

ORIGINAL RESEARCH

BIOACTIVE COMPOUNDS AND ANTIOXIDANT EVALUATION OF METHANOLIC EXTRACT OF HIBISCUS SABDARIFFA

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Abstract

Hibiscus Sabdariffa (Roselle) is renowned for its delicacy and healing properties. The study aimed at evaluating the bioactive compounds (phytochemicals) and antioxidant activity of leaf, stem, and calyx of roselle, extracted with methanol. Phytochemicals were identified using numerous test methods and quantified with a UV-Vis spectrophotometer. The screening test revealed a higher number of phytochemicals in the calyx extract than the leaf or stem. With the quantification, the highest concentration was recorded in the calyx, followed by the leaf and then the stem. The antioxidant activity of the investigated plant was in the order of calyx>leaf>stem. Higher recoveries, ranging between 90%-110%, was obtained for all compounds. Therefore, this study provides evidence that the leaf, stem, and calyces of roselle plants are the probable source of antioxidants and many bioactive compounds, justifying their ethnopharmacological use in conventional medicines.

KEYWORDS:

Antioxidant Evaluation, Bioactive Compounds, Hibiscus Sabdariffa, UV-Vis Spectrophotometer

1 | INTRODUCTION

The Hibiscus Sabdariffa plant, usually known as Roselle, which is innate to India and Malaysia, is presently found in several tropical and sub-tropical countries of Africa, Asia, and the Americas^[1]. Hibiscus Sabdariffa belongs to the family Malvaceae, which normally survives in comparatively poor soils^[2]. The plant is a yearly, erect, herbaceous sub-shrub with a deep root system. It is nurtured for its leaves, stem, seed, and calyces^[2].

The calyces of H. Sabdariffa have been the focus of many investigations and are identified to contain polyphenolic acids, flavonoids, and anthocyanins delphinidin-3-O-glucoside, delphinidin-3-O-sambubioside, and cyanidin-3-O-sambubioside^[3]. In Ghana, the hot water extract of roselle calyces is used in the production of a local non-alcoholic beverage (well known in the



FIGURE 1 Hibiscus Sabdariffa plant displaying leaf, stem, and gloomy red calyces.

local dialect as “sobolo”) and as food colorings, which is a good source of natural carbohydrate, protein and vitamin C^[4]. In traditional medicine systems, an infusion from the calyces is used as a diuretic and to treat gastrointestinal disorders, liver diseases, fever, hypercholesterolemia, and hypertension^[5]. The extract from the roselle plant has also been recounted to possess therapeutic value. It includes an antihypertensive, antiseptic, and astringent diuretic. The extract also has purgative activities remedy for cancer, abscesses, cough, dysuria, laxative, scurvy, and fever^[6]. Phytochemicals are bioactive elements establish in plants. They include tannins, alkaloids, saponins, flavonoids, steroids, and others^[7]. These bioactive elements of plants have lately become more popular and of great interest to researchers, owing to their countless medicinal uses. They have complementary and overlapping mechanisms of action in the body, including antioxidant effects, modulation of detoxification, and enzymes stimulation of the immune system, modulation of hormone metabolism, anti-bacteria, and antiviral effects^[8]. The quantity of bioactive components in plants is influenced by agricultural management in which they are grown (fertilization, cultivation practices, etc.). The efficacy of these phytochemicals depends on their concentration in the plants^[9].

Antioxidants are considered to be active inhibitors of carcinogenesis and other circumstances that are pathogenically linked with oxidative mechanisms. Reports have revealed that a lot of plants are a rich source of antioxidants. For instance, phenolic compounds such as flavonoids, tannins, and lignins in plants function as antioxidants^[10]. Several antioxidants perform a significant role in lessening inflammation, delaying aging, and averting cancers^[11]. Studies have revealed the bioactive compounds and antioxidant activity of Hibiscus sabdariffa, frequently in the calyces and occasionally in the plant’s leaf. However, little or no data on the stem has been reported, which parades the plant’s worthiness in all scopes. This study aims to identify and quantify bioactive compounds (phytochemicals) and evaluate the antioxidant activity in methanolic leaf, stem, and calyx extracts of *H. Sabdariffa*. A plate of *H. sabdariffa* (roselle) plant is shown in Figure 1.

2 | MATERIAL AND METHOD

2.1 | Collection and Identification of Plant Material

The plant materials (*Hibiscus Sabdariffa*) were collected on March 15th, 2018, from the Science market, University of Cape Coast, Cape Coast, Ghana. It was then identified and authenticated by a plant taxonomist at the herbarium of the Department of Agriculture, University of Cape Coast.

2.2 | Preparation of Plant Material

The freshly collected leaf, stem, and calyx of *Hibiscus Sabdariffa* were cautiously cleaned and air-dried in a ventilated room for five weeks. The dried leaf, stem, and calyces were pounded into a coarse powder with a mortar and a pestle. The powdered plant materials were reserved in sealed containers for extraction.

2.3 | Extraction of Plant Material

The plant materials were extracted using the cold maceration method of extraction, as described by Ncube et al.^[12]. Thirty grams (30 g) each of the coarsely powdered leaf, stem, and calyx of *H. Sabdariffa* was kept in contact with 250 mL of methanol in stoppered containers and allowed to stand at room temperature for three days with frequent agitation. The mixtures were then filtered through Whatman No.1 filter paper (125 mm) to get filtrate as extracts. The extracts were concentrated at 65 °C to one-quarter of the original volume using a rotary evaporator (Stuart RE 400, United Kingdom). The dried extracts were then stored in desiccators and labeled until usage.

2.4 | Phytochemical Examinations

We conducted three sequential examination processes. The three processes are preliminary phytochemical screening test, quantitative phytochemical analysis, and antioxidant activity determination. For the preliminary phytochemical screening tests, the standard qualitative methods by Trease and Evans^[13], Cannel^[14] and Adegoke^[15] were employed to screen for the presence of phenols, flavonoids, alkaloids, anthocyanins, anthraquinones, cardiac glycosides, terpenoids, tannins, steroids and saponins in the methanolic leaf, stem and calyx extracts of *Hibiscus sabdariffa*. The phytochemical screening tests were expressed as positive (+) for presence and negative (-) for the absence of a particular phytochemical. There are ten different tests.

The first test is the test for Phenols (Ferric Chloride Test). Two milliliters (2 mL) of 5% aqueous ferric chloride was added to 0.2 g of the extracts. The formation of bluish color indicated the presence of phenols in the extracts^[15]. The second test is for Flavonoids (Alkaline Reagent Test). Six drops of 2% NaOH solution was added to 0.2 g of each extract. The formation of intense yellow color, which developed into a colorless solution upon the addition of dilute acid, indicated the presence of flavonoids in the extract^[15]. The third test is for Alkaloids (Mayer's Test). Precisely 0.5 g of each extract was dissolved in 5 mL of 1% dilute HCl and filtered. The filtrate was treated with Mayer's reagent (Potassium mercuric iodide). The formation of a yellow-colored precipitate gave a positive result for alkaloids in the extract^[13, 15]. The fourth test is for Anthocyanins (Ammonia-HCl Test). To the 2 ml of the extract, 2 mL of 2 N HCl were added. The appearance of a pink-red color that turned purplish-blue after the addition of ammonia indicated anthocyanins^[13]. The fifth test is for Anthraquinones (Borntrager's Test). To the 10 mL of benzene, a small quantity of the extract was added and shaken. The content was filtered, and 5 mL of 10% ammonia solution was added to the filtrate, and the mixture then was shaken. No change in color in the ammoniacal layer (lower phase) indicates free anthraquinones^[14].

The sixth test is for Cardiac Glycosides (Kella-Killani Test). The five milliliters (5 mL) of glacial acetic acid containing a trace of ferric chloride was added to 0.5 g of the extract in a 20 mL test tube. The test tube was held at 45 °C, and 1 mL of concentrated H₂SO₄ was carefully added down the side. A purple ring color at the interface indicated the presence of cardiac glycoside^[14]. The seventh test is for Terpenoids (Salkowski's Test). The half a milliliter (0.5 mL) of chloroform was added to 0.1 g of extract, followed by 1 mL of concentrated sulphuric acid. The formation of reddish-brown precipitate gave the presence of terpenoids in the extract^[13]. The eighth test is for Tannins (Ferric Chloride Test). To the 0.2 g of the extract, an equal volume of distilled water was added in a test tube, followed by three drops of dilute ferric chloride. The formation of brownish blue or dark color indicated tannins in the extract^[15]. The ninth test is for Steroids (Liebermann-Burchard's Test). The half a gram (0.5 g) of each extract was mixed with 2 mL of chloroform. Two milliliters (2 mL) of concentrated sulphuric acid was then added to the mixture in a

test tube. The formation of red color in the lower chloroform layer showed steroids' presence in the extract^[15]. The last test is for Saponins (Foam Test). The six milliliters (6 mL) of distilled water was added to 0.2 g of the extract and shaken vigorously in a graduated cylinder for 15 minutes. The development of bubbles or persistent foam for 10 minutes indicated the presence of saponins in the extract^[14].

2.5 | Quantitative Phytochemical Analysis

The quantitative assay of the methanolic leaf, stem, and calyx extracts of *Hibiscus sabdariffa* was performed for total phenols, flavonoids, saponins, and alkaloids. There are four quantitative phytochemical analyses, i.e. the determination of total phenol content by Folin-Ciocalteu Assay, the determination of total flavonoid content by Aluminum Chloride Colorimetric Method, the determination of total saponin content by Vanillin-sulphuric Acid Colorimetric Assay, and the determination of total alkaloid content.

The determination of the total phenol content was carried out according to the method by Ainsworth and Gillespie^[16]. Twenty-five microliters (25 μ L) of gallic acid standard solutions (5, 10, 20, 30, 40, and 50 μ g/mL) or *H. sabdariffa* extracts were mixed with 40 μ L of Folin-Ciocalteu reagent. After 5 minutes, 200 μ L of sodium carbonate solution (7.5%, w/v) was added and allowed to stand at 20 °C in a water bath for 90 minutes. The reaction mixture was measured at 765 nm on a UV-VIS spectrophotometer (Shimadzu UV mini-1240, Ontario, Canada). The results were expressed as mg gallic acid equivalent/g of dry weight (mg GAE/g DW), based on the calibration curve.

The aluminum chloride colorimetric method, as described by Piyanete et al.^[17], was used to determine the total flavonoid content of *H. sabdariffa* extracts. Using Quercetin as standard, the flavonoid content was determined as a quercetin equivalent. The following concentrations (10, 25, 50, 75, and 100 μ g/mL) were prepared in methanol from the standard quercetin solution. A hundred microliters (100 μ L) of each of the quercetin dilution was mixed with 500 μ L of distilled water and then with 100 μ L of 5% sodium nitrate and allowed to stand for 6 minutes. Then 150 μ L of 10% aluminum chloride solution was added and allowed to stand for 5 minutes after which 200 μ L solution of 1 M sodium hydroxide was added sequentially. The reaction mixture was measured at 510 nm with a UV-VIS spectrophotometer (Shimadzu UV mini-1240, Ontario, Canada). It resulted expressed as mg/g quercetin equivalent (QE) of dry extract, based on the calibration curve.

The determination of the total saponin content was carried out according to the method as described by Moja et al.^[18] with slight modifications from Adusei et al.^[19]. A method based on vanillin-sulphuric acid colorimetric reaction. Exactly 5000 μ L of water was added to 100 μ L of Diosgenin. Five hundred microliters (500 μ L) of vanillin reagent (8 g of vanillin in 100 mL of 99.5% ethanol) was added. Five milliliters (5 mL) of 72% sulphuric acid were also added and mixed well. This solution was kept in a water bath at 60 °C for 10 minutes. After 10 minutes, it was cooled, and the absorbance read at 544 nm and recorded. All measurements were performed in triplicate for each analysis. The total saponin content determined from the linear equation of a standard curve prepared with Diosgenin and expressed as mg/g diosgenin equivalent (DE) of dry extract.

The total alkaloid content of *H. sabdariffa* extracts was determined according to a slightly modified method of Adusei et al.^[19]. One gram of crude extract of each leaf, stem, and calyx was weighed into a 100 mL beaker, and 50 mL of 10% hydrochloric acid in ethanol was added and covered and allowed to stand for 4 hours. The extract was filtered, and the filtrate was concentrated at 65 °C to one-quarter of the original volume using a rotary evaporator (Stuart RE 400, United Kingdom). Fifteen (15) drops of concentrated ammonium hydroxide were then added dropwise to the concentrate until the precipitation was complete. After 3 hours of mixture sedimentation, the supernatant was discarded, and the precipitates were washed with 20 mL of 0.1 M ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed. The percentage alkaloid was then calculated as the weight of residue x 100/ weight of sample taken and results expressed as %w/w of dry extracts.

2.6 | Antioxidant Activity Determination

The assay for antioxidant activity of *H. sabdariffa* was based on the reduction of Molybdate (VI) to Molybdate (V) by the extract and subsequent formation of green phosphate/MO (V) complex at acid pH as described by Akinmoladun et al.^[20], with slight modifications from Adusei et al. (2019). Ascorbic acid was used as a standard. One milliliter (1 mL) of 0.6 M sulphuric acid, 28 mM of sodium phosphate, and four mM of ammonium molybdate were added in 20 mL of distilled water and made up to the volume of 50 mL by adding distilled. One-third of a gram (0.3 g) of the extract was measured into three separate test tubes. One-third of a milliliter (0.3 mL) of the molybdate reagent solution was then added. These tubes were kept incubated at 95 OC

TABLE 1 Recoveries of analytes in methanolic extracts of Hibiscus Sabdariffa.

Analyte	Recovery
Phenols	110
Flavonoids	104
Alkaloids	90
Saponins	94
Antioxidant	99

for 90 minutes. After incubation, the tubes were normalized to room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 695 nm against a blank containing 100 μ L of methanol mixed with 900 μ L of reagent solution. The antioxidant activity was expressed as mg/g ascorbic acid equivalent (AAE) of the dry extracts.

2.7 | Statistical Analysis

The data obtained after the experiment were analyzed using GraphPad Prism version 5.01. The results were expressed as mean \pm standard deviation. A one-way ANOVA using Tukey: compared all pairs of columns, at a 95% confidence level was used to compare mean values among extracts. P-values less than 0.05 ($P < 0.05$) were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of the Cold Maceration Extraction Technique

For the recovery of bioactive compounds (secondary metabolites) from plant sources, extraction becomes the critical stage in the technique. The extract yield is dependent on certain factors such as the nature of the solvent used with varying polarity, extraction method, nature of phytochemicals, the existence of interfering substances, and solvent to sample volume ratio^[21]. The spike recovery experiment was a dependable technique to determine the correctness of the procedures used and the recovery of analytes (compounds of interest) from the plant material. With the spike recovery test, samples were divided into two portions, and a known quantity of a standard solution of analyte added to one portion. The analyte's concentration was determined for both the spiked, F, and unspiked portions, I, and the percent recovery, % R, was calculated as; $\% R = F - I / A * 100$. Where "A" is the concentration of analyte added to the spiked portion^[19]. The use of methanol as an extraction solvent presented a significantly high recovery ranging between 90% and 110%, as shown in table 1.

3.2 | Preliminary Qualitative Phytochemical Screening

The result of phytochemical screening on the methanolic leaf, stem, and calyx extracts of *H. sabdariffa* is displayed in Table 2. The calyx extract revealed the highest number (nine) of bioactive compounds (phenols, flavonoids, alkaloids, anthocyanins, anthraquinones, terpenoids, tannins, steroids, and saponins), except for cardiac glycosides being absent. The leaf extract also showed the presence of seven bioactive compounds (phenols, flavonoids, alkaloids, anthocyanins, tannins, steroids, and saponins), and the absence of three (anthraquinones, cardiac glycosides, and terpenoids). The stem extract revealed six compounds (phenols, flavonoids, alkaloids, anthocyanins, steroids, and saponins), and four (anthraquinones, cardiac glycosides, terpenoids, and tannins) being absent. This observation (many bioactive compounds revealed in the calyx extract than the leaf or stem) indicates the abundancy or richness of these compounds in the calyx than the leaf or stem. The presence and content of these bioactive compounds and other phytochemicals present in medicinal plants are mainly predisposed by the type of cultivation, genetic factors, environmental conditions, and the degree of maturation and the diversity of the plant^[21, 22]. Therefore, the result of this phytochemical test points out that *Hibiscus sabdariffa* possesses numerous biologically active compounds, which could serve as a possible source of drugs in herbal medicine.

3.3 | Quantitative Determinations

The quantitative examination was carried out for total phenols, flavonoids, saponins, alkaloids, and antioxidant activity in the methanolic leaf, stem, and calyx extracts using UV-Vis spectrophotometer with standard procedures.

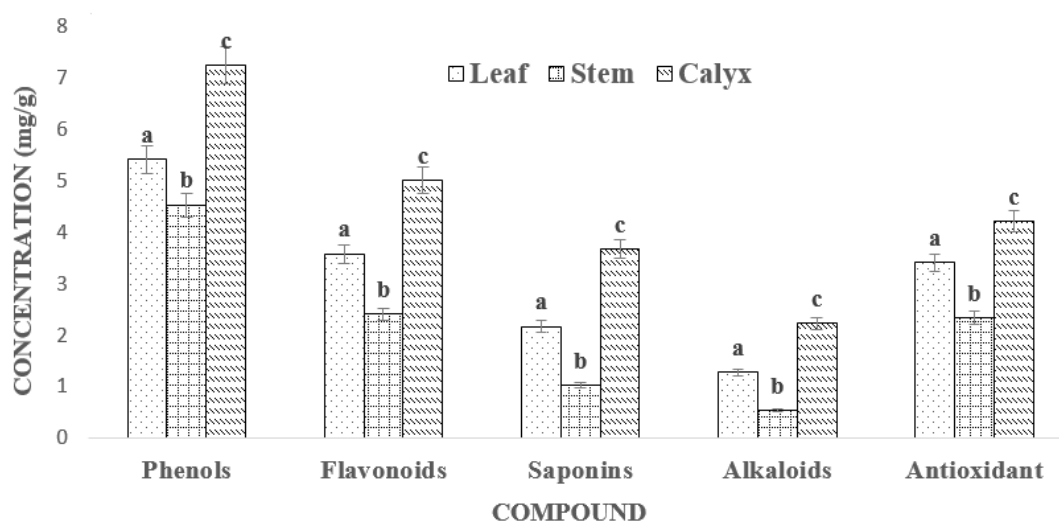
TABLE 2 Preliminary phytochemical analysis of methanolic extracts of *Hibiscus Sabdariffa*.

Bioactive compound	Test Used	Leaf	Stem	Calyx
Phenols	FeCl ₃ test	+	+	+
Flavonoids	Alkaline reagent test	+	+	+
Alkaloids	Mayer's test	+	+	+
Anthraquinones	Borntrager's test	-	-	+
Cardiac Glycosides	Kella-Killani test	-	-	-
Anthocyanins	Ammonia-HCl test	+	+	+
Terpenoids	Salkowski's test	-	-	+
Tannins	FeCl ₃ test	+	-	+
Steroids	Liebermann-Burchard's test	+	+	+
Saponins	Foam test	+	+	+

Key: (+) indicates present, and (-) signifies the absence of a specific bioactive compound

TABLE 3 Quantitative data on bioactive compounds and antioxidant activity of methanolic extract of *Hibiscus Sabdariffa*.

Compounds	Extract			P-Value
	Leaf	Stem	Calyx	
Phenols (mg GAE/g)	10.42 ± 0.04a	7.53 ± 0.01b	12.25 ± 0.03c	0.0215
Flavonoids (mg QE/g)	7.58 ± 0.01a	5.41 ± 0.03b	10.01 ± 0.02c	0.0324
Saponins (mg DE/g)	4.17 ± 0.02a	2.02 ± 0.02b	6.67 ± 0.01c	0.0432
Alkaloids (AA (mg AAE/g)	6.41 ± 0.03a	3.34 ± 0.01b	8.22 ± 0.04c	0.0251

**FIGURE 2** A bar chart represents bioactive compounds and antioxidant content of leaf, stem, and calyx extract of *Hibiscus Sabdariffa*.

The concentration of phenols, flavonoids, saponins, and alkaloids in methanolic leaf, stem, and calyx extracts of *Hibiscus sabdariffa* is shown in Table 3 and Figure 2. The abbreviations in column one of Table 3 can be describe as follows: GAE (Gallic Acid Equivalent), QE (Quercetin Equivalent), DE (Diosgenin Equivalent), w (Weight), AA (Antioxidant Activity), and AAE (Ascorbic Acid Equivalent). Each value is displayed as mean ± standard deviation (SD). Means in a row with different letter superscripts are significantly different ($P < 0.05$, Tukey's test). Figure 2 represents bioactive compounds and antioxidant content. The results were presented as mean ± SD of three independent experiments ($n = 3$). The different letters indicate a significant difference ($P < 0.05$). Significantly higher amounts of total phenols, flavonoids, saponins, and alkaloids were found in the calyx extract than the leaf or stem. This result implies that the above-mentioned bioactive compounds had been chiefly enriched in the calyx than the other tested parts of the plant. Although, many studies have reported the phytochemical constituents of *H. sabdariffa*, mostly in the leaves and calyces of the plant. However, this current study has disclosed the concentrations of the major phytochemicals (phenols, flavonoids, saponins, and alkaloids), not only in the calyx and leaf but also in the stem, with fewer data in the literature. For each gram of the extract, phenols appear to be the most abundant bioactive compound having the maximum concentration,

and with alkaloids being the least. Alkaloids have many pharmacological activities, including antihypertensive effects (many indole alkaloids), antiarrhythmic effects (quinidine), antimalarial activity (quinine), and anticancer actions (dimeric indoles, vincristine, vinblastine)^[23]. Phenols, the most lavish bioactive compound in *H. sabdariffa* of this present study, possess diverse biological activities, such as antiulcer, anti-inflammatory, cytotoxic, and antitumor activities^[24]. Flavonoids have also been reported to exert numerous biological property including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their ability to act as powerful antioxidants, which can safeguard the human body from free radicals and reactive oxygen species^[25]. Many saponins are well-known to be antimicrobial, inhibit mold, defend plants from insect attack, and act as antifungal and antiviral in humans^[26].

The antioxidant activity carried out on the methanolic leaf, stem, and calyx extracts of *H. sabdariffa* is displayed in table 3 and figure 2. The calyx extract was discovered to exhibit much antioxidant property (4.22 ± 0.04 mg/g) than the leaf (3.41 ± 0.03 mg/g) or stem (2.34 ± 0.01 mg/g). The antioxidant content of *H. sabdariffa* of this study higher than that reported by^[27]; (2.22 ± 0.04 mg/g leaves and 3.15 ± 0.01 mg/g calyces) may be due to the differences in growing medium (soil), environmental conditions, degree of maturation and the variety of the plant. However, this present study relates the antioxidant content in the leaf, stem, and calyx extracts, with limited fact in literature. The antioxidant content of the extracts of the roselle plant in this study may also be owed to a subgroup of flavonoids and anthocyanins, which have been reported in the literature as the main constituents of *H. sabdariffa*^[28]. The results of the antioxidant activity of *H. sabdariffa* of this present study corroborates that of the recent review by^[29], who gave an insight into the chemotherapeutic potential of *H. sabdariffa*. Several antioxidants play an essential role in diminishing inflammation, delaying aging, and reduces the oxidative destruction in foods by inhibiting oxidation produced by reactive oxygen species (ROS)^[11].

4 | CONCLUSION

Medicinal plants are an abundant source of secondary metabolites (phytochemicals). This study has revealed that Hibiscus Sabdariffa, a medicinal and food plant, is rich in many phytochemicals such as phenols, flavonoids, anthocyanins, and alkaloids, and many others, which are accountable for its pharmacological possessions. The antioxidant content was established to be higher in the calyx than the leaf or stem of the plant. Hence, the plant's calyces should be chosen for natural antioxidant roles and therapeutic purposes in traditional medicine systems. Therefore, Hibiscus Sabdariffa is a unique source of a broad spectrum of bioactive compounds having diverse medicinal assets, which could be a probable curative agent in plant medicine.

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