

Title: Studies on proximate analysis and biocidal potentials of extracts from *Hibiscus sabdariffa* calyx on bacterial strains associated with human infections

Short running title: proximate analysis and biocidal potentials of extracts from *Hibiscus sabdariffa* calyx

Abstract

Hibiscus sabdariffa calyx was assessed for nutritional value and biocidal potential. Proximate analysis revealed moisture content of 2.85%, while protein, fat and carbohydrate contents were 18.31%, 3.40% and 73.62% respectively. Phytochemical screening revealed presence of reducing sugars, flavonoids, steroids and cardiac glycosides. Inhibition zones due to sensitivity testing of the ethylacetate fraction (10 mg/ml) ranged between 10.33±0.58 and 17.17±0.76 mm against *Pseudomonas aeruginosa* (CISp1) and *Escherichia coli* (NCIB 86) respectively. The partially purified ethylacetate fraction (5 mg/mL) inhibition zones ranged between 12.00±1.00 (*P. aeruginosa* (CISp1)) and 24.00±0.00 (*P. aeruginosa* (NCIB 950)) while MIC ranged between 1.25 and 2.50 mg/mL. After 120min of direct contact between the fraction (1×MIC) and *Staphylococcus aureus*, total cell death was 74.7%. Absolute mortality rate was achieved 3×MIC concentration at 120 mins and 60 mins for *Staphylococcus aureus* and *Klebsiella pneumoniae*. Amount of K⁺ leakages at 1×MIC was 2.24µg/mL from *S. aureus* and 3.38µg/mL from *K. pneumoniae*. In general, leakages of cytoplasmic contents increase with increase in the concentration of the extract and the contact time. The green calyx of *H. sabdariffa* was found to have combined nutritional and medicinal values, thus, confirm its traditional application as food and in the treatment of infections.

Keywords: Killing rate; Nucleotide leakage; Solvent partitioning system; Crude protein

Introduction

The role of vegetables in protecting the body from disease and regulate proper functioning of physiology of human body cannot be over emphasized (Anon *et al.*, 2005). Vegetables are important for human health and serve as source of vitamins, minerals, phytochemical compounds, dietary fiber content and antioxidant vitamins. They are known to improve gastrointestinal health and reduced risk of heart disease and other chronic diseases such as cancer (Hanif *et al.*, 2006). In addition, vegetables are the reliable source for many dietary factors and are considered as protective supplementary food. They are known to neutralize the acids produced during digestion of proteins and fats.

Hibiscus sabdariffa calyx is known for delicacy and has been describe as one of the most nutritious plant. The calyx forms an important part of diet among some tribes in West Africa especially in Nigeria. *Hibiscus sabdariffa* calyx plays an important role in maintaining general good health due to the presence of minerals and vitamins in them.

Vegetables including *H. sabdariffa* are valuable ingredients in herbal medicine and also possess antioxidant properties that prevent oxidative processes in human. Thus, vegetables could serve as a source of herbal medicine to combat the emergence of superbugs which have developed resistant against available conventional antibiotics used as therapy to treat pathogens infections.

Occurrence of acquired resistance among pathogens of infectious diseases largely indicates a serious threat to the public health as it is often not limited to a specific antibiotic, but mostly extends to other members of the same class (Alayande *et al.*, 2018) This situation will undoubtedly increase the risk of spreading of multi-drug resistant strains of pathogens among human population and animals, hence, results in the outbreak of disease causing strains which may in turn be expensive and difficult to eradicate (Alayande *et al.*, 2020).

The scientific communities are thereby obliged to developing new drugs and/or seek alternative therapies in a bid to mitigate the overwhelming consequence of this phenomenon on public health. Natural products, most especially of plant origin, have been the most important source of bioactive molecules for valuable drug discoveries; they are relatively safe when compared to their synthetic counterparts and are derived from inexpensive raw materials through cheap technology, thus, readily available even in form of health functional foods (Pogorzelska-Nowicka *et al.*, 2018). Medicinal plants synthesize different kinds of secondary metabolites which have been exploited in a diverse array of applications; they are of great importance because of their innate phytochemicals that initiates a definite physiological action within human system (Akinpelu *et al.*, 2015). In addition, bioactive molecules are equally present, in small amount, in plant-based foods where they provide health benefits in addition to the basic nutritional values expected to derive from foods (Gry *et al.*, 2007).

Hibiscus sabdariffa belongs to the family Malvaceae and it is referred to as “isapa” among the Yorubas of southwestern part of Nigeria. The plant is extensively cultivated in many parts of Nigeria as a leafy vegetable and is regarded as perennial and generally 1-2 m tall. Its red calyx is used in the production of a local beverage known as “sobo” drink, while the seeds are used in the preparation of “daddawa”, a local delicacy (Falusi, 2007). Additionally, the leaves and green calyx

(roselle) are used in making soup and sauces and are very rich in vitamin B₂ and C among other major nutrient elements (Chen *et al.*, 2013). Beside the nutritional benefits, *Hibiscus sabdariffa* plant has been applied traditionally to provide folklore remedy (Lin *et al.*, 2007). This plant is used among many tribes in Africa for the treatment of ailments which include hypertension, liver diseases, cancer and fever (Da-Costa-Rocha *et al.*, 2014). The leaf of *H. sabdariffa* is rich in phenolics and has been demonstrated as viable antioxidant (Ochani, 2009), antitumor (Lin *et al.*, 2012), anti-hyperammonemic (Essa and Subramanian, 2007), anti-atherosclerotic (Chen *et al.*, 2013), anti-filarial (Saxena *et al.*, 2011) and anti-hyperlipidemic agent (Gosain *et al.*, 2010). *Hibiscus sabdariffa* extract was found to inhibit the growth of *Corynebacterium diphtheria*, *Staphylococcus aureus*, *Staphylococcus captis*, *Pseudomonas aeruginosa* and *Proteus mirabilis* (Eltayeib and Hamade, 2014).

This study, therefore, evaluates nutritional and phytochemical components of the green calyx of *H. sabdariffa* and further determines antimicrobial potentials as well as the mechanisms of biocidal effectiveness of its extract against panel of clinically important bacterial pathogens. This is with a view to providing information on the nutritional values and antibacterial effectiveness of the green calyx of *H. sabdariffa*.

Materials and methods

Microorganisms used in this study were obtained from culture collection of Prof. D.A. Akinpelu, Department of Microbiology, Obafemi Awolowo University, Ile Ife, Nigeria and Microbiology Laboratory at Seventh Day Adventist Hospital, Ile-Ife, Nigeria. The green calyces of *Hibiscus sabdariffa* were collected in the month of November, 2019 from Offa, Kwara State, Nigeria. The plant was identified in the Herbarium of Department of Botany, Obafemi Awolowo University, Ile Ife, Nigeria, where a specimen sample was deposited for future reference.

Preparation and extraction of the plant sample

The green calyx of *H. sabdariffa* was dried in hot-air oven at 45°C until a constant weight of the sample was obtained. The sample was ground into fine powder and exactly 1320 g of the powdered sample was extracted in methanol/sterile distilled water (3:2, v/v) for four days with regular agitation. The supernatant collected was filtered, and the filtrate was concentrated *in vacuo* in rotary evaporator. The aqueous part was lyophilized and the yield obtained was 240 g. This was kept in an air-tight container in the refrigerator at 5°C for further use.

Phytochemical screening of the extract

The dried extract of the green calyx was subjected to phytochemical screening using methods described by Harborne (1993) and Trease and Evans (2002) to test for alkaloids, tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycoside.

Proximate Analysis of the extract of green calyx of *H. sabdariffa*

The proximate analysis of the extract was carried out to determine nutrient contents of the green calyx following the standard procedure by Association of Official Analytical Chemists (AOAC, 2005).

Moisture content: Two grams of the extract (sample) in pre-weighed crucible was placed in an oven (105°C) for 24 h, cooled and re-weighed. The percentage moisture was calculated as follows:

$$\text{Moisture (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

W₁ is the weight of the crucible, *W₂* is the weight of the crucible plus sample before drying at 105°C, and *W₃* is the weight of the crucible plus sample after cooling in an airtight desiccator.

Crude protein: Crude protein content was determined using the micro-Kjeldahl method as described by Pearson (2010). A volume of 10 mL H₂SO₄ added to 0.2 g of the extract was digested

with a Kjeldahl digester (Model Bauchi 430) for a period of 90 min. A volume of 50 mL water was added and distilled using Kjeldahl distillation unit (Model unit B - 316) containing 40% concentrated NaOH and millipore water. Liberated ammonia was collected in 20 mL boric acid with bromocresol green and methyl red indicators and titrated against 0.04 N H₂SO₄. A blank (without sample) was likewise prepared. Percentage protein was calculated as:

$$\text{Crude protein (\%)} = \frac{\text{Sample titer} - \text{blank titer} \times 14 \times 6.25}{\text{Sample weight}}$$

14 = molecular weight of nitrogen; 6.25 = nitrogen factor

Crude fibre: A weighed crucible containing 2 g of defatted sample was attached to the extraction unit (in Kjeldahl, D-40599; Behr Labor- Technik GmbH, Dusseldorf, Germany). Exactly 150 mL of hot 1.25% H₂SO₄ was added to the defatted sample and digested for 30 min. The acid was drained and sample washed with hot distilled water for 90 min. The crucible was removed and oven-dried overnight at 105°C, cooled, weighed, and incinerated at 550°C in a muffle furnace (MF-1- 02; PCSIR Labs, Lahore, Pakistan) overnight. This was re-weighed after cooling. Percentage extracted fiber was calculated as:

$$\text{Crude fibre (\%)} = \frac{\text{Weight of digested sample} - \text{Weight of ashed sample} \times 100}{\text{Weight of sample}}$$

Lipid: Lipid content was estimated using TecatorSoxtec (Model 2043 Hilleroed, Denmark). A quantity of 2 g sample was weighed into a thimble and covered with absorbent cotton, while 40 mL of petroleum ether (40-60°C Bpt) was added to a pre-weighed cup. Both thimble and cup were attached to the extraction unit. The sample was extracted using ethanol for 30 mins and rinsed for 1½ h. Thereafter, the solvent was evaporated from the cup to the condensing column. Extracted fat in the cup was then placed in an oven for 1 h and cooled and weighed. Percent fat was calculated as:

$$\text{Lipid (\%)} = \frac{\text{Initial cup weight} - \text{Final cup weight}}{\text{Weight of sample}} \times 100$$

Ash: Exactly 2 g of the sample was added into a pre-weighed crucible and incinerated in muffle furnace at 600°C.

$$\text{Ash (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 10$$

W_1 is the weight of cleaned, dried, ignited and cooled crucible, W_2 is the weight of the crucible and sample after incinerating at 600°C and W_3 is the weight of the crucible and sample after cooling in an airtight homogenised vessel.

Carbohydrate: Carbohydrate content of the sample was determined by addition of all the percentages of moisture, fat, crude protein, ash and crude fiber, and then subtracted from 100%. This gave the amount of nitrogen-free extract otherwise known as carbohydrate.

$$\% \text{ Carbohydrate} = 100 - (\% \text{ Moisture} + \% \text{ Fat} + \% \text{ Ash} + \% \text{ Crude fibre} + \% \text{ Crude protein})$$

Solvent partitioning of the crude extract of *Hibiscus sabdariffa* calyx

The crude extract (100 g) was resolved in 200 mL of sterile distilled water and poured into in a separatory funnel and extracted with n-hexane (4 x 200 ml). The solvent showed no affinity for the bioactive content of the extract. The resultant aqueous phase was re-concentrated to drive out n-hexane leftover. The residue collected was further extracted with chloroform (4 x 200 ml). The chloroform fraction obtained was concentrated *in vacuo* to dryness and 29.80 g yield of the fraction was collected. The ethyl acetate fraction (18.55 g) and butanol fraction (25.60 g) fractions were also obtained using similar procedure. The remaining aqueous fraction was freeze-dried after re-concentrating in rotary evaporator to yield 20.79 g.

Susceptibility testing of the extracts against bacterial isolates

The susceptibility testing of the extracts was determined using agar-well diffusion method (Akinpelu *et al.*, 2015b; EUCAST, 2016). Standardised (0.5 McFarland standard) 18-h old inoculum was subcultured (0.2 ml) into cooled molten Mueller-Hinton agar (Lab M) and mixed gently before poured into a sterile Petri dish. This was allowed to set and wells were then bored into the inoculated medium using a sterile 6 mm cork borer. Each well was carefully filled with prepared solution of the extract and incubated at 37°C for 24 h. Thereafter, each plate was observed for zones of inhibition while effect of the extract on bacterial isolates was compared with that of streptomycin and ampicillin (1 mg/mL).

Determination of minimum inhibitory concentrations (MIC) of the extract

Minimum inhibitory concentrations of the extracts were determined as previously described by Akinpelu *et al.* (2015a). Two-fold dilution of the extract was prepared and 2 mL of different concentrations was added to 18 mL of pre-sterilized molten nutrient agar to give final concentrations regimes of 0.547 to 17.50 mg/mL. The medium was then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry before streaked with 18-h old standardized bacterial cultures. The plates were thereafter incubated at 37°C for 72 h after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration of the extract that prevented growth of the bacteria.

Determination of minimum bactericidal concentrations (MBC) of the extracts

Minimum bactericidal concentrations of the extract were determined as previously described (Akinpelu *et al.*, 2015b). Samples were taken from plates with no visible growth on the MIC plates, sub-cultured on to freshly prepared nutrient agar plates and then incubated at 37°C for 48 h. The MBC was taken as the lowest concentration of the extract that did not allow any bacterial growth.

Determination of rate of kill of the test isolates by active fractions

The rate of kill was performed using the method described by Odenholt *et al.* (2001) and Akinpelu *et al.* (2015a). Experiment was determined using each of the active fractions on the viability of *S. aureus* representing Gram-positive and *K. pneumoniae* representing Gram-negative organisms. Total viable counts of the test organisms were initially determined. A 0.5 mL volume of known cell density (by viable counts 10^6 cfu/mL) from each organism suspension was added to 4.5 mL of different concentrations of the extract. The suspension was thoroughly mixed and held at room temperature while the killing rate was determined over a period of 2 h. Exactly 0.5 mL of each suspension was withdrawn at the appropriate time intervals and transferred to 4.5 mL nutrient broth (Oxoid Ltd) recovery medium containing 3% “Tween 80” to neutralize the effects of the antimicrobial compounds carry-over from the test suspensions. The suspension was shaken properly then serially diluted in sterile physiological saline and 0.5 mL of the dilutions was plated out on nutrient agar (Oxoid Ltd) and incubated at 37°C for 72 h. Control experiment was set up without the inclusion of antimicrobial agent. Viable counts were made in duplicates for each sample and depression in the viable counts indicated killing by the antimicrobial agents.

Determination of potassium ions leakage from the test isolates by the active fractions

This was assessed following method described by Oladunmoye *et al.* (2007) and Akinpelu *et al.* (2015a). Eighteen hour old test cells were washed in 0.9% w/v NaCl (normal saline) and standardized. The Standardized suspension of washed bacterial cells (*S. aureus* and *K. pneumoniae*) cells were treated with various concentrations of the extract relative to the MIC at various time intervals for 2 h. Each suspension was then centrifuged at 10,000 rpm and supernatant collected was assessed for the presence of potassium ions using atomic absorption spectrophotometer. The percentage of potassium ions leaked was extrapolated using standard

curve. Sterile normal saline inoculated with the same quantity of test bacterial cells was used as control.

Determination of nucleotides leakage from the test isolates by the active fractions

The method described by Heipieper *et al.* (1992) and Alayande *et al.* (2017) was used to determine the leakage of the nucleotides from the test cells. Cells of *S. aureus* and *K. pneumoniae* from 18 h old nutrient broth culture were separately washed in 0.9% (w/v) NaCl. Standardized washed suspension of *S. aureus* and *K. pneumoniae* (inoculum size approximately 10^6 cells) were treated with various concentrations of the fractions relative to the MICs at various time intervals for 2 h. Each suspension was then centrifuged at 10,000 rpm and the optical density (OD) of the supernatant collected was measured at 260 nm wavelength using spectrophotometer. Sterile distilled water inoculated with the same quantity of inoculum was used as control.

Partial purification of the ethyl acetate fraction

The ethyl acetate fraction showed appreciable activities than other fractions and was therefore chosen for partial purification for further tests. This was carried out through thin-layer and column chromatography. The best solvent system for the elution of ethyl acetate fraction on column chromatography was determined by eluting the fraction with different solvent system on TLC plate. The solvent system determined for the elution base on the R_f values was in the order n-hexane, n-hexane-chloroform (9:1) and ethyl acetate-methanol (8:2). The active ethyl acetate fraction of the green calyx of *H. sabdariffa* were afterwards dissolved in minimal amount of ethyl acetate, adsorbed on a small quantity of silica gel of 230-400 mesh size and then allowed to dry. This was thereafter chromatographed on silica gel column (650 x 40 mm) and eluted with pre-determined solvent system. The column chromatography of ethyl acetate fraction yielded five

partially purified fractions (ETHYL A, ETHYL B, ETHYL C, ETHYL D and ETHYL E). The bulked fraction were concentrated to dryness *in vacuo* on rotary evaporator and the yields collected were stored in an air-tight container and kept in the refrigerator for further use.

Results

Phytochemical screening of the green calyx of *Hibiscus sabdariffa* extract revealed the presence of steroids, cardiac glycosides, reducing sugars and flavonoids (Table I). The proximate analysis of the extract equally showed the presence of carbohydrate, protein, crude fibre, fat and ash contents at different concentration (Table II).

Antibacterial activities of the extracts

All the test bacterial isolates are sensitive to the crude extract of *H. sabdariffa* green calyx at 35 mg/mL, except *Pseudomonas aeruginosa* (CISp1) and (CISp2); *Escherichia coli* (CIS1) and (CIS2); *Klebsiella pneumoniae* (CIW2); *Proteus mirabilis* (CIU2); and *Micrococcus luteus* (NCIB 196). When the extract was portioned into different fractions, only ethyl acetate fraction (10 mg/mL) maintained the antibacterial property effectively against 74% of the entire test isolates (Table II). None of the isolate was sensitive to the extract portioned into aqueous fraction. Chloroform fraction was only effective against *Klebsiella pneumoniae* (CIW 1) and *Pseudomonas aeruginosa* (ATCC 7700); and n-butanol fraction against *Escherichia coli* (ATCC 25922) at the same concentration of 10 mg/mL. About 26% of the isolates were resistant to both streptomycin and ampicillin (1 mg/mL) used as positive control while 74% were resistant to only ampicillin (Table II).

The minimum inhibitory concentrations determined for the active ethyl acetate fraction varies between 1.25 and 2.50 mg/mL throughout the entire test isolates, while that of the minimum bactericidal concentrations ranges between 2.50 and 5.0 mg/mL (Table IV). The active ethyl

acetate fraction was partially purified through column chromatography where it was fractionalized into ethyl A – ethyl E. Each collected fraction was tested at a concentration of 5 mg/mL. The fraction ethyl A proved to be the most active with effectiveness against 71% of the test isolates and exhibited zones of inhibition ranging between 12 ± 1.00 and 24 ± 0.00 (Table V).

Time-kill assessment

The extent and rate of killing of *Staphylococcus aureus* and *Klebsiella pneumoniae* by ethyl acetate fraction at the concentrations of $1 \times \text{MIC}$, $2 \times \text{MIC}$ and $3 \times \text{MIC}$ are shown in Fig. 1. The percentage of the bacterial cells killed by the fraction at $1 \times \text{MIC}$ after 15 min of contact with *S. aureus* was 15.1% and increased to 23.1% when the concentration was doubled. At the maximum test-time of 120 min, the total number of cell death was 74.7% at $1 \times \text{MIC}$. This increased with increase in the concentration of the fraction such that, 95.7% total cell death was recorded at $2 \times \text{MIC}$ while 100% was achieved at $3 \times \text{MIC}$. When the fraction was in contact with the *K. pneumoniae*; at the concentration of $1 \times \text{MIC}$, the percentage of the bacterial cells killed was 12.6% after 15 min of treatment. This increased to 19.5% and 23.3% after 30 and 60 min respectively. When the timing was increased to 90 and 120 min, the rate of cell death was 85.5% and 88.1% respectively. The same trend of increased death rate was observed with increase in the concentration and contact time. Total mortality rate was achieved after 60 min at a concentration of $3 \times \text{MIC}$.

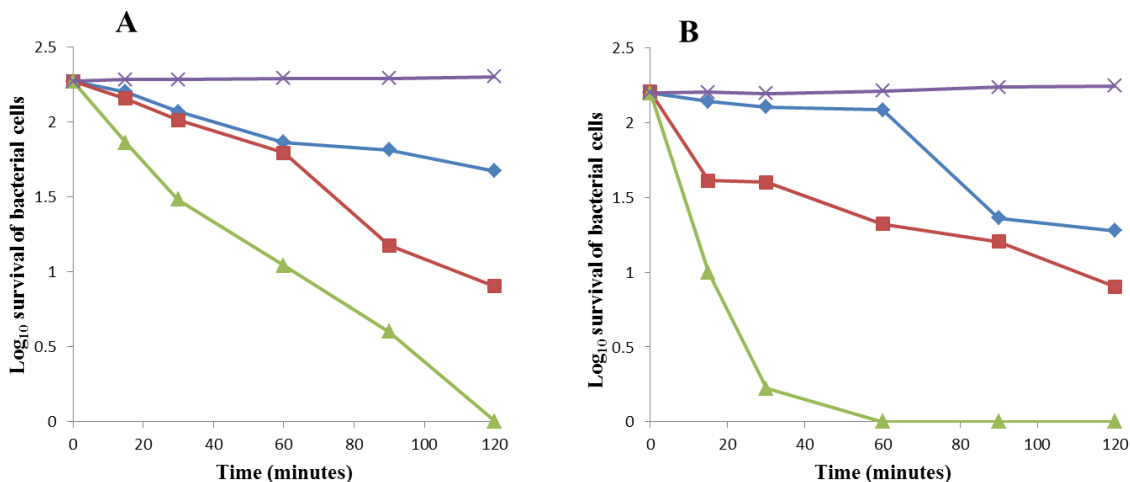


Fig. 1: The killing rate of *Staphylococcus aureus* (A) and *Klebsiella Pneumoniae* (B) cells by the ethyl acetate fraction at 1 × MIC (—◆—), 2 × MIC (—■—), 3 × MIC (—▲—) and control (—×—)

Leakages of cytoplasmic contents

The effect of ethyl acetate fraction on potassium ion leakage from *Staphylococcus aureus* and *Klebsiella pneumoniae* at different concentrations are shown in Fig. 2. The quantity of the potassium ion leaked away from the *S. aureus* cells after 15 minutes of contact time at 1 × MIC was 2.24 µg/ml, and 2.26 µg/ml after 30 min; the leaked quantity only slightly increased to 2.37 µg/mL after 120 min of contact time under the same condition. When the concentration of the fraction was increased to 2 × MIC, the maximum amount of potassium ion leaked away from the cells was 4.38 µg/mL after 120 min of contact time; and 7.42 µg/mL over the same period of time but at a concentration of 3 × MIC. On the other hand, the quantity of potassium ion leaked away from *K. pneumoniae* the cells when treated with the fraction at 1 × MIC was 3.38 µg/mL after 15 min of contact time. When the treatment time was increased to 30, 60, 90 and 120 min, there was very little difference in the amount of the leakages recorded under similar concentration and these are 3.73, 3.77, 3.87 and 3.88 µg/mL respectively. Similar pattern was observed when the concentration was increased to 2 × MIC and 3 × MIC, with initial leakages of 6.99 µg/mL and 11.32 µg/mL respectively after 15 min of contact time.

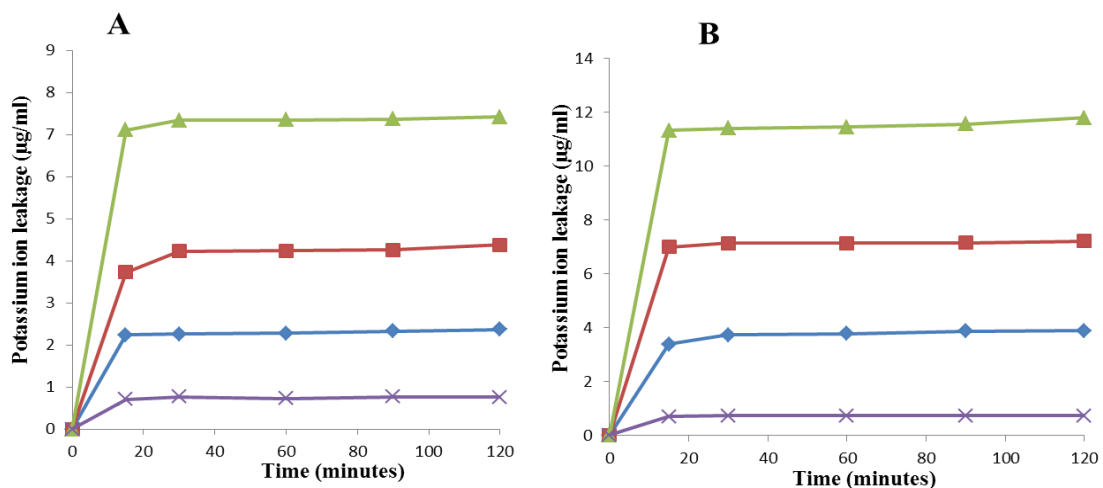


Fig. 2: Potassium ion leakage from *Staphylococcus aureus* (A) and *Klebsiella Pneumoniae* (B) cells by the ethyl acetate fraction at 1 × MIC (—◆—), 2 × MIC (—■—), 3 × MIC (—▲—) and control (—×—)

The effect of ethyl acetate fraction on nucleotide leakage from *S. aureus* and *Klebsiella pneumoniae* under different concentration of 1 × MIC, 2 × MIC and 3 × MIC are shown in Fig. 3. The amount of nucleotide leakage as observed in both isolates was increasing with increase in the concentration of the fraction and contact time. The maximum amount of nucleotide leakage (2.3 µg/mL) observed from *S. aureus* cells at a concentration of 3 × MIC and after 120 min of contact time. Likewise, from the *Klebsiella pneumoniae*, the maximum amount of nucleotide (2.49 µg/mL) was recorded under the same condition of concentration and time of exposure.

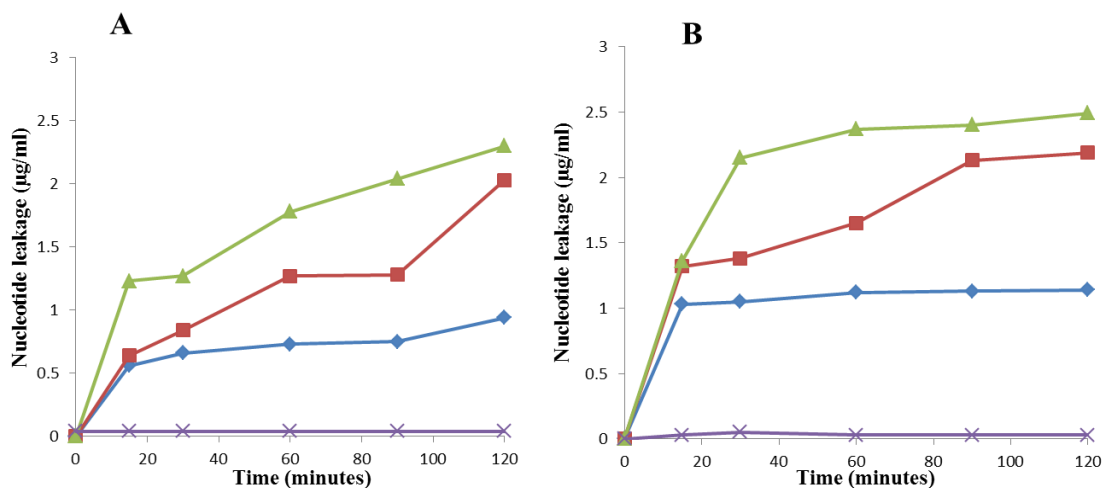


Fig. 3: Nucleotides leakage from *Staphylococcus aureus* (A) and *Klebsiella Pneumoniae* (B) cells by the ethyl acetate fraction at 1 × MIC (—◆—), 2 × MIC (—■—), 3 × MIC (—▲—) and control (—×—)

Discussion

The antibacterial potential of the extract obtained from green calyx of *H. sabdariffa* was investigated against some panel of bacterial strains comprising of both Gram-positive and Gram-negative. The extract partitioned into ethyl acetate was the only active fraction that showed appreciable level of activity against test bacterial strains, thus, indicates that ethyl acetate could be the best solvent for extraction of the bioactive components of *H. sabdariffa* calyx. The active fraction tested at a concentration of 10 mg/mL, inhibited 23 out of the 31 test strains while the partially purified Ethyl A fraction (5 mg/mL) inhibited the growth of 22 out of 31 test isolates with wider zones of inhibition. Considering the crude nature of the extract, it can be said that green calyx extract compared favourably with the streptomycin (1 mg/mL) which inhibited the growth of 20 bacterial strains and ampicillin (1 mg/mL) that was only effective against 8 bacterial strains among all test isolates. Therefore, the green calyx of *H. sabdariffa* appears to be a potential source of antibacterial compounds that could be of relevance in the treatment of bacterial infections of

various kinds. This supports the application of *H. sabdariffa* for the treatment of microbial infections in folklore remedies.

Among the pathogens susceptible to green calyx of *H. sabdariffa* extracts are *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis*. These pathogens are known as etiological agents for life threatening infections such as endocarditis, mastitis, osteomyelitis, chronic diarrhoea and other serious human infectious diseases. For instance, *E. coli* has been implicated in diarrhoea and urinary tract infection while *K. pneumoniae* is associated with pneumonia in humans (Pelczar *et al.*, 2006; Alayande *et al.*, 2017). This plant could, therefore, be a source of antimicrobial compounds of natural origin which may be useful in the treatment of infections caused by these pathogens.

The phytochemical analysis of green calyx extract of *H. sabdariffa* revealed the presence of flavonoids, steroids, reducing sugars and cardiac glycosides. These phytochemical compounds are known to play important roles in bioactivity of medicinal plants. The medicinal values of a plant depend on the kind of bioactive principles residing within the tissue of such plant (Akinpelu *et al.*, 2008). These bioactive metabolites have different medicinal potentials and mechanisms of action; largely depend on their molecular make up and structures. Flavonoids, for instance, are among the identified bioactive metabolites; they exhibit a wide range of biological activities which include antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic effects and antioxidant properties (Maikai, 2009). Flavonoids belong to a group of polyphenols which are structurally diverse, easily obtained in nature, and have been applied traditionally in the treatment of bacterial infections of various kinds (Farhadi *et al.*, 2019). They have been demonstrated to have wide range and credible efficiency against bacterial pathogens with very little toxicity towards human cells and no apparent haemolytic reaction when assessed for safety features (Wu *et al.*, 2019). This

safety attributes corroborate the fact that green calyx of *H. sabdariffa* is being used as nutrient source in form of vegetable in soup making and as local beverage; coupled with the identification of major nutritional components such as carbohydrate, protein, fat and fibre revealed through proximate analysis as integral phyto-components of this plant.

Damage to bacterial membrane often results in the release of cytoplasmic constituents of the cells and these may include: nucleic acids, potassium ions, sodium ions and other materials (Stojkovic *et al.*, 2013). The leakages of potassium ion and nucleic acids from the test bacterial cells demonstrated in this study, could suggest loss of structural integrity of the membrane which may have direct impact on selective permeability of the cytoplasmic membrane and thus allows escape of some important intracellular components and subsequent cell death. The results obtained from this study showed that *H. sabdariffa* calyx exerted its cidal effects on the test cells through disruption of their cell membranes. Leakage of nucleotides from the test cells

Conclusion

In conclusion, the green calyx of *H. sabdariffa* was found to have combined both nutritional and medicinal values. This, therefore, confirm its traditional application as food and in the treatment of infections. This study further suggests ethyl acetate as the appropriate solvent in the extraction of the antibacterial components of this part of *H. sabdariffa*. This will go a long way in the provision of alternative and affordable antibacterial agent for improve accessibility towards adequate and effective therapy against infectious diseases caused by bacterial pathogens.

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Table I: Phytochemical Screenings of the Green Calyx Extract of *Hibiscus sabdariffa*

Phytochemicals	Result
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Alkaloids	Absent
Reducing sugar	Present
Tannins	Absent
Flavonoid	Present
Steroids	Present
Saponins	Absent
Cardiac glycoside	Present

Table II: Proximate Analysis of the Calyx Extract of *Hibiscus Sabdariffa*

Sample	Quantity (%)
Dry matter	97.15
Moisture content	2.85
Carbohydrate	73.615
Protein	18.31
Crude fibre	0.015
Fat	3.40
Ash	1.81

Table III: Sensitivity patterns of the active extracts and standard antibiotics against test isolates

Bacterial isolates

Zones of inhibition **

	Crude Extract (35 mg/ml)	Ethyl acetate fraction (10 mg/mL)	Streptomycin (1 mg/ml)	Ampicillin (1 mg/ml)
<i>Pseudomonas aeruginosa</i> (CISp1)	0	10.33 ± 0.58	14.00 ± 1.73	0
<i>Pseudomonas aeruginosa</i> (CISp 2)	0	0	27.00 ± 0.87	0
<i>Pseudomonas aeruginosa</i> (CISp 3)	12.00 ± 0.00	10.67 ± 0.58	14.67 ± 0.58	0
<i>Escherichia coli</i> (CIU 1)	11.67 ± 2.90	11.67 ± 0.58	11.83 ± 0.29	0
<i>Escherichia coli</i> (CIU 2)	10.67 ± 1.15	0	15.33 ± 0.58	12.00 ± 0.00
<i>Escherichia coli</i> (CIU 3)	10.33 ± 0.58	10.33 ± 0.58	23.50 ± 1.50	21.67 ± 1.44
<i>Escherichia coli</i> (CIS1)	0	11.17 ± 2.02	16.67 ± 2.31	0
<i>Escherichia coli</i> (CIS2)	0	0	0	0
<i>Klebsiella pneumoniae</i> (CIW 1)	12.00 ± 2.00	10.33 ± 0.58	0	0
<i>Klebsiella pneumoniae</i> (CIW 2)	0	0	0	18.67 ± 1.15
<i>Klebsiella pneumoniae</i> (CIW 3)	10.33 ± 0.58	20.30 ± 0.29	0	0
<i>Klebsiella pneumoniae</i> (CIW 4)	11.00 ± 1.00	11.33 ± 1.15	0	0
<i>Proteus mirabilis</i> (CIU1)	10.50 ± 0.87	13.33 ± 1.15	13.33 ± 1.15	0
<i>Proteus mirabilis</i> (CIU2)	0	0	0	0
<i>Proteus mirabilis</i> (CIU 3)	10.67 ± 0.76	14.33 ± 1.04	0	0
<i>Proteus mirabilis</i> (CIU 4)	10.67 ± 0.58	11.17 ± 0.29	16.00 ± 0.00	10.67 ± 0.58
<i>Proteus vulgaris</i> (NCIB 67)	10.33 ± 0.58	0	18.67 ± 2.31	0
<i>Pseudomonas fluorescens</i> (NCIB 3756)	11.83 ± 0.29	13.50 ± 0.50	19.33 ± 1.15	0
<i>Pseudomonas aeruginosa</i> (ATCC 7700)	10.83 ± 1.04	11.67 ± 0.29	21.00 ± 1.00	0
<i>Pseudomonas aeruginosa</i> (NCIB 950)	10.67 ± 1.15	11.00 ± 0.58	0	0
<i>Klebsiella pneumoniae</i> (NCIB 418)	10.67 ± 0.58	14.17 ± 0.50	19.67 ± 0.58	0
<i>Klebsiella pneumoniae</i> (ATCC 4532)	11.00 ± 1.73	10.33 ± 0.58	18.33 ± 0.58	0
<i>Escherichia coli</i> (ATCC 25922)	15.70 ± 1.15	0	21.67 ± 1.44	12.00 ± 0.00
<i>Escherichia coli</i> (NCIB 86)	11.33 ± 1.15	17.17 ± 0.76	16.17 ± 2.50	20.00 ± 1.73
<i>Clostridium. sporogenes</i> (NCIB 532)	10.00 ± 0.00	10.67 ± 1.15	20.00 ± 1.73	16.00 ± 2.00
<i>Micrococcus luteus</i> (NCIB 196)	0	0	20.00 ± 0.00	0
<i>B. stearothermophilus</i> (NCIB 8222)	10.00 ± 0.00	12.33 ± 0.58	0	0
<i>Bacillus subtilis</i> (NCIB 3610)	10.67 ± 0.58	11.67 ± 0.58	20.00 ± 1.73	0
<i>Bacillus polymyxa</i> (LIO)	10.67 ± 1.15	13.83 ± 0.76	0	24.33 ± 1.15
<i>Bacillus cereus</i> (NCIB 6349)	11.33 ± 2.31	11.50 ± 0.86	20.83 ± 2.75	0
<i>Staphylococcus aureus</i> (NCIB 8588)	12.83 ± 0.29	15.17 ± 0.29	0	0

KEY: CISp = Clinical Isolates from Sputum, CIS1 = Clinical Isolates from Stool, CIU = Clinical Isolates from Urine, CIW = Clinical Isolates from Wound, NCIB = National collection of Industrial Bacteria, ATCC = American Typed Culture Collections, LIO = Locally Isolated Organism, ** = Mean of three replicates.

Table IV: The Minimum inhibitory concentrations and minimum bactericidal concentrations of the ethyl acetate fraction exhibited against the test isolates

Bacterial isolates	MIC (mg/mL)	MBC (mg/mL)
<i>Pseudomonas aeruginosa</i> (CISp 1)	2.50	5.00
<i>Pseudomonas aeruginosa</i> (CISp 2)	2.50	5.00
<i>Pseudomonas aeruginosa</i> (CISp 3)	2.50	5.00
<i>Escherichia coli</i> (CIU 1)	1.25	2.50
<i>Escherichia coli</i> (CIU 2)	1.25	5.00
<i>Escherichia coli</i> (CIU 3)	1.25	2.50
<i>Escherichia coli</i> (CISp 1)	1.25	2.50
<i>Escherichia coli</i> (CISp 2)	ND	ND
<i>Klebsiella pneumoniae</i> (CIW 1)	1.25	2.50
<i>Klebsiella pneumoniae</i> (CIW 2)	1.25	2.50
<i>Klebsiella pneumoniae</i> (CIW 3)	1.25	2.50
<i>Klebsiella pneumoniae</i> (CIW 4)	2.50	5.00
<i>Proteus mirabilis</i> (CIU 1)	1.25	2.50
<i>Proteus mirabilis</i> (CIU 2)	1.25	2.50
<i>Proteus mirabilis</i> (CIU 3)	1.25	2.50
<i>Proteus mirabilis</i> (CIU 4)	1.25	2.50
<i>Proteus vulgaris</i> (NCIB 67)	1.25	2.50
<i>Pseudomonas fluorescens</i> (NCIB 3756)	1.25	2.50
<i>Pseudomonas aeruginosa</i> (ATCC 7700)	2.50	5.00
<i>Pseudomonas aeruginosa</i> (NCIB 950)	1.25	5.00
<i>Klebsiella pneumoniae</i> (NCIB 418)	1.25	2.50
<i>Klebsiella pneumoniae</i> (ATCC 4532)	2.50	5.00
<i>Escherichia coli</i> (ATCC 25922)	1.25	2.50
<i>Escherichia coli</i> (NCIB 86)	2.50	5.00
<i>Clostridium. sporogenes</i> (NCIB 532)	2.50	5.00
<i>Micrococcus luteus</i> (NCIB 196)	1.25	2.50
<i>B. stearothermophilus</i> (NCIB 8222)	ND	ND
<i>Bacillus subtilis</i> (NCIB 3610)	ND	ND
<i>Bacillus polymyxa</i> (LIO)	ND	ND
<i>Bacillus cereus</i> (NCIB 6349)	1.25	2.50
<i>Staphylococcus aureus</i> (NCIB 8588)	1.25	2.50

KEY: CISp = Clinical Isolates from Sputum, CISp = Clinical Isolates from Stool, CIU = Clinical Isolates from Urine, CIW = Clinical Isolates from Wound, MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, NCIB = National Collection of Industrial Bacteria, ATCC = American Typed Culture Collections, LIO = Locally Isolated Organism, ND = Not Determined

Table V: Antimicrobial activities of partially purified fractions of the extract portioned into ethyl acetate

Zones of Inhibition (mm)**

Bacterial Isolates	ETHYL A (5 mg/mL)	ETHYL B (5 mg/mL)	ETHYL C (5 mg/mL)	ETHYL D (5 mg/mL)	ETHYL E (5 mg/mL)
<i>Pseudomonas aeruginosa</i> (CISp1)	12.00 ± 1.00	0	10.00 ± 0.00	0	0
<i>Pseudomonas aeruginosa</i> (CISp2)	20.00 ± 0.00	0	0	0	0
<i>Pseudomonas aeruginosa</i> (CISp3)	18.00 ± 1.00	0	12.00 ± 0.51	0	0
<i>Escherichia coli</i> (CIU1)	21.00 ± 0.50	0	0	0	0
<i>Escherichia coli</i> (CIU 2)	18.00 ± 0.85	0	0	0	0
<i>Escherichia coli</i> (CIU 3)	0	0	0	0	0
<i>Escherichia coli</i> (CIS t 1)	16.00 ± 1.50	0	0	0	0
<i>Escherichia coli</i> (CIS t 2)	0	0	0	0	0
<i>Klebsiella pneumoniae</i> (CIW 1)	18.00 ± 0.61	0	0	0	0
<i>Klebsiella pneumoniae</i> (CIW 2)	15.00 ± 0.00		0	0	0
<i>Klebsiella pneumoniae</i> (CIW 3)	14.00 ± 0.00	0	0	0	0
<i>Klebsiella pneumoniae</i> (CIW 4)	0	0	0	0	0
<i>Proteus mirabilis</i> (CIU 1)	0	0	0	0	0
<i>Proteus mirabilis</i> (CIU 2)	0	0	0	0	0
<i>Proteus mirabilis</i> (CIU 3)	0	0	0	0	0
<i>Proteus mirabilis</i> (CIU 4)	0	0	0	0	0
<i>Proteus vulgaris</i> (NCIB 67)	18.00 ± 0.00	0	0	0	0
<i>Pseudomonas fluorescen</i> (NCIB 3756)	20.00 ± 1.00	14.00 ± 1.50	13.00 ± 1.00	13.00 ± 0.55	0
<i>Pseudomonas aeruginosa</i> (ATCC 7700)	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> (NCIB 950)	24.00 ± 0.00	0	0	0	0
<i>Klebsiella pneumoniae</i> (NCIB 418)	18.00 ± 0.51	0	0	0	0
<i>Klebsiella pneumoniae</i> (ATCC 4532)	16.00 ± 0.52	10.67 ± 1.15	0	0	0
<i>Escherichia coli</i> (ATCC 25922)	17.00 ± 0.65	0	0	14.00 ± 1.73	0
<i>Escherichia coli</i> (NCIB 86)	16.00 ± 0.86	0	0	0	0
<i>Clostridium. sporogenes</i> (NCIB 532)	14.00 ± 0.00	0	0	0	0
<i>Micrococcus luteus</i> (NCIB 196)	15.00 ± 0.61	0	0	0	0
<i>B. stearothermophilus</i> (NCIB 8222)	20.00 ± 1.15	0	0	0	0
<i>Bacillus subtilis</i> (NCIB 3610)	0	0	0	0	0
<i>Bacillus polymyxa</i> (LIO)	18.00 ± 1.50	0	0	0	0
<i>Bacillus cereus</i> (NCIB 6349)	16.00 ± 0.51	10.00 ± 0.00	0	0	0
<i>Staphylococcus aureus</i> (NCIB 8588)	16.00 ± 0.00	0	0	0	0

KEY: CISp = Clinical Isolates from Sputum, CIS t = Clinical Isolates from Stool, CIU = Clinical Isolates from Urine, CIW = Clinical Isolates from Wound, NCIB = National Collection of Industrial Bacteria, LIO = Locally Isolated Organism, ETHYL A- ETHYL E = Partially Purified Fractions from Ethyl Acetate Fraction, ** = Mean of three replicates.