

In vitro Antimicrobial Activity of Ethanolic Leaf Extracts of *Hibiscus Asper* Hook. F. and *Hibiscus Sabdariffa* L. on some Pathogenic Bacteria

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Abstract

Purpose: The study aims to assess the antimicrobial activity of ethanolic leaf extracts of *Hibiscus asper* and *Hibiscus sabdariffa* against eight bacterial isolates.

Materials and Methods: An *in vitro* Antimicrobial activity of ethanolic leaf extract of the two plants against eight nosocomial and pathogenic bacteria viz; *Pseudomonas aeruginosa* (PAE), *Proteus vulgaris* (PVU), *Klebsiella aerogenes* (KAE), *Staphylococcus aureus* (SAU), *Bacillus cereus* (BCE), *Escherichia coli* (ECO), *Moraxella catarrhalis* (MCA) and *Salmonella typhi* (STY) was carried out using agar well diffusion assay with the concentration range of 3.13 – 100 mg/mL.

Results: *H. asper* and *H. sabdariffa* showed significant difference ($p < 0.05$) in antimicrobial activity against BCE over the rest of the isolates. Inhibition zone diameters exhibited by the isolates to ethanolic leaf extract of *H. asper* was in descending order of BCE (15.00 ± 1.00^a) > ECO (11.67 ± 0.58^b) > SAU (7.67 ± 0.58^c) > PAE (6.67 ± 0.58^d) > STY (5.67 ± 0.58^e) while that of *H. sabdariffa* was in the order BCE (15.33 ± 1.15^a) > MCA (11.33 ± 1.15^b) > SAU (11.00 ± 1.00^{bc}) > KAE (9.67 ± 0.58^c) > PAE (8.00 ± 1.00^d) > PVU (7.67 ± 0.57^e). PVU, KAE and MCA were resistant to the extract of *H. asper* while only STY was resistant to that of *H. sabdariffa*.

Conclusion: *H. sabdariffa* extract demonstrated higher antimicrobial activity against the selected bacterial isolates than *H. asper*. However, the two extracts minimum inhibition concentrations (MICs) ranged from 25 mg/mL to 12.5 mg/mL. This is worthy of further exploration by pharmacological industries in the formulation of potent broad spectrum antibiotics for combating the present health challenge due to antimicrobial resistance.

1. Introduction

Plants are nature's prodigious endowment for human and animals' boundless use. This usage among others encompasses nutritional and medicinal. Most of these plants grow luxuriantly in an attractive lush during raining season but rapidly faded away as soon as dry season sets in. Exploitation of their vast benefits will be a right step in the right direction towards the realization of millennial sustainability goals. Several authors have reported the antimicrobial potentials of medicinal plants overtime with the great possibility of acquiring bioactive compounds in them for drug formulation. According to the report of Ofongo and Ohimain (2019) aqueous extract of *Azadirachta indica* (neem) and *Vernonia amygdalyana*

(bitter leaf) were highly effective in lowering the population of enteropathogenic bacteria (*E. coli* and *Salmonella*) in the gut of broiler chickens. *Averrhoa carambola* otherwise called Star fruit is a sweet juicy and succulent fruit that is cherished and consumed by many people. The juice extract is also used in folkloric medicine in the treatment of certain ailments such as skin rashes, inflammatory conditions and diabetes. Oladipo *et al.* (2019) reported that the use of methanolic extract of *Annona muricata* bark led to gradual reduction in the blood sugar level. The plant showed a great potential when compared to standard anti-diabetic drug in the treatment of diabetes mellitus. Wumnokol *et al.* (2019) reported the inclusion of Acha (*Digitaria exilis* Staph) in the diet of broiler chickens as beneficial on the haematological parameters. It was reported by Ukorebi *et al.* (2019) that 10% dietary level of *Gongronema latifolia* (Utasi) supported normal health by enhancing the blood building capacity and performance of broiler chickens.

Hibiscus asper Hook. F. as reported by Baerts and Lehmann (2002) and Sunilson *et al.*, (2009) is a tropical plant belonging to the family Malvaceae of the order Malvales. Calyxes from *H. asper* are edible as a palatable vegetable after boiling. The plant has medicinal potentials to treat joint pain, anaemia, jaundice malaria, painful, irregular menstruation, leucorrhea, poison antidote, depression, gastrointestinal disorders, abscesses, urethritis, and dysmenorrhea (Burkill, 1985; Shippers *et al.*, 2004; Foyet *et al.*, 2011). The plant is also relevant in veterinary medicine for the treatment of ecto-parasites (Hritcu *et al.*, 2011). Prepared aqueous and methanolic extracts of the leaf of *H. asper* possess significant antidiarrheal activity in experimented rats while the methanolic fraction had antibacterial activity on human enterobacteria (Ateufack *et al.*, 2014). *Hibiscus sabdariffa* (Roselle) belongs to the same family with *H. asper* and probably native to West Africa (Encyclopedia Britannica, 2020) and grown for its edible calyx. This plant is a good source of lipid-soluble antioxidants, (gamma-tocopherol) polyphenols, flavonoids, and their derivatives (Mohamed *et al.*, 2007 and Jing *et al.*, 2015). It possesses the pharmacological potentials of being antiviral, anticancer, antimicrobial, antifungal, anti-anaemic, hypotensive, anti-urolithiasis etc. (Bireswar &Charu, 2018). The two plants are rich in phytochemicals and this might probably be responsible for their potent medicinal values recorded in literatures.

Pseudomonas aeruginosa is of the family pseudomonadaceae. It is a very common Gram negative bacterium implicated to be one of the causative agents of nosocomial infections. It is an opportunistic bacterium that is highly resistant to many commercial disinfectants (Hugo&Russel, 1998; Verma *et al.*, 2014). *Proteus vulgaris* has been documented as one of the zoonotic pathogenic bacteria that are multi-drug resistant and this can be traceable to drug abuse and indiscriminate prescriptions of antibiotics. It is a Gram-negative bacterium in the family enterobacteriaceae that has been reported as a disease causing agent in poultry and humans (Debasis *et al.*, 2015; Ogunleye&Carlson, 2016). *Klebsiella aerogenes*, (formerly known as *Enterobacter aerogenes*) is an important opportunistic Gram-negative rod-shaped pathogenic bacterium in the family Enterobacteriaceae with drug resistance characteristics. The bacterium has been commonly reported as one the causative agent of disease outbreaks in intensive care unit with high epidemic potential (Adel *et al.*, 2019). *Staphylococcus aureus* is a Gram-positive bacterium implicated as a coloniser of the pharynx and nasal cavity causing endocarditis, bacteremia etc. Its infection is not limited to a particular site of the body and can also cause food poisoning (Shamshul *et al.*, 2019). *Bacillus cereus* is a spore forming motile

Gram-positive bacterium causing two major types of food poisoning through its production of one emetic toxin and three different enterotoxins: the emetic type normally results into vomiting and the other type culminating into diarrhoea (Kramer and Gilbert 1989; Granum 1994; Lund& Granum 1997; Per&Terje, 1997). *B. cereus* is one of the bacteria responsible for the possible risk of food-borne infections/intoxications in both plant and animal food origin. This organism found these food sources to be favourable substrates for its development and replication (Ana *et al.*, 2019). *Escherichia coli* (a rod-shaped Gram-negative bacterium) is from the family Enterobacteriaceae within the class Gammaproteobacteria (Jang *et al.*, 2017). *E. coli* comprises commensal and pathogenic strains responsible for several human ailments aggravating to millions of deaths annually (Kaper *et al.*, 2004). Many of the water borne outbreaks in the World have been attributed to Shiga toxin-producing and enteropathogenic *E. coli* (Chandran& Mazumder, 2015). *Moraxella catarrhalis* is a pathogenic gram-negative bacterium coccus of the Neisseriae family usually colonises the upper respiratory tract and thereby causing infection ranging from otitis media in children and downstream respiratory infection in adult. This bacterium has become greatly resistant to penicillin antibiotic (Savitha *et al.*, 2020). *Salmonella typhi* is a Gram-negative, aerobic, rod-shaped bacterium known to be involved in salmonellosis and the disease is usually contracted in contaminated food stuffs (especially in meat) by this organism (Yang *et al.*, 2010). Salmonellosis is a worldwide disease that is zoonotic and causing gastroenteritis in human (Lee, 2015). Stale air and overcrowded environments are predisposing conditions for spreading the disease. The diseases caused by these pathogenic bacteria, coupled with their resistance to many available commercial antibiotics and the dearth of information concerning the utilization of the leaf extract of *H. asper* and *H. sabdariffa* as antimicrobial agents are the considerable factors in designing and carrying out this research work.

2. Methodology and Procedures

Collection of experimental plants

Fresh and disease free leaves of *Hibiscus asper* and *Hibiscus Sabdariffa* were harvested in November 2019 from a farm at Goshen Community, off Aule GRA Akure, Nigeria, Latitude 7:16.06N and Longitude 5:8.85E.

Identification and authentication

The plants were identified and authenticated by an experienced Taxonomist with identification numbers FPI 2278 and FPI 2280 for *Hibiscus asper* and *Hibiscus Sabdariffa* respectively at Faculty of Pharmacy Herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria.

Plants' preparation

The plants were rid of dust particles by gentle rinse in distilled water. Crispiness of the leaves was achieved in eight days under shade drying and thereafter pulverized into powder

using Bajaj Twister Mixer Electric Grinder (3 Jar QC. NO: HP/14/001/0064). The samples were preserved in air-tight plastic containers at 4°C prior to subsequent assignment.

Preparation of the extracts

Cold maceration method was adopted in the preparation of the leaf extracts. Exactly 50 g each of the powdered leaves was soaked in 500 mL of 80% ethanol (w/v) kept and kept for 72 hours in a tightly sealed plastic container at room temperature. Filtration was done with muslin cloth and later re-filtered with Whatman filter paper No 1(125 mm). The wet extract was concentrated into pasty form using a crude method for bacterial susceptibility test experiment.

Calculation of the leaf processing percentage yield

The leaf processing percentage yield (LPPY) was calculated using the formula:

$$\text{LPPY} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

Test Microorganisms

Clinical strains of *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Moraxella catarrhalis* and *Salmonella typhi* were obtained from the Microbiology Department of a reputable Specialist Hospital in Akure, Ondo State, Nigeria while *Bacillus cereus*, *Escherichia coli* and *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) were isolated from drinking water from Ekiti State, Nigeria. The organisms were carefully maintained on nutrient slants at 4⁰ C throughout the period of the experiment.

Standardization of the extracts

The two extracts were reconstituted with 10 % DMSO (Dimethylsulphoxide) and distilled water following manufacturer's recommendation. Six fold serial dilutions were carried out before obtaining six different concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.50 mg/mL, 6.25 mg/mL, and 3.13 mg/mL.

Standardization of the inocula

Mueller-Hinton Agar (MHA) served as a medium for sub-culturing the isolates on newly purchased sterile plates. The plates were incubated at 37°C for 24 hours and thereafter preserved at 4°C for subsequent overnight culturing from which isolates were later suspended in broth culture for antimicrobial assay. The broth cultures of the isolates were adjusted to the exact turbidity equivalent to 0.5 McFarland Standard as recommended by NCCLS, (1998).

Antimicrobial Assay

Antimicrobial activity of the extracts was determined using agar well diffusion assay method (Thitilertdecha *et al.*, 2008 and Ameya *et al.*, 2016). Mueller Hinton Agar (MHA) was

prepared as recommended by the manufacturer and allowed to cool to 50°C before pouring on pre-labelled sterile petri plates on a level surface. One petri-plate was prepared per organism and done in triplicates except that of the positive test control using Maxi discs that was done in duplicate. Sterile 6 mm borer was used to punch seven equidistant wells: wells 1 – 6 for different concentrations of extracts (3.13 mg/mL – 100 mg/mL). Well number seven (7) was bored at the centre of the plates for the solvent used (negative control). A dip with sterile swabbing sticks of overnight broth cultures of each of the isolates was streaked on the surface of the prepared MHA plates. The volume of 0.2 mL of the extracts at preset concentration was introduced to each of the wells and the plates were allowed to rest (and set) on the laboratory bench for 45 minutes allowing for proper pre-diffusion of the extracts before 24 hours incubation at 37°C.

Antibacterial activity

The antimicrobial activities of the extracts were assessed by measuring the inhibition zones around the wells with the aid of a graduated transparent metre rule. There was no observed antimicrobial activity against the test organisms in the solvent used (DMSO 10%) for the extracts' reconstitution as indicated by zero inhibition zones.

Minimum Inhibitory Concentrations (MICs)

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after an overnight incubation. The MICs were determined by tube dilution (12-fold) method (Benger *et al.*, 2004, Manilal *et al.*, 2010 and Kaya *et al.*, 2012) with slight modifications.

3. Results and Discussion

The results of the leaf processing percentage yield (LPPY), mean values for the antimicrobial activity and Minimum Inhibitory Concentrations (MICs) of the ethanolic leaf extract of *H. asper* and *H. sabdariffa* are presented in Table 1 to 4. Leaf processing percentage yield was higher in *H. asper* (22.86 %) than *H. sabdariffa* (15.83%) as seen in Table 1. There is virtually no adduceable reason(s) for this as at now, because researchers in medicinal plants have not been reporting this aspect of work in literatures.

Hibiscus asper

Antimicrobial activity of ethanolic leaf extract of *H. asper* was more evident in BCE having the highest inhibition zone (Table 2) with significant difference ($p < 0.05$) over other isolates in descending order of BCE (15.00 ± 1.00^a) > ECO (11.67 ± 0.58^b) > SAU (7.67 ± 0.58^c) > PAE (6.67 ± 0.58^d) > STY (5.67 ± 0.58^e). The susceptibility of ECO and STY to the extract of *H. asper* in the present study agrees with earlier report of its antibacterial property against some Gram-negative bacteria (Ateufack *et al.*, 2014). The extract can be described as broad spectrum because of its antibacterial effect on the Gram-positive (BCE and SAU) and Gram-negative isolates (ECO, PAE and STY) in this study. The sensitivity of SAU here disagrees with the resistance of SAU to the extracts of *H. asper* as reported by Ateufack *et al.* (2014).

The extract did not show any activity on PVU, KAE and MCA in the present study as shown in Table 2.

Hibiscus sabdariffa

The extract of *H. sabdariffa* was active on all the tested bacterial isolates (Table 3) with the exception of STY. The antibacterial potential of this plant as witnessed in the present study agrees with the reports on it against ECO, PAE, PVU, and SAU (Khalid *et al.*, 2015), ECO (Fullerton *et al.*, 2011), ECO and SAU (Higginbotham *et al.*, 2014). The highest mean zone of inhibition displayed by the tested bacteria was found in BCE (15.33 ± 1.15^a) followed by MCA (11.33 ± 1.15^b), SAU (11.00 ± 1.00^{bc}), KAE (9.67 ± 0.58^c), PAE (8.00 ± 1.00^d) and PVU (7.67 ± 0.57^e). This highest activity on BCE (Gram-positive) corroborates the earlier report on the extract of *H. sabdariffa* to be more active on Gram-positive bacteria (Khalid *et al.*, 2015). Only STY showed resistance to the ethanolic leaf extract of *H. sabdariffa*. The earlier report of the preliminary phytochemical screening of *H. asper* and *H. sabdariffa* indicated the presence of tannins, flavonoids, saponins, steroids and terpenoids in both samples and the absence of phlobatannins, alkaloids and anthraquinones (Arogbodo and Ajayi, 2020). The absence of alkaloids in *H. sabdariffa* agrees with the phytochemical report of Adamu and Ngwu (2015). Cardiac glycosides were present in *H. asper* but absent in *H. sabdariffa*. The phytochemicals (qualitatively and quantitatively) were found to be higher in *H. asper* than *H. sabdariffa* (Arogbodo and Ajayi, 2020). The observed antibacterial potential of ethanolic leaf extracts of *H. asper* and *H. sabdariffa* on both Gram-positive and Gram-negative bacteria can be attributed to their rich phytochemicals like the flavonoids (Elmanam *et al.*, 2011; VimalinHena, 2010).

Antimicrobial resistance

PAE, PVU, SAU, ECO, MCA and STY were all resistant to the commercial antibiotic Septrin used as positive control in this experiment while only KAE and BCE were sensitive. On the other hand, all the tested bacteria were resistant to Amoxicillin (positive control) with the exception of PAE. The multidrug resistance of PAE, ECO and SAU to Septrin and that of ECO and SAU to Amoxicillin in this study agrees with the findings of Osuntokun and Adesemoye (2019), which is the current global problem (antimicrobial resistance).

Minimum inhibitory concentrations (MICs)

The minimum inhibitory concentration for PAE in both extracts (Table 4) was 25 mg/mL. PVU, KAE and MCA were resistant to *H. asper* but all had the same MIC of 25 mg/mL in the extract of *H. sabdariffa*. SAU, BCE and ECO MICs were 25 mg/mL, 6.25 mg/mL, and 25 mg/mL in *H. asper*, but 12.25 mg/mL, 12.25 mg/mL and 50 mg/mL in *H. sabdariffa* respectively. STY MIC in *H. asper* was 25 mg/mL but showed no activity in the extract of *H. sabdariffa*. The MIC of 25 and 50 mg/mL for ECO in this study agrees with that of Jung *et al.* (2013) who reported same for ECO in aqueous extract of *H. sabdariffa*

Table 1: Leaf processing percentage yield (LPPY) of *H. asper* and *H. Sabdariffa*

Medicinal plants	Fresh weight	Dry weight	Yield %
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<i>H. asper</i>	280	64	22.86
<i>H. sabdariffa</i>	417	66	15.83

Table 2: Mean values (mm) of inhibition zone diameters of ethanolic leaf extract of *H. asper* against the selected pathogenic bacteria

SN	Isolates	Mean zones of inhibition (mm)								DMSO 10 %
		C1	C2	C3	C4	C5	C6	SXT (30µg)	AM (30µg)	
1	PAE	6.67 ± 0.58 ^d	4.33 ± 0.58 ^{bc}	2.33 ± 0.58 ^{bc}	-	-	-	R	14	NIL
2	PVU	R	R	R	R	R	R	R	R	NIL
3	KAE	R	R	R	R	R	R	12	R	NIL
4	SAU	7.67 ± 0.58 ^c	3.33 ± 0.58 ^c	2.33 ± 0.58 ^{bc}	-	-	-	R	R	NIL
5	BCE	15.00 ± 1.00 ^a	13.67 ± 0.58 ^a	12.33 ± 0.58 ^a	10.00 ± 1.00	6.33 ± 0.58	-	12	R	NIL
6	ECO	11.67 ± 0.58 ^b	8.33 ± 0.58 ^b	5.67 ± 0.58 ^b	-	-	-	R	R	NIL
7	MCA	R	R	R	R	R	R	R	R	NIL
8	STY	5.67 ± 0.58 ^e	4.33 ± 0.58 ^{bc}	2.67 ± 0.58 ^{bc}	-	-	-	R	R	NIL

Mean values with different superscript along the same row are significantly different from each other ($p < 0.05$). Values are mean ± Standard deviation of triplicate determination of inhibition zones less the diameter of borer. Where C1=100 mg/mL, C2=50 mg/mL, C3=25 mg/mL, C4=12.5 mg/mL, C5=6.25 mg/mL, C6=3.13 mg/mL, PAE= *Pseudomonas aeruginosa*, PVU = *Proteus vulgaris*, KAE = *Klebsiella aerogenes*, SAU = *Staphylococcus aureus*, BCE = *Bacillus cereus*, ECO = *Escherichia coli*, MCA = *Moraxella catarrhalis*, STY = *Salmonella typhi*, SXT= Septrin, AM = Amoxicillin, DMSO= Dimethylsulphoxide and R = Resistant.

Table 3: Mean values (mm) of inhibition zone diameters of ethanolic leaf extract of *H. sabdariffa* against the selected pathogenic bacteria

SN	Isolates	Mean zones of inhibition (mm)								DMSO 10%
		C1	C2	C3	C4	C5	C6	SXT (30µg)	AM (30µg)	
1	PAE	8.00 ± 1.00 ^d	7.67 ± 0.58 ^{cd}	6.33 ± 0.58 ^{bc}	-	-	-	R	14	NIL
2	PVU	7.67 ± 0.57 ^e	6.00 ± 0.00 ^e	4.33 ± 0.58 ^d	-	-	-	R	R	NIL
3	KAE	9.67 ± 0.58 ^c	8.00 ± 0.00 ^c	4.00 ± 0.00 ^d	-	-	-	12	R	NIL
4	SAU	11.00 ± 1.00 ^{bc}	6.67 ± 1.15 ^{de}	6.00 ± 0.00 ^c	3.00 ± 1.00 ^b	-	-	R	R	NIL
5	BCE	15.33 ± 1.15 ^a	13.33 ± 1.15 ^a	7.33 ± 1.15 ^a	3.00 ± 1.00 ^a	-	-	12	R	NIL

6	ECO	3.00 ±1.00 ^f	2.00 ± 0.00 ^f	-	-	-	-	R	R	NIL
7	MCA	11.33 ± 1.15 ^b	9.33 ± 1.15 ^b	4.67 ± 1.15 ^d	-	-	-	R	R	NIL
8	STY	R	R	R	R	R	R	R	R	NIL

Mean values with different superscript along the same row are significantly different from each other ($p < 0.05$). Values are mean ± Standard deviation of triplicate determination of inhibition zones less the diameter of borer. Where C1=100 mg/mL, C2=50 mg/mL, C3=25 mg/mL, C4=12.5 mg/mL, C5=6.25 mg/mL, C6=3.13 mg/mL, PAE = *Pseudomonas aeruginosa*, PVU = *Proteus vulgaris*, KAE = *Klebsiella aerogenes*, SAU = *Staphylococcus aureus*, BCE = *Bacillus cereus*, ECO = *Escherichia coli*, MCA = *Moraxella catarrhalis*, STY = *Salmonella typhi*, SXT= Septrin, AM= Amoxicillin, DMSO= Dimethylsulphoxide and R = Resistant.

Table 4: Minimum inhibitory concentrations (MICs) of *Hibiscus asper* and *Hibiscus sabdariffa* on the selected bacterial isolates

SN	Bacterial isolates	<i>H. asper</i> (mg/mL)	<i>H. sabdariffa</i> (mg/mL)
1	<i>Pseudomonas aeruginosa</i> (PAE)	25	25
2	<i>Proteus vulgaris</i> (PVU)	R	25
3	<i>Klebsiella aerogenes</i> (KAE)	R	25
4	<i>Staphylococcus aureus</i> (SAU)	25	12.5
5	<i>Bacillus cereus</i> (BCE)	6.25	12.5
6	<i>Escherichia coli</i> (ECO)	25	50
7	<i>Moraxella catarrhalis</i> (MCA)	R	25
8	<i>Salmonella typhimurium</i> (STY)	25	R

R = Resistant

4. Conclusion and Suggestion

The two-plant species demonstrated antibacterial potential on both Gram-positive and Gram-negative bacteria in this study. However, *H. sabdariffa* was more active as it showed antibacterial activity on seven bacteria as against *H. asper* that was on five out of the eight experimented isolates. The antibacterial effect of the extracts from both plants was greatest on BCE with mean inhibition zones diameter of 15.00 ± 1.00^a mm and 15.33 ± 1.15^a mm in *H. asper* and *H. sabdariffa* respectively. The MIC of BCE was lower in *H. asper* (6.25 mg/mL) than *H. sabdariffa* (12.5 mg/mL). Based on the outcome of this experiment, *H. asper* and *H. sabdariffa* are therefore suggested as potential anti-bacteria sources to be reckoned with for further research in herbal antimicrobial drug formulation by pharmaceutical industries both for human usage and in animal husbandry. Testing of the bacterial isolates with higher extracts' concentrations can be evaluated for the possibility of better results.

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Conflict of Interest

The authors have no competing interest to declare.

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