initial study examined the total phenolic contents of HPE while subsequent studies examined the phenolic nature of HPE using spectrophotometric assays, thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and liquid chromatography – mass spectrophotometery (LC-MS).

1. MATERIALS AND METHODS

2.1. CHEMICALS

All chemical used were of analytical grade. Special reagents were 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 (C-stock-solution) = 0.568mmol/L), Catechin hydrate, Salicyclic acid; Ferulic acid; Potassium Peroxodissulfate; Quercetin; *p*-Coumaric acid; Caffeic acid; Rutin hydrate; and all other chemicals were purchased from Sigma -Aldrich Company Limited, Dorsert, United Kingdom.

2.2. Plant Material

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

2.3. Preparation of *Hibiscus sabdariffa* Polyphenol Rich Extract (HPE)

HPE was prepared according to the method of Lin *et al*. [9]. Briefly, 100 g of *Hibiscus sabdariffa* calyx were extracted three times with 300 ml of methanol at 50 ^{0}C for 3 hours. The samples were filtered after each extraction and the solvent was removed from the combined extracts with a vacuum rotary evaporator. The residue was then dissolved in 500 ml of water (50 ^{0}C) and extracted with 200 ml hexane to remove some of the pigments (i.e. chlorophyll, caratenoids). The aqueous phase was extracted three times with 180 ml ethyl acetate, and the ethyl acetate was evaporated under reduced pressure. The residue was re-dissolved in 250 ml water and was lyophilized to obtain approximately 1.5 g of HPE and stored at -20 ^{0}C before use.

2.4. Determination of Total Phenolic Compounds in HPE

The content of total phenolic compounds in HPE was determined by Folin–Ciocalteu method as described by Miliauskas *et al*. [14]. Briefly, l ml aliquots of 0.024, 0.075, 0.0105 and 0.3 mg/ml ethanolic gallic acid solutions were mixed with 5 ml Folin-ciocalteu reagent (diluted ten-fold) and 4 ml (75 g/L) sodium carbonate. The absorption was read after 30 min at 20 ^{0}C at 765 nm and the calibration curve was drawn. One ml of HPE (1 mg/ml) was mixed with the same reagents as described above, and after l hour 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, in g.

2.5. Thin Layer Chromatography (TLC) Analysis of HPE

HPE and standard compounds were dissolved in methanol to a concentration of 1 mg/ml. Diluted 10 µl of HPE and standards were loaded onto silica gel 60 F_{254} TLC plates and left to dry. The plates were run for ~1h in an ethylacetate : methanol : water (10:2:1, v/v/v) solvent system. The plates were dried for 15 minutes at 115 ^{0}C, left to cool, and then visualized using UV light, iodine vapour and ferric chloride spray reagent (2.7% w/v in 2 M HCl). In all instances, the distance moved by the sample/standard was divided by the distance moved by the solvent front to obtain the R_f Value.

2.6. HPLC Analysis of HPE

The HPLC method employed a 5 µ R*P*-18 column. *Hibiscus sabdariffa* polyphenol rich extract (HPE) and various polyphenolic standards were filtered through a 0.45 µm filter disc and 20 µl were injected onto the column. The chromatography was monitored at 280 nm. The mobile phase contained two solvents (A, 0.1 % formic acid; B, 100 % methanol) run by bi-gradient method at room temperature as follows: 5 % B to 5 % B for the first 10 minutes, 5 % B to 50 % B for the next 20 minutes and maintained at 50 % B for final 20 minutes. The flow rate was 1 ml/minute.

2.7. Trolox Equivalent Antioxidant Capacity with Potassium Persulfate

The assay was performed essentially as described by Re *et al*. [15]. 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 hours before use. The $ABTS^{\bullet+}$ solution was diluted with water and adjusted to an absorbance of 0.700 ± 0.020 at 734 nm. For the photometric assay, 1 ml of the $ABTS^{\bullet+}$ solution and eluent of peaks obtained during HPLC analysis of HPE were mixed for 45 seconds and measured immediately after 1 minute at

734 nm. The antioxidant activity of each peak was calculated by determining the decrease in absorbance by using the following equation:

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

2.8. LC-MS Analysis of HPE

The analyses of polyphenol in HPE were carried out on a Waters Alliance 2695 HPLC system (Waters, Milford, MA), Micromass (Manchester, U.K.) LCT mass spectrometer equipped with Z-spray ESI source and MassLynx Software version 4.0 (Micromass, Manchester, U.K.). The separation was performed using a symmetry shield RP 18 column (80 A, 5 µ). The mobile phase for the HPLC analysis of HPE consisted of two solvents (A, 0.1 % formic acid; B, 100 % methanol) run by bi-gradient method at room temperature as follows: 5 % B to 5 % B for the first 10 minutes, 5 % B to 50 % B for the next 20 minutes and maintained at 50 % B for the final 20 minutes. The flow rate was 1 ml/minute while the UV-vis spectra were detected at 280 nm. The MS detector operated at capillary voltage 3500 V, extractor voltage 1 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas flow (N_2) 17 L/h, desolvation gas flow (N_2) at 600 L/h and mass acquisition between 100 and 1500 Da. The identification of HPE constituents was carried out by comparison of retention times, UV-vis, and MS spectra with standards protocatechuic acid, catechin, caffeic acid, rutin and literature data.

Statistical analysis

Results are expressed as means ± SEM. One way analysis of variance followed by Tukey's test was used to analyze the results with $p<0.05$ considered significant.

3. RESULTS

3.1. The Phenolic Content of HPE

The total amount of phenolic compounds present in HPE was found to be 97.9 ± 1.31 mg/g.

3.2. Analysis of Phenolic Compounds in HPE by TLC

HPE was well resolved and five bands were detected with ferric chloride and four well resolved blue fluorescent bands detected with UV light (Table 1). The fluorescence bands obtained further indicated that they were phenolic in nature. When iodine vapour was used to visualise the plates twelve visible brown bands were observed (Table 1).

Table 1. The R_f values for standard phenolic compounds and HPE sample visualized with Ferric Chloride, UV light and Iodine Vapour.

Phenolic standards	Ferric Chloride		UV Light		Iodine Vapour	
	Standards	HPE	Standards	HPE	Standards	HPE
Chlorogenic acid	0.13	0.13	0.12	0.13	0.13	0.13
Caffeic acid	0.67	0.65	0.68	0.67	0.66	0.67
Rutin	0.33	0.24	0.32	0.26	0.28	0.26
Epigallocatechingallate	0.58		0.55	0.58	0.56	0.58
Gallic acid	0.62	0.60	0.63		0.62	0.63
Salicyclic acid	0.42	0.37	0.42		0.45	0.44
Quercetin	0.76		0.75		0.75	0.73
Procatechuic acid	0.65		0.66		0.66	0.18
Ferulic acid	0.69		0.70		0.67	0.33
Coumaric acid	0.70		0.71		0.68	0.39
Catechin	0.71		0.72		0.69	0.47
Naringenin	0.84		0.80		0.81	0.53

3.3. Analysis of phenolic compounds in HPE by HPLC.

Fourteen main peaks were obtained when HPE was analysed with HPLC (Figure 1). The fourteen peaks were grouped into three major fractions (Fraction 1 (peaks 1-6), fraction 2 (peaks

7-9) and fraction 3 (peaks 10-14)) base on result obtained from the plot of antioxidant activity against the peak number, for ease of measurement (Figure 2**)**. When retention times of HPE peaks were compared with the retention times of standard plant phenolic compounds: (1) Gallic acid, 4.02 (2) Protocatechuic acid, 4.82 (3) Chlorogenic acid, 17.66 (4) Catechin hydrate, 18.28 (5) Caffeic acid, 19.31 (6) EGCG, 22.15 (7) *p*-Coumaric, 23.02 (8) Ferulic acid, 25.04 (9) Naringenin, 29.65 (10) Rutin, 30.22 (11) Quercetin, 34.24; the result suggest possible presence of Protocatechuic acid, *p*-Coumaric acid, Naringenin and Rutin in HPE (Figures 1 & 3).

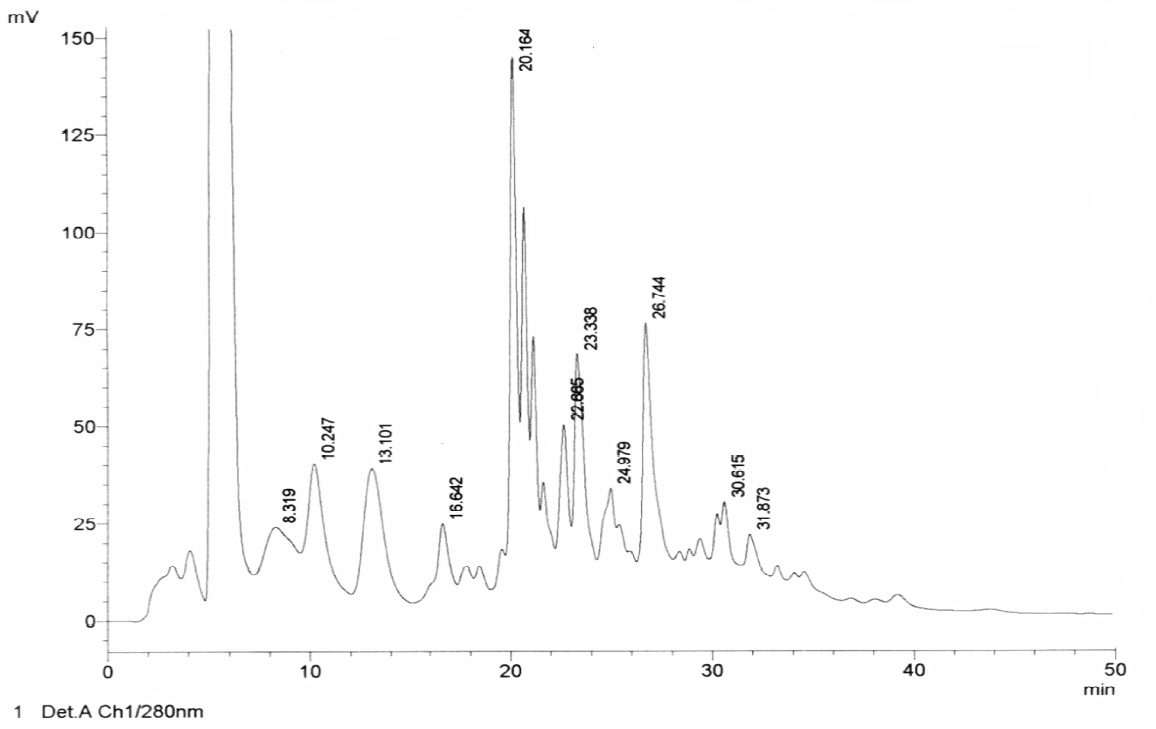

Figure 1. A typical HPLC analysis of HPE diluted with methanol.

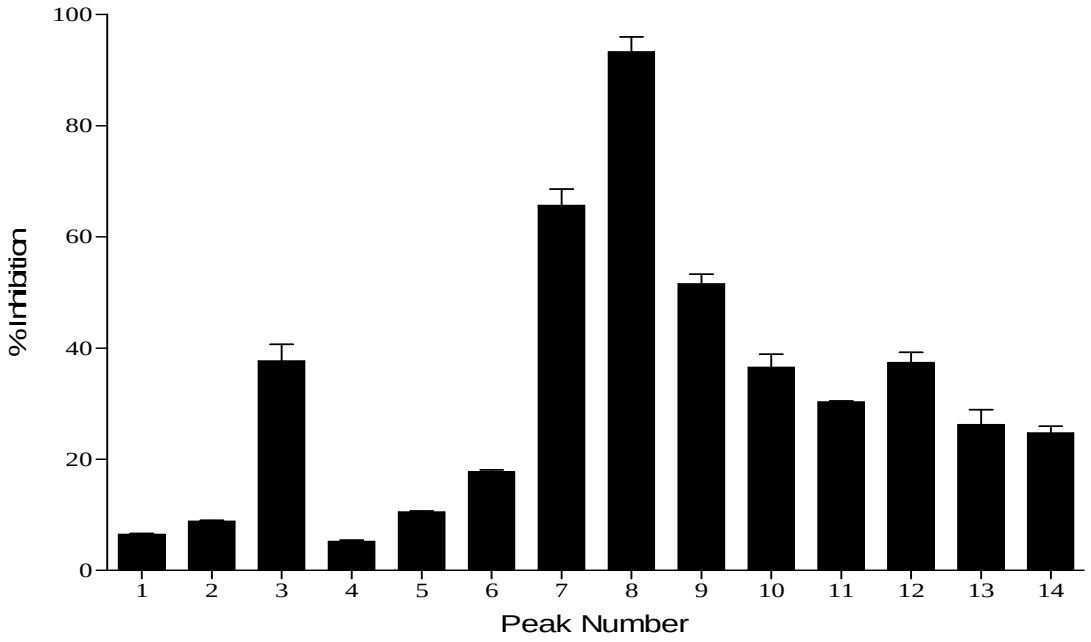

Figure 2. Antioxidant activity of HPE peaks obtained in HPLC analysis

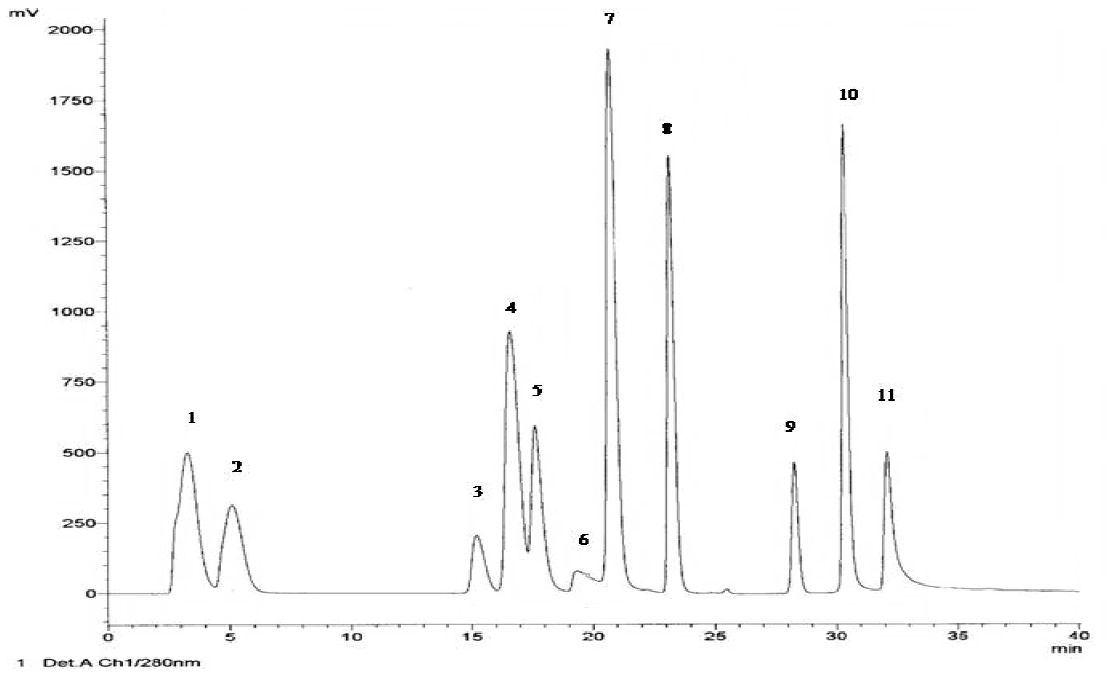

Figure 3. A typical HPLC chromatogram of standard phenolic compounds.

3.4. Analysis of phenolic compounds in HPE by LC-MS

Positive ESI-MS was used to identify the major compounds in Fraction 1, 2 and 3 collected from HPLC analysis of HPE. Authentic caffeic acid, catechin and rutin were analyzed first to determine their retention times and positive mass spectra values. At reasonable experimental conditions, m/z 611, 291 and 181 $[M + 1]^+$ were always observed in the positive mode for rutin, catechin and caffeic acid respectively (Table 2). Compounds in HPE were identified on the basis of their chromatographic retention times and positive mass spectra by comparison with reference standards or with literature data. Fraction 2 gave a retention time 18.6 minutes and MS peak at m/z 355 $[M + 1]^+$ value characteristic of chlorogenic acid, the same fraction 2 also gave a retention time 21.3 minutes and MS peak at m/z 195 $[M + 1]^+$ value characteristic of ferulic acid, while fraction 3 gave retention times 24.5, 26.6 and 31.3 minutes with corresponding MS peak at m/z 273, 611, and 303 $[M + 1]^+$ values characteristic of naringenin, rutin and quercetin respectively (Table 3).

Table 2. The m/z positive values and retention time of standard phenolic compounds.

Standards Phenolic Compounds	Retention time	M + 1 Values
Caffeic acid	17.5	181.0
Catechin	16.4	291.0
Rutin	26.1	611.0

Table 3. Spectra properties of HPE constituents.

Fraction Number	RT (min)	λ_{max} (nm)	m/z $(M+1)^+$	Compound
2	18.6	235, 325	355	Chlorogenic acid
2	21.3	235, 325	195	Ferulic Acid
3	24.5	290, 330	273	Nanringenin
3	26.6	290, 330	611	Rutin
3	31.3	290, 330	303	Quercetin

1. DISCUSSION

The results presented here identified the presence of different types of polyphenolics in HPE after several analyses with different analytic techniques. It is likely that Chlorogenic acid, Ferulic acid, Naringenin, Rutin and Quercetin are present in HPE. Although, the presence of flavan-3-ols compounds (catechin, epigallocatechin, epigallocatechingallate) earlier identified to be present in HPE by Lin *et al.* [9] cannot be completely inferred from this study, their presence however, in HPE cannot be totally excluded as peaks and elution volume corresponding to the standards of these phenolic compounds and that of protocatechuic acids, caffeic acid and *p*-coumaric were obtained in the HPLC study. Although many other studies have identified phenolic compounds in *H. sabdariffa*, it is noteworthy to state that most of the compounds previously detected were identified by reverse phase-HPLC based only on retention values and UV spectra [8] [9]. The present report makes use of HPLC-MS for the peak identification.

All the phenolic compounds identified in HPE are known for possessing one pharmacological properties or the other, for example Ferulic acid is a well known natural protector against

ultraviolet radiation known to cause skin disorder such as cancer and aging of the skin [19], and its antidiabetic, anticarcinogenic, hepatoprotective and hypotensive properties have also been demonstrated in many previous studies [20] [21]. It is believed these therapeutic effects of ferulic acids are due to its antioxidants and anti-inflammatory activity [22]. Chlorogenic acid has been reported to possess anxiolytic, antibacterial, antimutagenic, anti-inflammatory, antitumor and antiviral properties, as well as acting as an antioxidant by radical-scavenging and metal chelation [23] [24] [25], while naringenin has been shown to protect against doxorubicin-induced apoptosis, and doxorubicin-induced cardial toxicity in rats [26] effects which could underlie its use as therapeutic agent for treating or preventing cardiomyopathy associated with doxorubicin [27]. It has also been demonstrated to protect against cisplatin induced nephrotoxicity in rat [28] and inhibit tumor growth in various cancer cell lines and sarcoma S-180-implanted mice [29]. Recently, naringenin has been shown to protect against UVB-induce apoptosis, making it a promising natural flavonoid in preventing against skin aging and carcinogenesis [30].

Both quercetin and rutin have been demonstrated to have neuroprotective effects against spatial memory impairment and neuronal death induced by repeated cerebral ischemia in rats [31]. They have also been demonstrated to have anti-inflammatory, antioxidant and antidepressant activities in many studies [32] [33]. Therefore, it can be inferred that the presence of these antioxidants phenolic compounds (ferulic acid, chlorogenic acid, naringenin, rutin and quercetin) in HPE would explain the medicinal principle behind the ethnomedical practice associated with *H. sabdariffa*. Nevertheless, other phenolic constituents not identified in this study could be working synergistically with these phenolic compounds giving a larger antioxidant effect.

Based on the results obtained in this study polyphenols are by far the major antioxidant constituents of *H. sabdariffa*, therefore this class of compounds appears to be of major relevance for the observed preventive effects of this plant. Ascorbic acid, due to its high bioavailability and concentration (141.09mg/100g) [34], might also contribute, although its contribution to the observed effects may not be of major relevance. All attempts to determine β-carotene and lycopene in crude methanolic extract of *H. sabdariffa* used for this work did not achieve the desired goals despite using several techniques. These lipid soluble dietary antioxidants have been shown to be present in low concentrations in *H. sabdariffa*, i.e. β-carotene (1.88mg/100g) and lycopene (164.34µg/100 g) [34]. The inability to find any lycopene and β-carotene in this study could be due to several factors which could include, sensitivity of techniques used for determination, use of dried calyx, instead of fresh calyx as well as use of lower quantity of *H. sabdariffa*, i.e 2 g as against 100 g used by the previous workers [34]. Therefore, medicinal values of *H. sabdariffa* cannot be ascribed to β-carotene and lycopene.

Many bioavailability studies on polyphenols have indicated a rather poor absorption from the gastrointestinal tract on the basis of measuring parent compound concentrations in plasma and urine [35]. In addition, polyphenols are subject to phase II metabolism after absorption, yielding methoxylated, glucuronidated, and sulfated compounds [36]. This may greatly influence their bioactivity, but only a few studies have examined this to date. Therefore further studies would be needed to know if the amount of polyphenols absorbed through consumption of *H. sabdariffa* is sufficient to exert significant antioxidant effects.

5. CONCLUSION

The results of phytochemical analysis in HPE through several chromatographic analyses indicates that phenolic compounds such as ferulic acid, chlorogenic acid, naringenin, rutin and quercetin may be present in HPE. The presences of these antioxidants phenolic compounds in HPE gives an insight into understanding the mechanism of antioxidant activities of *H. sabdariffa* and explain the medicinal principle behind the ethnomedical practices associated with the plant.

CONSENT

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist

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