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Antibacterial activity of leave extracts of *Nymphaea lotus (Nymphaeaceae) on Methicillin* resistant *Staphylococcus aureus (MRSA) and* Vancomycin resistant *Staphylococcus aureus (VRSA) isolated from clinical samples*

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Abstract

Ethanolic extracts of Nymphaea lotus leaves were tested for antibacterial activity against methicillin resistant Staphylococcus aureus (MRSA) and vancomycin resistant Staphylococcus aureus (VRSA) isolated from wounds and urine using disc diffusion method(DDM) .The results of the phytochemical analysis of the extracts showed the presence of bio-active compounds such as tannins, terpenes, flavonoids, alkaloids, anthraquinones, saponins, cardiac glycosides and phenolics. The results also showed that both MRSA and VRSA were susceptible to Nymphaea lotus at different concentrations (5, 10, 20, 40, 80) mg/ml). The zones of inhibition ranged from 8.0 ± 0.5mm to 24.0 ± 1.0mm in MRSA and from 8.0 ± 0.5mm to 27.0± 1.0mm. The results also showed that the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) ranging from 5 to 15 and 10-30 for MRSA and VRSA respectively. The antibacterial activity of Nymphaea lotus extracts against MRSA and VRSA showed the plant active compounds as a possible potential antibiotic substance for the treatment and management of staphylococci infections.

Key words: *Nymphaea lotus*, Susceptibility, Wounds, Urine, Phytochemical, Staphylococcus.

INTRODUCTION

Plants have long formed the basis of sophisticated traditional medicine systems and natural products and purportedly provide excellent leads for new drug developments [1]. Approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care and plants also play an important role in the health care system [2]. The rediscovery of the connection between plants and health is responsible for the launching of a new generation of multi-component botanical drugs, dietary supplements and plant-produced recombinant proteins [3]. Moreover, the emergence of multi-drug resistant (MDR) bacteria is of great concern to both clinicians and the pharmaceutical industries, since it is a major cause of treatment failure in many infectious diseases [4-6].

Staphylococcus aureus is recognized as one of the major causes of infections in humans occurring in both the communities and the hospitals. Over time, the widespread use of antibiotics has led some *S. aureus* to become more resistant to antibiotics especially methicillin and vancomycin Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Staphylococcus aureus* have become major nosocomial pathogens [7]. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in the 1960s and it is presently endemic while vancomycin-resistant *Staphylococcus aureus* (VRSA) is a strain of *Staphylococcus aureus* that has become resistant to the glycopeptides antibiotic called vancomycin. With the increase of staphylococcal resistance to methicillin, vancomycin was the only effective treatment for severe methicillin-resistant *Staphylococcus aureus* (MRSA) infection strains till 1996 when an intermediate resistance to vancomycin (VISA: vancomycin-intermediate *S. aureus*), In 2002, a newly reported VRSA was isolated from the catheter tip of a renal dialysis patient in Michigan.

Although, there has been an increased interest in the investigation of natural materials as a source of new antibacterial agents over the past two decades, yet there is still an urgent need to identify novel substances that are active towards pathogens with high resistance [8]. One of the possible strategies towards this objective involves the identification of plants with their bio-active phytochemicals for antibacterial activity [1, 9]. Screening of compounds obtained from plants for their pharmacological assay has indeed been the vast source of innumerable therapeutic agents representing molecular diversity engineered by nature. One of the ways to prevent antibiotic resistance of pathogenic species is to use new compounds that are not based on existing synthetic antimicrobial agents [10].

Nymphaea lotus belongs to Nymphaeaceae family, It is a perennial plant that grows up to 45cm in height; it is herbaceous aquatic plant, whose leaves float or submerged in water [11]. This plant prefers clear, warm, still and slightly acidic water and is localized to Central and Southern Europe, Asia, the Middle East, Northern Africa, tropical mountains in Africa and West Africa especially in Nigeria. Many bioactive and pharmacologically important compounds have been obtained from *Nymphaea spp* and used in medicine and pharmacy [12]. Hence, the present study was initiated to evaluate the antibacterial activity of the ethanolic leave extracts of *Nymphaea lotus (Nymphaeaceae) against* methicillin resistant *Staphylococcus aureus (MRSA) and* vancomycin *resistant Staphylococcus aureus (VRSA).*

MATERIALS AND METHODS

Plant materials

The leaves of *Nymphaea lotus* were collected from the Itu stream at Itu village, near Uyo, Akwa Ibom State. The plant leaves were cleaned of extraneous matter, and necrotic parts removed by rinsing in fresh water. The leaves were further repeatedly rinsed thoroughly with running distilled water for further analysis.

Plant Identification

The plant was identified and confirmed as *Nymphaea lotus* by Dr. Ubom of Botany and Ecological Studies Department of University of Uyo, Akwa Ibom State, Nigeria.

Preparation of plant extracts

A sample (50 g) of the shade-dried powdered leaves of *Nymphaea lotus* was soaked in 95% ethanol (200 ml) for 72 h and was filtered using Whatman No.1filter paper. The filtrate was then concentrated in a vacuum at 30^{o}C. After complete evaporation, the extract was weighed and preserved aseptically at 5^{o}C .The graded concentrations (5 mg/ml, 10 mg/ml, 20 mg/ml,40 mg/ml and 80 mg/ml) of the extracts were prepared and then subjected to antibacterial activity assays.

Microbial cultures

The methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) strains were isolated from urine and wounds of subjects attending University of Uyo Teaching Hospital from July 2008 to September, 2008. Stock cultures were maintained on nutrient agar slant at 4^{o}C. The isolates were identified as *S. aureus* according to colonial and microscopic morphology, growth on Mannitol Salt agar, 5% blood sheep agar, positive catalase, DNase production and coagulase production [13]. Methicillin and vancomycin resistance was carried out using the disk diffusion method [14]. Methicillin (5µg) and vancomycin (5ug) disks (Oxoid, UK) were used, and the inhibitory zones were determined in accordance with procedures of the National Committee for Clinical Laboratory Standard [15].

Bioassay

The ethanol extracts were tested for anti-MRSA and anti- VRSA by the disc diffusion method [15,16] using Oxoid- Mueller Hinton agar (Difco Laboratories, Detroit, Mich) supplemented with 2% NaCl. The Mueller – Hinton agar (MHA) was sterilized in flasks cooled to 45 – 50^{o}C and then poured into sterilized Petri dishes. Sterile filter paper discs of 6 mm diameter were impregnated with extract solution of graded concentrations (5 mg/ml, 10 mg/ml, 20 mg/ml,40 mg/ml and 80 mg/ml) and then placed on to the agar plates which had previously been inoculated with the tested microorganisms (MRSA and VRSA). Control experiments comprising streptomycin were set up. The plates were then incubated at 37^{o}C for 24 h .The diameters of the inhibitory zones were measured in millimeters. Assays were performed in triplicate and the data are shown as the mean ± standard deviation (SD).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of the extracts was determined for each of the test organisms (MRSA and VRSA) in test tubes. 0.5ml each of the test isolate was added to the following varying concentrations of the extracts 5 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml and 80 mg/ml containing 2ml of nutrient broth. Similar tubes were set containing streptomycin was as the control group. The cultures were then incubated at 37^{o}C for 24 h. After incubation the tubes were then examined for microbial growth by observing for turbidity. The tube containing the least concentration of extract showing no visible sign of growth was considered as the minimum inhibitory concentration .To determine the MBC, for each of the test isolate 1ml of the broth was collected from the tubes that showed no growth and inoculated onto sterile nutrient agar [17]. The plates were then incubated at 37^{o}C for 24 h. After incubation the concentration that showed no visible growth was considered as the Minimum Bactericidal Concentration (MBC). Both MIC and MBC for each of the test bacteria were determined in triplicate assays and the data were shown as the mean ± SD.

Phytochemical screening

The preliminary phytochemical analysis of the plant extracts was performed to screen for the presence of bio-active components present in the leaves of the *Nymphaea lotus* [18, 19, 20]

Test for Tannins

i.) 1 cm^3 of freshly prepared 10% KOH was added to 1 cm^3 of the extract. A dirty white precipitate indicated the presence of tannins.

ii.) Powdered plant leaves of the test plant (1.0 g) were weighed into a beaker and 10 ml of distilled water added. The mixture was boiled for five minutes. Two drops of 5% $FeCl_3$ were then added. Production of a greenish precipitate was an indication of the presence of tannins.

Test for alkaloids

i) The extract of the plant leaves sample (0.5 g) was stirred with 5 ml of 1% HCl on a steam bath. The solution obtained was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract

ii) A few drops of freshly prepared Drangendorff's reagent were added to 0.5g of the plant extract in the test tube and colour change to brown was observed.

iii) A few drops of freshly prepared Picric reagent were added to 0.5g of the plant extract in the test tube and colour change to brown was observed.

Test for saponins

0.5 g of plant extract was introduced into a tube containing 5.0 ml of distilled water, the mixture was vigorously shaken for 2 min, and formation of froth indicated the presence of saponins.

Test for flavonoids
A small piece of magnesium ribbon was added to ethanolic extract of the plant material, this was followed by the drop wise addition of concentrated hydrochloric acid. Colours varying from orange to red indicated flavones, red to crimson indicated flavonoids, crimson to magenta indicated flavonones.

Test for Terpenes
To 0.5g of the extract, 3.0ml chloroform was added and filtered, 10 drops of acetic anhydride and 2 drops of H_2SO_4 were added to the filtrate and the colour change from blue to green was observed.

Cardiac glycosides:
0.5g of the plant extract was dissolved in 2ml dissolved in 2ml of acetic anhydride and cooled in ice bath. Concentrated H_2SO_4 was carefully added drop by drop . A colour change from violet to blue to green indicated the presence cardiac glycoside. Also 0.5g of the plant extract was dissolved in 2ml of chloroform, Concentrated H_2SO_4 was carefully added drop by drop to form a lower layer. A reddish- brown colour at the interface indicated the presence of cardiac glycoside

Anthraquinones
0.5g of plant extract was shaken with 10ml of benzene and filtered and 5ml of 10% ammonia was added to the filtrate. The mixture was shaken and the presence of pink, red or violet colour indicated the presence of anthraquinones

RESULTS

The results of the *in vitro* antimicrobial activity of the ethanolic crude extracts of *Nymphaea lotus* leaves at different concentrations (5, 10, 20, 40, 80) mg/ml using agar diffusion method on the isolates varied as shown in Tables 1 and 2. The mean zones of inhibition the extract ranged from 8.0 ± 0.5mm to 24.0 ± 1.0mm against MRSA (Table1) and from 8.0 ± 0.5mm to 27.0± 1.0mm against VRSA (Table 2). The extracts showed the highest zone of inhibition (24 ± 2.0 mm) against VRSA at 80mg/ml and highest (24 ± 1.0 mm) against MRSA at 80mg/ml (Tables 1 and 2). The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the plant extracts on both MRSA and VRSA ranged from 5 to 15 and 10-30 respectively (Tables 3 and 4). The results of the preliminary phytochemical analysis of the extracts showed the concentrations of bio-active compounds in the ethanolic crude extracts of *Nymphaea lotus* leaves (Table 5). The following bioactive compounds were identified from the ethanolic crude extracts of *Nymphaea lotus* leaves; tannins, flavonoids, alkaloids, anthraquinones, saponins, terpenes, cardiac glycosides and phenolics (Table 5). The anthraquinones, terpenes, and cardiac glycosides were the most prominent bioactive compounds in the extract.

Table 1: Antibacterial activity of *Nymphaea lotus* against Methicillin Resistant *Staphylococcus aureus* (MRSA)

	Mean zones of Inhibition (MM) ±SD					
Bacteria	**5mg/ml**	**10mg/ml**	**20mg/ml**	**40mg/ml**	**80mg/ml**	**Streptomycin (30ug)**
MR-SA 1	8.0±1.0	12.0 ± 1.0	12.0 ± 2.0	20.0 ± 2.5	23.0 ± 1.0	24.0 ± 2.0
MR-SA 2	10.0±1.5	15.0 ± 2.0	17.0 ± 1.5	20.0 ± 1.5	22.0 ± 1.5	20.0 ± 2.0
MR-SA 3	8.0±0.5	8.0 ± 0.5	10.0 ± 1.0	15.0 ± 1.0	20.0 ± 3.0	18.0 ± 3.0
MR-SA 4	NZ	8.0 ± 1.0	12.0 ± 2.0	12.0 ± 2.0	15.0 ± 3.0	18.0 ± 1.0
MR-SA 5	8.0±0.5	8.0 ± 1.0	10.0 ± 1.0	13.0 ± 1.0	15.0 ± 1.5	21.0 ± 2.0
MR-SA 6	8.0 ±1.0	10.0 ± 2.0	15.0 ± 1.0	18.0 ± 3.0	20.0 ± 2.0	20.0 ± 1.0
MR-SA 7	NZ	8.0 ± 1.0	8.0 ± 1.0	10.0 ± 1.5	15.0 ± 1.0	12.0 ± 2.0
MR-SA 8	NZ	9.0 ± 0.5	12.0 ± 1.0	15.0 ± 2.0	22.0 ± 1.0	20.0 ± 1.0
MR-SA 9	8.0±1.0	12.0 ± 0.5	12.0 ± 1.0	15.0 ± 2.0	24.0 ± 1.0	22.0 ± 1.0
MR-SA 10	8 .0±0.5	10.0 ± 0.5	12.0 ± 1.0	20.0 ± 1.0	20.0 ± 1.0	26.0 ± 1.0

Key: MR-SA 1-5: Methicillin Resistant *Staphylococcus aureus* isolated from wounds
MR-SA 6-10: Methicillin Resistant *Staphylococcus aureus* isolated from urine
NZ - No Zone of Inhibition

Table 2: Antibacterial activity of *Nymphaea lotus* against Vancomycin Resistant *Staphylococcus aureus* (VRSA)

	Mean zones of Inhibition (MM) ±SD					
Bacteria	5mg/ml	10mg/ml	20mg/ml	40mg/ml	80mg/ml	Streptomycin (30ug)
VR-SA 1	8.0±1.0	8.0 ± 1.0	10.0 ± 2.0	20.0 ± 2.5	25.0 ± 1.0	22.0 ± 3.0
VR-SA 2	9.0±1.5	12.0 ± 2.0	14.0 ± 1.5	18.0 ± 1.0	21.0 ± 1.5	21.0 ± 2.0

VR-SA	3	8.0±0.5	8.0 ± 0.5	10.0 ± 1.0	15.0 ± 1.0	20.0 ± 2.0	20.0 ± 3.0
VR-SA	4	10.0± 0.5	10.0 ± 1.0	12.0 ± 2.0	15.0 ± 2.0	15.0 ± 3.0	20.0 ± 1.0
VR-SA	5	10.0±0.5	15.0 ± 1.0	15.0 ± 1.0	15.0 ± 2.0	22.0 ± 1.5	19.0 ± 2.0
VR-SA	6	8.0 ±1.0	12.0 ± 1.5	15.0 ± 1.0	21.0 ± 3.0	24.0 ± 2.0	22.0 ± 1.0
VR-SA	7	8.0±0.5	8.0 ± 1.0	12.0 ± 1.5	12.0 ± 1.5	21.0 ± 1.0	25.0 ± 3.0
VR-SA	*8*	8 .0±0.5	10.0 ± 0.5	10.0 ± 1.0	14.0 ± 2.0	18.0 ± 1.0	20.0 ± 1.5
VR-SA	*9*	10 .0±0.5	13.0 ± 0.5	18.0 ± 1.0	20.0 ± 2.0	27.0 ± 1.0	23.0 ± 1.0
VR-SA	*10*	NZ	9.0 ± 0.5	12.0 ± 1.0	12.0 ± 2.0	15.0 ± 1.0	15.0 ± 1.0

Keys; VR-SA 1-5: Vancomycin Resistant *Staphylococcus aureus* isolated from wounds
VR-SA 6-10: Vancomycin Resistant *Staphylococcus aureus* isolated from urine
NZ - No Zone of Inhibition

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of extracts of *N. lotus on* Methicillin Resistant *Staphylococcus aureus* (MRSA)

Bacteria	MIC	MBC
MR-SA 1	5.0±1.0	10.0 ± 1.5
MR-SA 2	10.0±1.5	20.0 ± 2.0
MR-SA 3	10.0±0.5	10.0 ± 3.0
MR-SA 4	5.0 ± 1.0	10.0 ± 1.0
MR-SA 5	10.0±0.5	20.0 ± 1.5
MR-SA 6	10.0 ±0.5	30.0 ± 1.0
MR-SA 7	10.0 ±0.5	20.0 ± 1.5

MR-SA 8	5.0± 1.5	20.0 ± 0.5
MR-SA 9	10 .0±1.0	30.0 ± 1.0
MR-SA 10	5 .0±1.0	20.0 ± 0.5

Keys; MR-SA 1-5: Methicillin Resistant *Staphylococcus aureus* isolated from wounds
VR-SA 6-10: Methicillin Resistant *Staphylococcus aureus* isolated from urine

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of extracts of *N. lotus on* Vancomycin Resistant *Staphylococcus aureus* (VRSA)

Bacteria	MIC	MBC
VR-SA 1	5.0±1.0	10.0 ± .0
VR-SA 2	10.0±1.5	30.0 ± 2.0
VR-SA 3	5.0±0.5	20.0 ± 3.0
VR-SA 4	5.0 ± 1.0	15.0 ± 1.0
VR-SA 5	5.0±0.5	10.0 ± 2.0
VR-SA 6	10.0 ±1.0	30.0 ± 1.0
VR-SA 7	10.0 ±0.5	20.0 ± 3.0
VR-SA 8	10 .0±0.5	30.0 ± 1.0
VR-SA 9	5 .0±0.5	10.0 ± 1.0
VR-SA 10	10 .0±0.5	10.0 ± 1.0

Keys; VR-SA 1-5: Vancomycin Resistant *Staphylococcus aureus* isolated from wounds
VR-SA 6-10: Vancomycin Resistant *Staphylococcus aureus* isolated from urine

Table 5: Phytochemical analysis of the ethanol extracts of *Nymphaea lotus* leaves

Plant Constituents	Tests used	Occurrence
Alkaloids	Drangendorff's test	++
	Mayer's Reagent test	+
	Picric Acid test	-
Flavonoids	General test	++
Phenolics	Frothing test	++
Saponins	General test	+
Tannins	General test	+
Anthraquinones	General test	+++
Cardiac glycosides	Liberman' s test	+++
	Salkowski test	+++
Terpenes	Chloroform test	+++

Keys: + = Present in small concentrations
++ = Present in moderately high concentrations
+ + + = Present in high concentrations

DISCUSSION

The worldwide emergence of both MRSA and VRSA can have severe public health implications as these organisms can cause a variety of nosocomial and community-acquired infections and potentially life-threatening disease. It is important and valuable to find compounds that potentiate antimicrobial activity against MRSA and VRSA. While the battle between man and microbes continues, starting with the defeat suffered by penicillin, methicillin to the emergence of vancomycin resistant microorganisms [21, 22]. Preliminary phytochemical screening of *Nymphaea lotus* revealed the presence of alkaloids, tannins, phenolics, saponins, cardiac glycoside, terpenes and flavonoids. Some workers have also attributed to their observed antimicrobial effect of plant extracts to the presence of these secondary plant metabolites and it is responsible for their use as herbs [23] Hence, the presence of the secondary metabolites such as anthraquinones, cardiac glycosides, saponins, tannins, alkaloids, flavonoids and phenolics in *Nymphaea lotus* may be responsible for its potential use as drug against MRSA and VRSA. Antimicrobial activities of alkaloids and flavonoids have been reported [24, 25].Tannins are important in herbal medicine in treating wounds and to arrests bleedings [26]. Phyto-constituents such as saponins and phenolics compounds have been reported to inhibit bacterial growth. The results obtained showed that ethanolic extracts of *Nymphaea lotus* exhibited inhibitory activities against MRSA and VRSA bacteria with different degrees as demonstrated by measuring the diameters of inhibition zones and these results are in conformity with the results obtained by [6,11]

In conclusion, *S. aureus* is a major cause of serious nosocomial infections colligated with morbidity and mortality. Emergence of MRSA and VRSA to antibiotics will make infections difficult to treat. Therefore, is need to consider the use of this potent extracts that have shown some measures of antimicrobial potency, judging by the antimicrobial activity, activity index (A.I), Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration

(MBC) results of the ethanolic crude extracts in controlling MRSA and VRSA infections as well as provide a new strategy to treat reemerging infections.

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