RESEARCH ARTICLE

Phytochemical Constituents and Antibacterial Activities of *Hibiscus sabdariffa* L. Calyces (Zobo Flower) Extracts on *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Introduction: *Hibiscus sabdariffa* plant has been used in many ways both for industrial purposes and medicinal uses. It has been discovered to have antimicrobial properties.

Aim and objective: The present study was aimed to investigate the phytochemical and antimicrobial activities of methanolic and aqueous extracts of *H. sabdariffa* calyces on *Staphylococcus aureus* and *Escherichia coli*.

Materials and methods: The phytochemical analysis was carried out using standard methods. The antibacterial activity of the plant extracts was determined using the agar well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts on the test isolates were determined using the micro broth dilution method.

Results: The phytochemical analysis showed that terpenoids, phenols, flavonoids, glycosides, tannin, saponin, alkaloids, and anthraquinolones were present in varying concentrations of the different extracts. The methanol extract of *H. Sabdariffa* calyces possesses more antimicrobial activity (13–24 mm) in a concentration-dependent manner than the aqueous extract (7–20 mm). The MIC of different extracts of *S. aureus* was between 25 mg/mL and 50 mg/mL while that of *E. coli* was also between 12.5 mg/mL and 25 mg/mL. The MBC of different extracts of *S. aureus* was between 25 and 50 mg/mL while that of *E. coli* was between 6.25 and 50 mg/mL.

Conclusion: It can be concluded that some secondary metabolites present in *H. sabdariffa* calyces was responsible for the observed inhibition of the bacteria seen in this study. The methanol extract of *H. sabdariffa* calyces possesses more antimicrobial activity in a concentration-dependent manner than the aqueous extract. Therefore, the test plant could be used to manufacture drugs that could be used to treat infections caused by the test organisms.

Keywords: Aqueous, *Escherichia coli*, *Hibiscus sabdariffa*, Methanol, *Staphylococcus aureus*.

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Introduction

The plant *Hibiscus sabdariffa*, commonly called "Roselle" and "Zobo" in Nigeria, belongs to the family Malvaceae.[1] It is an annual or perennial herb or woody-based subshrub, growing to 2–2.5 m (7–8 feet) tall. The leaves are deeply three- to five-lobed, 8–15 cm (3–6 in.) long, arranged alternately on the stems.

The flowers are 8–10 cm (3–4 in.) in diameter, white to pale yellow with a dark red spot at the base of each petal, and have a stout fleshy calyx at the base, 1–2 cm (0.39–0.79 in.) wide, enlarging to 3–3.5 cm (1.2–1.4 in.), fleshy and bright red as the fruit matures. They take about 6 months to mature.[2]

Among the Yoruba in southwest Nigeria, Roselle is known as *isapa*, and *yakuwa* by the Hausa people of northern Nigeria who also call the seeds as *gurguzu* and the capsule cover as *zoborodo* or *zobo*.[1]

Hibiscus sabdariffa plant has been used in many ways both for industrial purposes and medicinal uses. It has been incorporated in the treatment of many ailments such as hypertension, arterioclerosis, neurosis, cancer, etc.[3] It has also been used for many industrial products. In India, the plant is primarily cultivated for the production of bast fiber used in cordage, made from its stem.[4] The fiber may be used as a substitute for jute in making burlap.[4] *Hibiscus*, specifically roselle, has been used in folk medicine as a diuretic and mild laxative.[4]

The red calyces of the plant are increasingly exported to the United States and Europe, particularly Germany, where they are used as food colorings. It can be found in markets (as flowers or syrup) in places, such as France, where there are Senegalese immigrant communities.[5] The green leaves are used like a spicy version of spinach. They give flavor to the Senegalese fish and rice dish *thieboudienne*. Proper records are not kept, but the Senegalese government estimates national production and consumption at 700 t (770 short tons) per year.[5]

A tea made from *Hibiscus* flowers is known by many names around the world and is served both hot and cold. The beverage is known for its red color, tart, flavor, and vitamin C content.[6]

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In Nigeria, rosella jam has been made since colonial times and is still sold regularly at community fetes and charity stalls.[7] It is similar in flavor to plum jam, although more acidic. It differs from other jams in that the pectin is obtained from boiling the interior buds of the rosella flowers. It is thus possible to make rosella jam with nothing but rosella buds and sugar.[8]

The plant has been used in the treatment of many ailments and used in many folk medicines.[9] The *Hibiscus* leaves are a good source of polyphenolic compounds. The major identified compounds include neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, caffeoylshikimic acid, and flavonoid compounds such as quercetin, kaempferol, and their derivatives.[9] The flowers are rich in anthocyanins, as well as protocatechuic acid. The dried calyces contain the flavonoids gossypetin, hibiscetine, and sabdaretine.[10] The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), chrysanthenin (cyanidin 3-monoglucoside), and delphinidin are present. Roselle seeds are a good source of lipid-soluble antioxidants, particularly gamma-tocopherol.[11]

Previously published studies have shown that it has significant antibacterial activity against *Bacillus cereus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Klebsiella pneumonia*, *Proteus vulgaris*, etc.[12]

Therefore, the aim of this study is to evaluate the phytochemical and antibacterial activities of *H. sabdariffa* leaf extracts on *E. coli* and *S. aureus*.

Materials and Methods

Sample Collection

The flowers of *H. sabdariffa* (Zobo flowers) were bought from Ogbete market in Enugu State, Nigeria, and were authenticated by a botanist at the Nnamdi Azikiwe University Herbarium with a voucher number of NAUH 27A.

The preparation was done at the microbiology laboratory of Spiritan University, Nneochi, Abia State. The fresh flowers of *H. sabdariffa* were washed thoroughly with clean water, cut into bits, and placed in a washed tray to air dry.[13] With the help of a pestle and mortar, it was crushed to coarse powder. Different containers were used to label and store the powdered form of the leaves.

Test Organisms

The antibacterial test organisms used were bacterial cultures of *E. coli* and *S. aureus* obtained from the laboratory section of the Department of Microbiology, Nnamdi Azikiwe University, Anambra State, Nigeria. Standard methods, i.e., cultural, morphological, and biochemical tests, were used to confirm their identity.[14] Agar slants were used to maintain the bacterial isolates at 4°C until further use.

Biochemical Identification of the Test Organism

Escherichia coli

The *E. coli* from the test culture was placed on Eosine Methylene Blue (EMB) agar for 24 hours. A positive result for *E. coli* was indicated with the appearance of colonies with green metallic sheen. The distinct colonies with metallic green sheen on EMB agar were picked and confirmed by streaking onto the Chromagar *E. coli* medium (Oxoid, Basingstoke, UK). Colonies with a blue/violet appearance were selected and analyzed further by gram staining and biochemical tests.[14]

Staphylococcus aureus

Staphylococcus aureus that showed positive result for the catalase test was subcultured on blood agar and incubated at 37°C for 24 hours. Then, the single colonies were placed on Mannitol Salt Agar (MSA) for 24 hours. A positive result was indicated by smooth circular colonies with yellow color.[14]

Standardization of the Tests Organisms

The test organisms (*E. coli* and *S. aureus*) were standardized by the use of 24-hour-old broth cultures prepared by inoculating the test organisms into 5 mL of nutrient broth and the culture was incubated for 2 hours. The growth of the organism was indicated by the turbid change in color of the nutrient broth, which was adjusted to match the color of the 0.5 McFarland turbidity equivalent standards.[14]

Preparation of Methanolic and Aqueous Extracts

The extracts were prepared according to the method described by Akinnibosun.[13]

Preparation of Aqueous Extract

Ten (10) g of dried grinded flower powder was measured out using an electronic weighing balance. It was dissolved in 100 mL of distilled water for 24 hours. Using a Whatman's filter paper No. 1, the resulting mixture was filtered to obtain a solid-free solution. A water bath was use to evaporate the filtrate to dryness and the resulting extract was collected using a sterile universal bottle. It was stored at 4°C in a refrigerator until when it is required for further use.[13]

Preparation of Methanol Extract

Ten (10) g of dried grinded flower powder were suspended in 100 mL of 95% methanol for 24 hours. Using a Whatman's filter paper No. 1, the resulting mixture was filtered to obtain a solid-free solution. The filtrate was evaporated to dryness and stored at 4°C until it is required for further use.[13]

Extract Dilution

After the extracts were prepared as described by Akinnibosun, the aqueous and methanol extracts were reconstituted using sterile distilled water to obtain the following concentrations: 200, 100, 50, 25, 12.5, 6.25, and 3.13 mg/mL.[13]

Sterility Test of Leaf Extract

The extracts (methanol and aqueous) were tested for growth of contaminants according to the methods of Cheesebrough.[14] Standard extract (1 mL) was inoculated aseptically onto nutrient agar and incubated at 37°C for 24 hours. The plates were observed for sign of growth. No growth on the plates signified sterility of the extracts.

Phytochemical Screening of *H. sabdariffa*

The sample was screened for the following compounds: alkaloids, phenols, terpenoids, saponins, tannins, flavonoids, glycosides, steroids, anthraquinones, etc. This was done following standard methods.[15]

Test for Tannins

Two (2) g of each extract were dissolved in 10 mL of distilled water in separate test tubes. Three (3) drops of 10% ferric chloride ($FeCl_3$) was added to 2 mL of the solution. The appearance of blackish-blue or blackish green coloration indicates the presence of tannins.

Test for Saponins

About 0.1 g of each extract was dissolved in 5 mL of distilled water. The solution was shaken vigorously. The presence of saponin was indicated by the formation of frothing bubbles, which lasted for 10 minutes.

Test for Alkaloids

About 0.5 g of each extract was dissolved in three drops of Dragendoff's reagent. The presence of alkaloid was indicated by the formation of orange precipitate.

Test for Flavonoids

About 0.2 g of each extract was added to 2 mL of sodium hydroxide solution. The resulting solution was shaken vigorously to dissolve. The presence of flavonoids was indicated by the occurrence of a yellowish solution, which disappears on addition of hydrochloric acid (HCl).

Test for Glycoside

Half (0.5 g) of each extract was added to 3 mL of Fehling solution and dissolved. The presence of glycosides was indicated by the formation of a brick red precipitate.

Test for Steroids

Five (5) drops of concentrated H_2SO_4 was added to 0.1 g of each extract in the test tube. The presence of steroids was indicated by the formation of a reddish brown coloration in the test tube.

Terpenoids

Four milligrams (4 mg) of extract were treated with 0.5 mL of acetic anhydride and 0.5 mL of chloroform. Then concentrated sulfuric acid solution was added slowly and red violet color was observed for terpenoid.

Anthraquinone

To 10 mg of the dissolved extract, magnesium acetate solution was added. Pink color developed, which indicates the presence of anthraquinone and no color change indicates negative.

Antibacterial Assay

The agar-well diffusion technique was used to carry out the antibacterial susceptibility of the plant flower extracts in comparison with standard antibiotic gentamicin (20 mg/mL) *in vitro* on the isolates according to the methods of National Committee for Clinical Laboratory Standards.[16] Pure culture of the bacteria was grown on nutrient agar. Three colonies of each organism were pick into the Mueller Hinton broth (Oxoid, England), incubated for 4 hours at 37°C, and diluted with sterile saline to a density visually equivalent to the MacFarland standard. Using a sterile 6-mm-diameter cork borer, four (4) wells were cut in the agar to which the two extracts of *H. sabdariffa* calyces were added, as well as the standard drug, gentamicin (GEN, 20 mg/mL) and sterile water separately, which served as the positive and negative controls, respectively. For pre-diffusion to occur, the plates were placed on the bench for 30 minutes after which they were subsequently incubated for 48 hours at 37°C. The zones of inhibition were then measured with the use of a calibrated ruler.

Determination of Minimum Inhibitory Concentration

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of *H. sabdariffa* flower extracts against the test organisms. To each 5 mL of the various extracts in different test tubes, 5 mL of nutrient broth each was added and serially diluted out to various concentrations ranging from 200 to 3.13 mg/mL. A loop full of each test bacteria was inoculated into each of the test tubes and subsequently incubated for 24 hours at 37°C. The lowest concentration of the leaf extracts that inhibited the growth of the organism was the MIC.[14]

Determination of Minimum Bactericidal Concentration

Briefly, 1 mL bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes, which did not show any growth and were subcultured onto nutrient agar. They were subsequently incubated at 37°C for 24 hours. This was obtained by streaking out the samples from the MIC tubes that showed no visible growth on nutrient agar plates. The least concentration of the sample that showed no growth was noted and recorded as the minimum bactericidal concentration (MBC).[14]

Results

Table 1 shows the phytochemical components of methanol and aqueous extracts of *H. sabdariffa* extracts. Phenols, anthraquinolones, terpenoids, tannins and glycosides were present in both extracts at varying concentrations. Alkaloids and flavonoids were present in high amount in methanol extracts and absent in aqueous extracts. There was absence of steroids in both extracts.

The antibacterial efficacy of both extracts of *H. sabdariffa* on *S. aureus* and *E. coli* is found in Table 2. The diameters of the zone of inhibition of *S. aureus* on AE at concentrations of 200, 100, 50, 25, and 12.5 mg/mL are 20, 18, 1, 1, and 10 mm, respectively. While the diameters of THE zone of inhibition of *S. aureus* on ME at concentrations of 200, 100, 50, 25, and 12.5 mg/mL are 24, 22, 20, 17 and 15 mm, respectively. The diameters of the zone of inhibition of *E. coli* on same concentrations of AE and ME are found in Table 2.

The MIC and MBC of leaf extracts of *H. sabdariffa* extracts on *S. aureus* and *E. coli* are found in Table 3. The MIC and MBC of AE on *S. aureus* are both 50 mg/mL while the MIC and MBC of AE on *E. coli* are 25 and 50 mg/mL, respectively. That of the methanol extract on *S. aureus* and *E. coli* is found in Table 3.

Discussion

The phytochemical analysis indicates the presence of flavonoids, terpenoids, alkaloids, saponins, phenol, anthraquinolones, and tannins in varying concentrations in the methanol and aqueous extracts of *H. sabdariffa*. Alkaloids, saponins, tannins, flavonoids,

Table 1: Phytochemical composition of *H. sabdariffa* extracts

Phytochemical components	*Methanol extracts*	*Aqueous extracts*
Phenols	+++	++
Flavonoids	+++	–
Steroids	–	–
Anthraquinolones	++	+
Terpenoids	+	+
Glycosides	++	++
Tannins	+	+
Saponins	–	++
Alkaloids	+++	–

–, absence; +, slightly present; ++, moderately present; +++, highly present

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Table 2: Antibacterial activities of methanol and aqueous extract of *H. sabdariffa* on *S. aureus* and *E. coli*

	Extract concentrations (zone diameter of inhibition in mm)							
Isolates	*200*	*100*	*50*	*25*	*12.5*	*+C*	*−C*	*Extracts*
S. aureus	20	18	16	12	10	17	0	AE
S. aureus	24	22	20	17	15	17	0	ME
E. coli	17	11	8	7	7	18	0	AE
E. coli	22	20	18	15	13	18	0	ME

AE, aqueous extract; ME, methanol extract; +C, positive control (gentamicin, 20 mg/mL); −C, negative control (sterile water)

Table 3: Minimum inhibitory and bactericidal concentrations of *H. sabdariffa* extracts on *S. aureus and E. coli*

	Concentration of extracts (mg/mL)		
Isolates	*MIC (mg/mL)*	*MBC (mg/mL)*	*Extracts*
S. aureus	50	50	AE
S. aureus	25	25	ME
E. coli	25	50	AE
E. coli	12.5	6.25	ME

AE, distilled water extract; ME, methanol extract; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

phenols, and several other aromatics have the capability to resist microbial invasion.[17] It is documented that the chemical structure of the phytochemicals plays an important role in determining antibacterial activity. For instance, flavonoids that are hydroxylated phenolic substance are produced by plants in response to microbial infection. They complex with extracellular and soluble proteins and also with the bacterial cell wall.[18]

Saponins too possess detergent-like properties, which may increase the permeability of bacterial cell membranes thereby facilitating antibiotic influx through the bacterial cell wall.[19]

Similarly, basic character of tannins enables them to bind with proteins and damage the bacterial cell membrane. Tannins also hinder microbial growth by precipitating microbial protein and make nutritional proteins unavailable.[20] Thus, *H. sabdariffa* has been found to be the reservoir of phytochemicals and capable of exhibiting antibacterial activity against number of diseases.[20]

The absence of some phytochemicals might be due to differences in the polarity of the solvents, as the types of solvent used determined the kind of biologically active compounds that can be extracted from the plant.[21]

It has been reported that different solvents have different extraction capabilities.[22] Ashok et al.[22] reported that the best way to extract broad-spectrum antimicrobial compound from plant is by the use of methanol solvents. The differences observed between antibacterial activities of the extracts could be explained by their abilities to dissolve in different solvents.[22]

Results from this study show that the methanol extracts of *H. sabdariffa* calyces inhibited the growth of both test organisms than the aqueous extract in a concentration-dependent manner. The variation in the antibacterial activities is due to difference in the quantity of compounds present in those plant extracts.[23] Similar result was discovered in the work of Ewansiha.[24]

Result from this work showed a greater zone of inhibition produced by the methanolic extracts (13–24 mm) of *H. sabdariffa* calyces at all concentrations used compared to that produced by the distilled water extract (7–20 mm). This indicates the possibility that *H. sabdariffa* could serve as a better and alternative drug to treat infections caused by *S. aureus* and *E. coli* compared to most conventional antibiotics used presently in the world. Similar result was discovered in the work of Fullerton et al.[25]

The MIC obtained shows that different concentrations were effective against the two test organisms. The generally low MIC and MBC values of methanol extracts against *S. aureus* and *E. coli* are an indication of their antibacterial potential.[26] The different values obtained from the MBC of the leaf extract of *H. sabdariffa* calyces on *E. coli* and *S. aureus* confirmed that both extracts have varying phytochemical properties, and hence exhibit different inhibitory effects and bactericidal effects on the test organisms but less when compared to the standard used[27] (Abubakar and Usman 2016). This could be further explained by the differences in the chemical composition of these extracts.[23] Similar results were obtained from the works of Okereke et al.[28] and Mungole and Chaturvedi,[29] but contrasted with the work of Arvind and Alka,[30] who posited that *H. sabdariffa* extracts exhibited bactericidal effects very well when compared to standard antibiotics.

Conclusion

From this study, it can be concluded that some secondary metabolites such as phenols, flavonoids, glycosides, tannin, saponin, terpenoids, alkaloids, and anthraquinolones present in *H. sabdariffa* calyx were responsible for the inhibition of the bacteria observed in this present study and could also justify its use as an antimicrobial agent.

It can also be concluded that the methanol extract of *H. sabdariffa* calyx possesses more antimicrobial activity (13–24 mm) in a concentration-dependent manner than the aqueous extract (7–20 mm).

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