JOPAT Vol. 15(1) 38 - 49, January – December, 2015.

ISSN 1118 - 1028

PRELIMINARY PHYTOCHEMICAL AND ANTIMICROBIAL SCREENING OF THE CALYX OF GREEN *HIBISCUS SABDARIFFA* (LINN) (MALVACEAE)

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ABSTRACT

Successive extraction of air-dried calyces of *Hibiscus sabdariffa* Linn (green roselle) in cold maceration using hexane, ethyl acetate and methanol gave the corresponding extracts. The phytochemical screening of these extracts revealed the presence of carbohydrates, tannins, phlobatannins, terpenes and sterols. Antimicrobial screening of the extracts showed activity in the ethyl acetate extract at 2000 µg/ml against all the test organisms namely: *Esherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeroginosa* (ATCC 27853) and clinical isolates of *Salmonella typhi*, *Candida albicans* and *Bacillus subtilis*. One of the ethyl acetate chromatographic fractions exhibited the most significant antimicrobial activity against *Candida albicans* at MIC of 16 µg/ml, while the other fractions gave MIC ranging between 250 µg/ml and 500 µg/ml against all the test organisms. The methanol extract showed activity against *Escherichia coli* and *Candida albicans* but only one of the fractions exhibited partial antimicrobial activity at 500 µg/ml. The hexane extract displayed no activity against all the test organisms. The minimum inhibitory concentration (MIC) of both the ethyl acetate and methanol extracts was 500 µg/ml against *Esherichia coli*. As a standard, chloramphenicol had an MIC of 50 µg/ml against five of the test organisms excluding *Candida albicans*. The results provide scientific justification and support for the ethnomedicinal use of the plant as a traditional remedy for camel sores, bleeding gum, respiratory tract infections and typhoid fever.

Keywords: Green *Hibiscus sabdariffa,* phytochemical constituents, antimicrobial activity.

INTRODUCTION

Hibiscus sabdariffa Linn is a shrub belonging to the family–Malvaceae. It is a popular vegetable in Indonesia, India, West Africa and many tropical regions [1]. The plant is widely grown in tropics like Caribbean, Central America, India, Africa, Brazil, Australia, Hawaii, Florida and Philippines as a home garden crop. The name "*sabdariffa"* originated from the Philippine Islands [2]. In addition to Roselle, in English-speaking regions it is called Rozelle, Sorrel, Green sorrel, Jamaica sorrel, Indian sorrel, Guinea sorrel, Sour-sour, Queensland jelly plant, Jelly okra, lemon bush and Florida cranberry. In Nigerian languages it is called Yakwua (Hausa) which refers to the whole plant [2], *Amukan* (Yoruba) and *Okworoozo* (Ibo) [3].

The vegetable is also widely grown in the North-Eastern, Western and middle belt regions of Nigeria [4]. The plant has been found to thrive in a wide range of soil conditions. It can perform satisfactorily on relatively infertile soils, but for economic purposes, a soil well supplied with organic materials and essential nutrients is essential [1]. It can tolerate relatively high temperature throughout the growing and fruiting periods. The plant requires an optimum rainfall of approximately 45-50 cm distributed over a 90-120 day growing period [1]. In Nigeria, two botanical varieties are recognized, the calyx of the red variety are used for the preparation of "Sobo" drink, while the calyx of the green variety is used to cook soup, stew and sauces. The calyx of roselle (green) is very rich in vitamin C and riboflavin with some major minerals present [5]. Roselle calyces are used as a digestive and purgative agent and a folk remedy for abscesses, bilious conditions, cough, dysuria, scurvy, stangury, cancer, hypertension etc [6]. In Western, Northern and Middle belt regions of Nigeria, roselle calyces are used in cooking vegetable soup. It is usually prepared by steeping it with wood ash overnight or parboiled with wood ash and washed thoroughly prior to its being used for the preparation of soup. Ethno-botanical survey revealed that a decoction of the leaves of the green roselle is used to treat bleeding gum in children, improve appetite of malnourished children and increase breast milk in lactating mothers.

The plant is about 3.5 m tall and has a deep penetrating taproot. It has a smooth or nearly smooth, cylindrical, typically dark green stems (Figure **1**). The leaves are alternate, 7.5-12.5 cm long, green with veins long or short petioles (Figure **1**). The leaves of young seedlings and upper leaves of older plants are simple while the lower leaves are deeply 3 or 5 or even 7-lobbed and the margins are toothed. The flowers, borne singly in the leaf axils, are up to 12.5 cm wide and are yellow (Figure **1**) [7, 8, 9]. The species *H. sabdariffa* comprises a large number of cultivated types which, on the basis of their growth habit or end use, are classified broadly under two varieties, *H. sabdariffa var. sabdariffa* (*ruber* and

intermedius) and *H. sabdariffa var. altissima* Wester which is not found in Nigeria.This study aims to confirm the traditional medicinal uses of *Hibiscus sabdariffa* Linn (green roselle, *intermedius*).

MATERIALS AND METHODS

Materials

Analytical grade organic solvents were used in this study for extraction. The media used in the antimicrobial assays were nutrient agar and nutrient broth, all of Oxoid Limited Basingstroke, Hampshire. England. Chloramphenicol was used as a standard antimicrobial agent.

The microorganisms used in this study included standard strains, *Escherichia coli* (Ec) (ATCC 25922), *Staphylococcus aureus* (Sa) (ATCC 29213), *Pseudomonas aeroginosa* (Ps) (ATCC 27853) obtained from the Université du Benin, Cotonou. and clinical isolates of *Salmonella typhi* (St), *Candida albicans* (Ca) and *Bacillus subtilis* (Bs), obtained from National Institute for Pharmaceutical Research and Development (NIPRD), Idu-Abuja, Nigeria.

Plant collection and preparation

The calyces of *Hibiscus sabdariffa* Linn (Green Roselle-*intermedius*) were harvested from Omegede Otutubatu, Omala Local Government Area of Kogi State, Nigeria, in November. The plant was identified by a taxonomist and a voucher specimen with the number, NIPRD/H/6150, was deposited at the herbarium, National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria. The collected plant materials were checked for foreign matters, which were removed. The calyces were thereafter air-dried in a shade for two weeks. The dried calyces were pulverized in a mortar using a pestle and kept in an air tight cellophane bag and preserved in the dark until required.

Extraction

The pulverized dried sample of *Hibiscus sabdariffa* (*intermedius*) (5.0 kg) was macerated successively in n-hexane, ethyl acetate and methanol for 24 hours each [10] at room temperature (28 ± 2^{o}C). The extracts were filtered and the filtrates concentrated *in vacuo* using a rotary evaporator at 45^{o}C to give crude hexane extract (oily light dark solid, 10 g), the ethyl acetate extract (black creamy solid, 42.7 g) and the methanol extract (black creamy sticky solid, 266.8 g). The crude extracts were kept in sealed containers in the dark until required.

Phytochemical Screening

The crude hexane, ethyl acetate and methanol extracts were screened for the presence of secondary metabolites using a standard method [10]. The metabolites screened for included carbohydrates, tannins, phlobatannins, saponins, terpenes, sterols, flavonoids alkaloids and anthroquinones.

Fractionation of Ethyl Acetate and methanol Crude Extracts

10 g each of ethyl acetate and methanol extracts were fractionated using silica gel column chromatography (60 g). With gradient elution of n-hexane, EtOAc and MeOH (hexane-EtOAc, 90:10 to hexane-EtOAc, 0:100 to EtOAc-MeOH, 90:10 in 10% increasing polarity of the solvent). Twelve and eighteen major fractions respectively were collected based on similarities of their thin layer chromatography (TLC) profiles.

Preparation of the test organisms (inocula)

A wire loop-full (wire loop flamed red hot and cooled) of each test organism was taken aseptically from their respective slants and sub cultured into Mac-Cartney bottles containing five milliliters (5 ml) of freshly prepared nutrient broth and placed in the incubator for 24 hours at 37^{o}C. The 24 hour cultures were sub-cultured using a wire loop-full into freshly prepared broth and incubated at 37^{o}C for 3 h (containing approximately 1.25 X 10^{6} – 1.25X 10^{7} colony forming units (cfu)). This is equivalent to half McFarland standard.

Antimicrobial screening

The extracts were screened for antimicrobial activity against *Escherichia coli* (Ec) (ATCC 25922), *Staphylococcus aureus* (Sa) (ATCC 29213), *Pseudomonas aeroginosa* (Ps) (ATCC 27853) and clinical isolates of *Salmonella typhi* (St), *Candida albicans* (Ca) and *Bacillus subtilis* (Bs) using agar dilution method [11]. 1 ml of each of the crude extract (containing 32000 µg) was introduced into 15 ml of molten nutrient agar placed in water at 45^{o}C. These were mixed properly and poured into sterile petri dishes to give a final concentration of 2000 µg/ml. The dishes which were prepared in duplicates and then allowed to gel and thereafter, the test organisms were inoculated by streaking onto the nutrient agar using a wire loop. Control plates were also set up containing only agar and test organisms (Organism Viability Control), plates containing agar and dimethylsulphoxide (DMSO) and plates containing agar and sterile water, also served as controls. The petri dishes were incubated over night at 37^{o}C (20-24 hours) after which they were observed for microbial growth inhibition. Both water and DMSO showed no inhibitory effects on the test organisms. All procedures were done aseptically in the biosafety cabinet to avoid the introduction of unwanted micro-organisms from the environment.

The crude extracts that inhibited microbial growth at 2000 µg/ml including chloramphenicol (used as standard) and the chromatographic fractions were diluted to lower concentrations following the same procedures to determine the minimum inhibitory concentration (MIC).

RESULTS AND DISCUSSION

The cold maceration of *H. sabdariffa* (green roselle) using various solvents gave yields of extracts which increased from hexane to methanol according to polarity (0.2%, 2.14% and 13.34%).

The various extracts were subjected to phytochemical screening which revealed the presence of terpenes and sterols in all the extracts and the presence of carbohydrates, tannins and phlobatannins in the methanol extract only (Table **1**).

Table 1: Phytochemical screening of the crude extracts of *H. sabdariffa*

Phytochemical components	Extracts		
	Hexane	Ethylacetate	Methanol
Alkaloids	-	-	-
Anthraquinones	-	-	-
Carbohydrate	-	-	+
Flavonoids	-	-	-
Phlobatannins	-	-	+
Saponins	-	-	-
Sterols	+	+	+
Tannins	-	-	+
Terpenes	+	+	+

+; Present, -; Absent

The presence of flavonoids, alkaloids, saponins, carbohydrates and sterols is consistent with previous reports for *Hibiscus sabdariffa* (red roselle) [9, 12], *Hibiscus taiwanensis* [13] and *Hibiscus vitifolius* [14] which are other members of Malvaceae family.

The extracts were subjected to antimicrobial screening to determine their activity against the test organisms. These results (Table **2**) showed that at 2000 µg/ml concentration, the hexane extract exhibited no activity against the six microorganisms used in this study. The ethyl acetate extract showed activity by inhibiting the growth of all the six test organisms while the methanol extract exhibited activity against three organisms, *Escherichia coli, Candida albicans* and *Pseudomonas aeroginosa*. From these results,

the ethyl acetate extract was the most active crude extract with activity spectrum covering both Gram-negative and Gram-positive organisms.

Table 2: Results of antimicrobial screening of the crude extracts

Extracts	Microorganisms/MIC(µg/ml)					
	Bs	Ec	Ca	Ps	Sa	St
Hexane	-	-	-	-	-	-
Ethylacetate	2000	500	1000	2000	2000	2000
Methanol	-	500	2000	2000	-	-
DMSO/Acetone	-	-	-	-	-	-
Chloramphenicol	50	50	-	50	50	50

+; Activity, -; No activity, Bs; *Bacillus subtilis,* St; *Salmonella typhi,* Ec; *Esherichia coli* (ATCC 25922), Ca; *Candida albicans,* Ps; *Pseudomonas aeroginosa* (ATCC 27853), Sa; *Staphylococcus aureus* (ATCC 29213)

As controls, growth of all the microorganisms were observed in plates bearing the agar alone and solvent (DMSO, acetone) used to dissolve the extracts. This was an indication that the organisms were viable during the study and that the activities observed were due to the presence of the extracts. Previous studies have also shown that the stem [15] of *Hibiscus taiwanensis*, the flowers [16] of *Hibiscus vitifolius* and the flowers [17] and calyces [18] of *H. sabdariffa* (red roselle) possess antimicrobial activities. The ethyl acetate extract of the calyces of *H. sabdariffa* (red roselle) exhibited antibacterial activity against *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), while the methanol extract, in the same study, exhibited activity against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 15380), *Haemophilus influenza* (ATCC 10211), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 12344) [17]. From literature [10], extracts that showed activity at 2000 µg/ml were diluted and tested against the same set of microorganisms to determine their MICs. Table **1** also showed that the MIC of the ethyl acetate extract was 1000 µg/ml and 500 µg/ml against *Candida albicans* and *Escherichia coli,* respectively. The methanol extract had MIC of 500 µg/ml against *Escherichia coli* only. Thus, both the ethyl acetate and the methanol extracts have demonstrated reasonable activity in this study.

Table 3: Minimum inhibitory concentration of the crude ethylacetate extract and column chromatographic fractions

	Microorganisms/concentration/µg/ml					
Extracts	Sa	St	Bs	Ps	Ca	Ec
Fraction 1	-	400	-	-	16	400
Fraction 2	-	1000	-	-	1000	1000
Fraction 3	ND	ND	ND	ND	ND	ND
Fraction 4	1000	ND	130	1000	500	1000
Fraction 5	-	1000	250	1000	1000	1000
Fraction 6	-	1000	500	1000	1000	500
Fraction 7	-	1000	500	1000	1000	1000
Fraction 8	1000	1000	--	1000	1000	1000
Fraction 9	-	370	-	370	370	370
Fraction 10	-	1000	-	1000	1000	1000
Fraction 11	1000	1000	-	1000	1000	1000
Fraction 12	-	1000	-	1000	1000	1000
Controls DMSO/ Acetone (separately)	-	-	-	-	-	-

-; No activity ND; Not done Bs; *Bacillus subtilis,* St; *Salmonella typhi*, Ec; *Esherichia coli* (ATCC 25922), Ca; *Candida albicans,* Ps; *Pseudomonas aeroginosa* (ATCC 27853), Sa; *Staphylococcus aureus* (ATCC 29213)

Table **3** showed the MIC of the ethyl acetate chromatographic fractions. Fractions from crude extracts are supposed to show antimicrobial activity at lower concentrations. The fractions were diluted starting from 1000 µg/ml. From the result (Table **3**), a broad spectrum of activity against both Gram-positive and Gram-negative organisms was observed at lower concentrations just like the crude ethyl acetate extract (Table **2**). Fraction 1 exhibited the highest activity at 16 µg/ml against *Candida albicans.* This indicated that fraction 1 was probably responsible for the activity exhibited by the ethyl acetate extract in Table **2** against *Candida albicans.* It has been previously reported that a compound, hibicuslide C, isolated from the stem of *Hibiscus taiwanensis*, possessed potent activities toward various fungal strains and exerts its

effect by membrane-active mechanism in *Candida albicans* [15]. The difficulties associated with the management of *Candida* infections necessitate the discovery of new antifungal agents, in order to widen the spectrum of activity against *Candida* and combat strains expressing resistance to available antifungal agents [19]. Plant-derived natural products, such as fraction 1 in this study, may offer potential lead to new antifungal agent which could act on these fungi [20].

Table 4: Antimicrobial screening of the column chromatographic fractions of methanol crude extract

	Microorganisms			
Extracts/µg/ml	Ca	Ec	St	Sa
Fraction 1/500	-	-	-	-
Fraction 2/500	-	-	-	-
Fraction 3/500	-	-	-	-
Fraction 4/500	-	⊥	-	⊥
Fraction 5/500	-	-	-	-
Fraction 6/500	-	-	-	-
Fraction 7/500	-	-	-	-
Fraction 8/500	-	-	-	-
Fraction 9/500	-	-	-	-
Fraction 10/500	-	-	-	-
Fraction 11/500	-	-	-	-
Fraction 12/500	-	-	-	-
Fraction 13/500	-	-	-	-
Fraction 14/500	-	-	-	-
Fraction 15/500	-	-	-	-
Fraction 16/500	-	-	-	-
Fraction 17/500	-	-	-	-
Fraction 18/500	-	-	-	-
DMSO	-	-	-	-
Distilled water	-	-	-	-

-; No activity ⊥; Partial activity St; *Salmonella typhi* Ec; *Esherichia Coli* (ATCC 25922)

Ca; *Candida albicans* Sa; *Staphylococcus aureus* (ATCC 25923)

Table **4** showed the antimicrobial activity of the chromatographic fractions of the methanol crude extract. From the result, only fraction 7 showed partial activity against *Escherichia coli* and *Staphylococcus aureus* at 500 µg/ml. This again showed that fraction 7 may be responsible for the activity observed in the methanol crude extract (Table **2**). It may be possible that the components of the the methanol crude extract worked in synergy and produced significant antimicrobial activity (Table **2**) but when seaparated, lost the activity (Table **4**).

CONCLUSION

The search for new antimicrobial agents led to the selection of a Nigerian ethnomedicinal plant, *Hibiscus sabdariffa* (green roselle calyces), claimed as a traditional remedy for diseases. Phytochemical screening of the extracts revealed the presence of carbohydrates, tannins, phlobatannins, terpenes and sterols. The antimicrobial screening showed that the ethyl acetate extract was the most active which exhibited significant activity against all the test organisms used in this study.

The methanol extract exhibited activity against three of the six organisms while the hexane extract did not show any activity. On fractionation, one the fractions of the ethyl acetate extract showed the highest activity at MIC of 16 µg/ml against *Candida albicans* while only one of the fractions of the methanol extract showed partial activity against *Escherichia coli* and *Staphylococcus aureus* at 500 µg/ml. These results corroborate the traditional medicinal uses of the plant. Further work on the ethyl acetate extract, with its broad spectrum of activity, may provide leads for the development of new antimicrobial agents.

ACKNOWLEDGEMENT

The authors are grateful to the taxonomist, Dr. (Mrs) Jemilat Ibrahim, who identified the plant and stored a herbarium specimen and to the ethonobotanist, Mr. Muazzam Ibrahim Wudil, who provided the ethnobotanical information on the plant and Mr. John Atogwe who planted and raised the plant for capturing and documentation, all at the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja, Nigeria.

Figure 1: The green roselle, *Hibiscus sabdariffa* Linn, *var. sabdariffa* (*Intermedius*) [7]

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