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Phytochemical and *in vitro* anti-bacterial properties of *Hibiscus sabdariffa* L (Roselle) juice

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The anti-bacterial properties of aqueous and ethanol extracts of the calyces of *Hibiscus sabdariffa* on five bacteria genera, namely, *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shigella dysenteriae* and *Streptococcus mutans* were evaluated using agar well and disc diffusion methods. Six bioactive compounds were identified from phytochemical analysis of the juice extract of *H. sabdariffa*. Analysis revealed the presence of the following in the water and ethanol extracts: saponins (1.46%), alkaloids (0.09%), tannins (0.19%), total phenols (0.07%), flavonoids (2.41%) and glycosides (0.13%). The results revealed that both water and ethanol extracts had significant anti-bacterial effects (P ≤ 0.05) against the tested pathogenic bacteria genera with the ethanol extract of Roselle having higher anti-bacterial effects against all the pathogens. *S. aureus* and *E. coli* were the most sensitive to Roselle juice extract (47.9 and 45. 8 mm zones of inhibition, respectively). The minimum inhibitory concentration (MIC) for the ethanol extract of *H. sabdariffa* was between 0.26 and 1.03 mg/ml. The results observed in this study contribute to scientific baseline data on the anti-bacterial activities of *H. sabdariffa* juice extract.

Key words: Antimicrobial activity, minimum inhibitory concentration, pathogenic bacteria, phytochemical, *Hibiscus sabdariffa.*

INTRODUCTION

Hibiscus sabdariffa is an herbaceous annual of the family Malvaceae. The plant originated in West Africa, though it has been grown in Asia for several centuries and now has a wide distribution throughout the tropics (David and Adam, 1985). There are two main types of Roselle: *H. sabdariffa var altissima* Wester and *H. sabdariffa* var *sabdariffa*.

H. sabdariffa var *altisimma* Wester is more widely cultivated for its jute-like fibre in India, the East Indies, Nigeria and to some extent, in tropical America (Yayock et al., 1988). The flowers are yellow and calyces red or green, non fleshy, spiny and not used for food (Morton, 1987). The other distinct type of Roselle, *H. sabdariffa* var *sabdariffa* is a bushy branched sub-shrub with red or green stem and red or pale yellow inflated edible calyces. Both Roselle types grow well on fairly fertile sandy to loamy soil being usually propagated by seeds, but also growing readily from cuttings.

The increased cultivation of Roselle in many areas is centered more on its pharmaceutical, rather than its food potential. All the aforementioned ground-parts of the Roselle plant are valued in native medicine (David and Adam, 1985). Infusions of the leaves, petals and other parts are regarded as diurectic, vasorelaxative, cholerectic, febrifugal and hypotensive in folk medicine (Odigie et al., 2003; Sarr et al., 2009; Mozaffari-Khosravi et al., 2009).

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Pharmacognosists in Senegal recommend Roselle extract for lowering blood pressure (Chopra et al., 1986), while the mechanisms of blood pressure lowering effect of the calyx extract has been studied in rats (Adegunloye et al., 1996). In experiments with domestic fowl, Roselle extract decreased the rate of absorption of alcohol thereby reducing intoxication arising from alcohol consumption (Morton, 1987). As such, the extract has been found to be a favorite remedy for after-effects of drunkenness. The calyx extract is used in the treatment of debility, hypertension, dyspepsia and heart ailments. The extracts of the leaves and flowers of the Roselle plant are used internally as tonic tea for digestive and kidney functions (Bown, 1995). However, there appears to be limited information on the scientific basis for many of the acclaimed health benefits of this plant. The present study was aimed at assessing the phytochemical properties of *H. sabdariffa* var *sabdariffa* and evaluation of its antibacterial properties as a step towards providing scientific justification for its pharmaceutical uses.

MATERIALS AND METHODS

Collection and processing of samples

Dried calyces of Roselle *(H. sabdariffa*) were purchased from Kuto market in Abeokuta, South Western Nigeria. The calyces were identified by a botanist in the Department of Biological Sciences, University of Agriculture, Abeokuta, Nigeria.

Preparation of *H. sabdariffa* extracts

Extraction of juice from calyces of *H. sabdariffa* was done with water and ethanol. Replicate 100 g of dried blended calyces of Roselle were soaked in 200 ml of ethanol and distilled water separately. The beakers were covered with aluminum foil and kept at ambient temperature (29±1°C) for 12 h. The extracts were then filtered through Whatman filter paper No. 42 (125 mm) and stored at 4°C until required for analyses.

Test microorganisms

The microorganisms used in this study comprised clinical isolates of five pathogenic bacteria (*Escherichia coli, Staphylococcus aureus*, *Salmonella typhi, Shigella dysenteriae* and *Streptococcus mutans*) obtained from University College Hospital, Ibadan, Nigeria. These organisms are known to cause diseases such as diarrhoea, dysentery, oral and dental infections. Selected biochemical tests were carried out on the test cultures to confirm their identities before use. The test organisms were sub-cultured in nutrient broth (Oxoid Ltd, UK) at 37°C and stored at 4°C until required for the study. They were used for antimicrobial testing by seeding in agar plates at ca. log 7.0 CFU ml^{-1}.

Phytochemical analyses of *H. sabdariffa* extracts

Screening of *H. sabdariffa* aqueous and ethanol extracts was carried out to identify the phytochemical constituents by standard procedures (Trease and Evans, 1989; Harborne and Harborne, 1998).

Determination of saponins

Octanol (100 ml) was added to 2 g of each extract, shaken for 5 h to ensure uniform mixing before filtering through a Whatman No.1 filter paper. Twenty millilitres of 40% saturated solution of $MgCO_3$ was added to neutralize the filtrate obtained. The mixture was again filtered to obtain a clear colourless solution. To 1 ml of the clear solution 2 ml of 5% $FeCl_3$ solution was added before making up to 50 ml with distilled water. The mixture was allowed to stand for 30 min for the blood red colouration to develop. The absorbance of the samples were read with a spectrophotometer (Spectronic 20) at a wavelength of 30 nm and compared with 0 to 10 ppm standard saponin solutions. Percent saponin present in the samples was calculated as follows:

% Saponin = Absorbance of sample × Average gradient × Dilution factor/10,000

Determination of alkaloids

An amount of 2 ml of each extract was warmed with 2% H_2S0_4 for 2 min. Each extract was filtered and a few drops of Dragendorff's reagent were added. Orange red precipitation indicated the presence of alkaloids after which quantification was estimated by mixing 10 g of each sample with 20 ml of 80% alcohol. After thorough mixing, more 80 ml of 80% alcohol was added to make the mixture up to 100 ml before 1 g magnesium oxide was added. The mixture was digested in a boiling water bath for 1½ h under a reflux air condenser with occasional shaking. The residue was returned to the flask and re-digested for 30 min with 50 ml alcohol after which the alcohol was evaporated and 20 ml of hot water was added. 2 to 3 drops of 10% HCl was added and the solution was transferred into a 150 ml volumetric flask and mixed thoroughly with 5 ml each of zinc acetate and potassium ferric cyanide solutions consecutively. The mixture was allowed to stand for a 5 min before being filtered through a dry No. 1 Whatman filter paper. 10 ml of filtrate was transferred into a separator funnel and the alkaloids present were extracted by shaking vigorously with five successive 30 ml portions of chloroform. The residue obtained was dissolved in hot water and transferred into a Kjeldahl flask with the addition of 0.2 g Selenium for digestion to a colourless solution to determine the percentage nitrogen by Kjeldhal distillation method. The percentage nitrogen obtained was multiplied by dilution factor 6.26 to get percentage total alkaloid.

Alkaloid (%) = Nitrogen percentage × 6.26

Determination of tannins

An amount of 2 ml of each extract was mixed with distilled water in a separate tube and heated in a water bath. The mixture was filtered and ferric chloride reagent was added to each of the filtrates. Dark green coloration indicated the presence of tannins. To determine the amount of tannins present, 100 ml of 4:1 solvent mixture (that is, 80 ml of acetone + 20 ml of glacial acetic acid) was used to extract tannin from 2 g of each sample. After allowing the mixture to soak for 5 h, the sample was filtered using a double layered Whatman No.1 filter paper. The filtrate was made up to the 100 ml mark with distilled water and mixed thoroughly. One millilitre of sample extract was pipetted into 50 ml volumetric flask, 20 ml water was added as well as 2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 before mixing thoroughly. The mixture was made up to mark with water, mixed again and allowed to stand for 20 min. Bluish-green colouration indicated positive result for tannins. Standard tannic acid solution of range 0 to 10 ppm was treated similarly as 1 ml of sample earlier. The absorbance of the tannic

acid standard solution as well as sample was read after colour development on a spectrophotometer (Spectronic 20) at a wavelength of 760 nm. Percentage of tannin was calculated using the formula:

$$\text{Tannin (\%)} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{10{,}000}$$

Determination of total polyphenols

An amount of 10 g of samples were soaked in 20 ml of distilled water for 4 days. The samples were filtered and each filtrate was made up to 100 ml with distilled water. One millilitre of filtrate from each sample was measured into a test-tube, 3 ml of each 0.008 N potassium hexacyanoferrate (iii) and 0.01 N of iron (iii) chloride were added into each filtrate. The absorbance of each filtrate was read on a spectrophotometer after 10 min. The percentage total polyphenol was determined using the formula:

$$\text{Total polyphenol (\%)} = \frac{\text{Absorbance} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Determination of flavonoids

To 2 ml of each extract was added 2 ml of dilute sodium hydroxide and hydrochloric acid. Yellow solutions that turned colorless indicated the presence of flavonoids which were quantified by extracting 5 g of each sample with 100 ml solvent - 1% aluminum chloride solution in methanol concentrated HCl, magnesium turnins, and potassium hydroxide solution (Earnsworth et al., 1974). After extraction, absorbances of filtrates were read on a spectrophotometer at 380 mm wavelength and compared with 0.10, 0.15, 0.20, 0.25 and 0.30 ppm standards. The slope was calculated from the standard curve as follows:

$$\text{Flavonoid (\%)} = \frac{\text{Metre reading} \times \text{Slope} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Determination of glycosides

Five grams of each sample were weighed into 250 ml conical flask along with 50 ml of ethanol and 20 ml of distilled water. The mixture was sonicated for 5 min before the addition of 8 ml of concentrated hydrochloric acid. It was refluxed for 3 h and cooled to room temperature. After the extraction, filtration was done using double layer of filter paper. Each extract was transferred into a two-necked 50 ml flask connected with steam generator. This was steam-distilled with saturated sodium bicarbonate solution contained in a 50 ml conical flask for 60 min. One millilitre of starch indicator was added to 20 ml of each distillate and was titrated with 0.2 m of iodine solution. The percentage glycoside was calculated thus:

$$\text{Hydrocyanide (\%)} = \frac{\text{Titre value} \times 10 \times 0.27}{1000 \times \text{weight of sample}} \times 100$$

Antimicrobial testing

The antibacterial activities of aqueous and ethanol extracts of *H. sabdariffa* were determined by paper disc and agar well diffusion methods (Omenka and Osuoha, 2000; Mahesh and Satish, 2008).

Disc diffusion method

Sterile filter paper discs (5 mm diameter) were soaked in different extracts of known concentration (20 mg/ml per disc) for 2 h. The discs were carefully placed at the centre of labeled seeded plates. The plates were incubated at 37°C for 24 h after which the diameters of the zones of inhibition were measured in millimeters with a ruler. Standard discs of the antibiotic chloramphenicol at a concentration of 10 mg served as positive antibacterial control.

Agar well diffusion method

In this method, 0.1 ml of 24 h broth culture of each of the test microorganisms containing ca. log 7.0 CFU ml^{-1} was aseptically seeded into nutrient agar plates and allowed to solidify. The dried plates were then punched with sterile cork borer (5.0 mm diameter) to make open wells. Five hundred microlitres of the test extracts were introduced into the wells before they were incubated at 37°C for 24 h. The zones of inhibition were measured and recorded as for the paper disc diffusion method.

Minimum inhibitory concentration (MIC)

Different concentrations of the extracts of the calyces of *H. sabdariffa* were prepared to obtain 12.8, 6.4 and 0.20 mg/ml. Three drops of overnight broth culture of the test organisms were inoculated into the different dilutions and incubated at 37°C for 24 h. The MIC of the concentrated extracts against each test organism was taken as the lowest concentration of the extract that inhibited the test organism.

RESULTS AND DISCUSSION

The ethanol extracts of *H. sabdriffa* had higher concentrations of bioactive metabolites than the water extracts except for alkaloids. This result agrees with the work of Glass (1991) who showed that the non-polarity of organic solvents could be responsible for the more efficient extraction of the metabolites. However, saponins were generally well extracted by either water or ethanol.

Flavonoids were found to be the most abundant bioactive agent in *H. sabdriffa* followed by saponins, while polyphenols were the least abundant (Table 1). The flavonoid constituents of *H. sabdariffa* have been reported to show good effect on peroxidase and protease activity in human blood which confirmed the benefit of Roselle as an antioxidant and anti-aging plant, in addition to indications through *in vitro* and *in vivo* studies that flavonoids hold great potential as anticancer substances (Nhung et al., 1998).

Despite the small amounts present, polyphenols extracted from *H. sabdariffa* have been found to have anti-inflammatory effects *in vitro* and *in vivo* thereby improving anti-oxidative conditions (Erl-Shyh et al., 2009). The phytochemical composition of the various extracts was comparable to previous reports of Lin et al. (2005), Olaleye (2007) and Kao et al. (2009).

Results obtained from the anti-bacterial studies presented in Table 2 showed that the calyces of *H. sabdriffa* have significant inhibitory effects on the test pathogens.

Table 1. Concentration of bioactive metabolites in extracts of *Hibiscus sabdriffa* juice.

Bioactive compound	Roselle extract	
	Water extract	Ethanol extract
Saponins (%)	1.13 ± 0.03	1.46 ± 0.02
Alkaloids (%)	0.09 ± 0.01	0.08 ± 0.01
Tannins (%)	0.07 ± 0.01	0.19 ± 0.01
Total phenols (%)	0.05 ± 0.00	0.07 ± 0.00
Flavonoids (%)	1.08 ± 0.01	2.41 ± 0.02
Glycoside (%)	0.05 ± 0.00	0.13 ± 0.01

Values are represent means of three replicates ± Standard Deviation

Table 2. Antimicrobial activities of *Hibiscus sabdriffa* juice by disc and agar well diffusion methods.

Test organism	Paper disc diffusion			Open well diffusion		
	Aqueous extract	Ethanol extract	Chloramphenicol	Aqueous extract	Ethanol extract	Chloramphenicol
Escherichia coli	41.2 ± 0.2^{b}	43.2 ± 0.2^{c}	40.4 ± 0.4^{a}	43.5 ± 0.5^{c}	45.8 ± 0.2^{d}	41.2 ± 0.2^{b}
Salmonella typhi	39.1 ± 0.3^{c}	42.0 ± 0.3^{e}	36.0 ± 0.2^{b}	40.0 ± 0.2^{d}	43.8 ± 0.2^{f}	35.1 ± 0.1^{a}
Shigella dysentariae	27.9 ± 0.3^{a}	32.8 ± 0.2^{c}	30.2 ± 0.2^{b}	35.8 ± 0.3^{d}	43.2 ± 0.2^{e}	32.9 ± 0.3^{c}
Staphylococcus aureus	43.2 ± 0.2^{c}	45.6 ± 0.6^{e}	38.9 ± 0.2^{b}	44.7 ± 0.3^{d}	47.9 ± 0.2^{f}	37.8 ± 0.3^{a}
Streptococcus mutans	26.9 ± 0.4^{c}	28.1 ± 0.1^{d}	32.1 ± 0.3^{e}	22.2 ± 0.2^{a}	23.3 ± 0.3^{b}	33.2 ± 0.2^{f}

Values are zones of inhibition measured in mm ± standard deviation. Means with the same superscript are not significantly different at 5% level of significance along the rows.

Generally, the organisms were more sensitive to alcoholic extracts than aqueous extracts. In addition, the open well diffusion method was more sensitive going by the wider inhibition zones recorded for most of the test organisms. The agar well diffusion method was used because it is known to allow better diffusion of the extracts into the medium, thus enhancing contact with the test organisms and ensuring more accurate results. This observation agrees with the work of Omenka and Osuoha (2000) who showed that open well diffusion method allows better diffusion of the extracts into the medium, thus enhancing contact with the organisms as the organisms are introduced directly into the wells. Paper disc may acts as barrier between the extract and the organism thus preventing total diffusion of active components absorbed by the discs into the medium and may be responsible for the observed differences. However, the contrary was the case for *S. mutans* which was also the least sensitive to the extracts recording the lowest inhibition zones (22.2 to 28.1 mm) for both methods (Plate 1).

The widest zone of inhibition (47.9 mm) was observed by the ethanol extract of *H. sabdariffa* against *S. aureus* in the open well diffusion method (Plate 2). This value was followed by 45.8 mm observed in the open well diffusion method for the ethanol extract of Roselle against *Escherichia coli*. A beverage containing *H. sabdariffa* L.

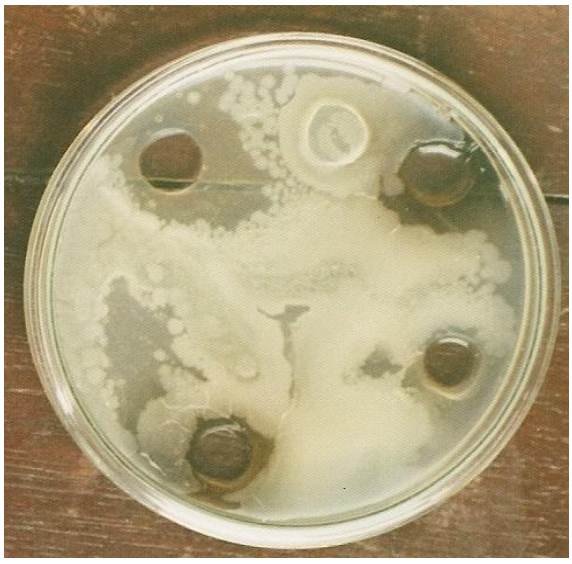

Plate 1. Inhibition of *Streptococcus mutans* by ethanol extract of *Hibiscus sabdariffa* using open well diffusion method.

was previously shown to possess bactericidal activity against *E. coli*, *Bacillus subtilis*, *S. typhi* and *K. pneumoniae* (Alian et al., 1983). An inhibitory effect of *H.*

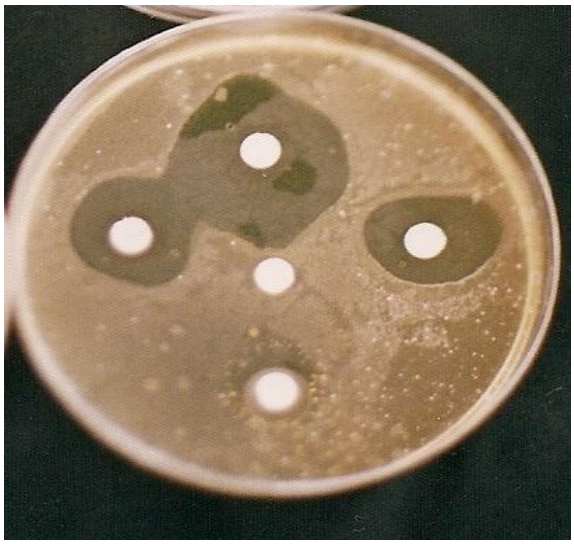

Plate 2. Inhibition of *Staphylococcus aureus* by ethanol extract of *H. sabdariffa* using disc diffusion method.

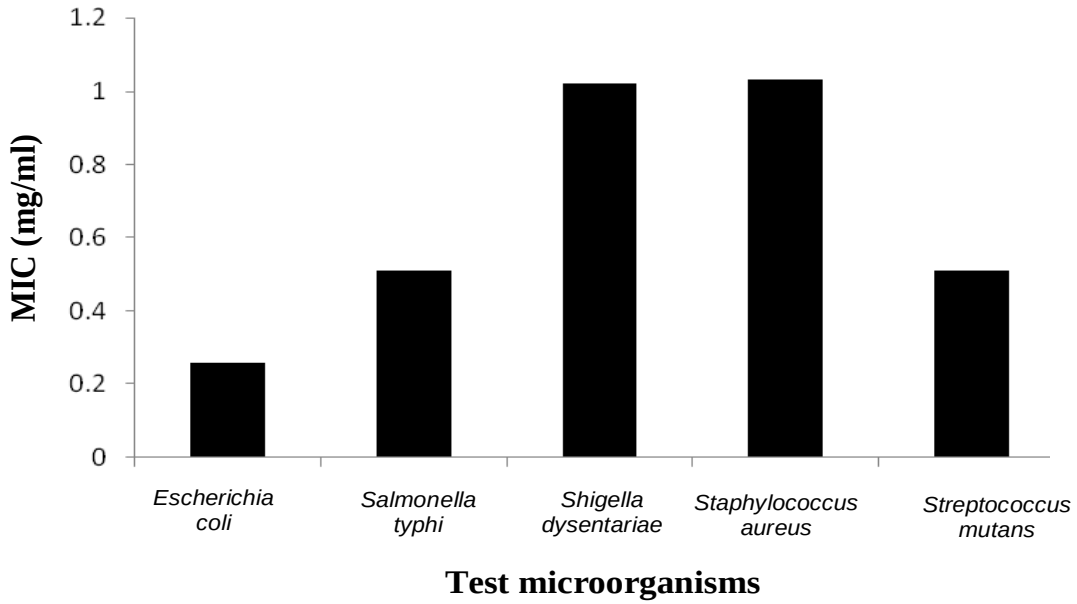

Figure 1. Minimum inhibitory concentration (mg/ml) of test bacteria by ethanol extract of Roselle.

corroborate the antibacterial effects of Roselle extracts against the test pathogens. The values obtained showed that the highest activity was recorded against *E. coli* in ethanol extracts of *H. sabdariffa* juice extract (Figure 1).

The anti-microbial activities demonstrated by the extracts of Roselle justify some of the ethno-pharmacological claims about this plant in the treatment of diseases caused by some of the test pathogens such as diarrhoea, dysentery, oral and dental infections.

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