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Antibacterial Effects of Methanol Extract of *Bryophyllum pinnatum* L on Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from Urine

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Authors' contributions

The work was carried out in collaboration by all the authors. Author CEU designed the study and wrote the first draft of the manuscript while author CO managed the analysis of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

There are major concerns about rising levels of methicillin resistant *Staphylococcus aureus* (MRSA). This is due to the difficulties in treating the infections which they cause and .the ease with which they spread in hospitals. This has necessitated the continuous search for alternative anti-MRSA agents. Efforts in this study was therefore directed at isolation of MRSA from the urine of patients and its susceptibility to the methanol extract and aqueous fraction of *Bryophyllum pinnatum* . Urine samples from the urine of patients were screened for the presence of *Staphylococcus aureus* using conventional microbiological methods. Confirmed isolates were screened for methicillin resistance by confirming their susceptibility or otherwise to 30µg cefoxitin. Detection of Mec A gene by Polymerase chain reaction (PCR) was further used to confirm some MRSA isolates. Conventional susceptibility testing methods were used to compare the activity of both methanol extract of *Brophyllum pinnatum* and its aqueous fraction on the MRSA isolates. Results obtained confirmed the susceptibility of the MRSA isolates to the extracts and that their activity was time dependent. It also showed that the extract was only moderately toxic with an LD_{50} of 866.03mg/kg body weight

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and that at the MIC and 2xMIC their activity was only bacteriostatic. Results obtained are intended to be used to prove that in the search for alternative anti- MRSA agents from natural sources, *Bryophyllum pinnatum* will be a possible candidate for further investigation.

Keywords: Methicillin; Bryophyllum pinnatum; bacteriostatic; susceptible; alternative.

1. INTRODUCTION

Staphylococcus aureus is a facultative anaerobic, Gram positive cocci. It is found as part of normal skin flora, in the nostrils [1] and as a normal inhabitant of lower reproductive tract of women [2]. *Staphylococcus aureus* is a versatile human pathogen that causes diseases ranging from relatively mild infections of skin and soft tissue to life-threatening sepsis in humans. It is also known to cause cause both hospital and community-associated infections. These infections occur as a result of a breach in the mucosal barriers of the body. It also takes advantage of suppressed inert and active immunity of an individual to cause infections [3].

Staphylococcus aureus quickly develops resistance and is capable of producing many resistant strains [3]. It can acquire resistance genes through horizontal gene transfer mechanisms which enable them to show resistance against antimicrobial agents and spread worldwide [4]. Presently, a large percentage of the infections caused by *Staphylococcus aureus* are due to methicillin-resistant strains of *Staphylococcus aureus*.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a specific strain of the *Staphylococcus aureus,* which is resistant to methicillin and all β-lactams [5]. It has been associated with many infection sites including bones and joints, lungs, and the urinary tract. It also causes bacteremia which possibly leads to endocarditis osteomyelitis [6]. MRSA is associated with high morbidity and mortality rates because of the development of multidrug antibiotic resistance [7]. Resistance to methicillin is due to the presence of mecA gene, which is a part of a large cluster called staphylococcal cluster cassette chromosome mec (SCCmec) [8]. The mecA gene encodes an altered penicillin binding protein 2a having reduced affinity for β-lactams thereby providing resistance to practically all β-lactams antibiotics [9].

Bryophyllum pinnatum (*Lam.*) *Kurz* (Crassulaceae) also known as Ndodob or Afiayo among the Ibibio people of southern Nigeria,is a perennial herb growing widely and used in folkloric medicine in tropical Africa, tropical America, India, China and Australia. A number of its specie are cultivated as ornamentals and are popular tropical house plants. It is popularly known as miracle plant or life plant.

B. pinnatum is used in ethno medicine generally for the treatment of ear ache, cough, diarrhea, dysentery, abscesses, ulcer, insect bites, heart-troubles, epilepsy, arthritis, dysmenorrhea and whitlow [9] also reported the use of the leaves and leaf juice traditionally as anti-inflammatory, antipyretic, antimicrobial antioxidant, antitumor, antidiabetic, antiulcer, antiseptic, hypocholo-sterolemic and cough suppressant. Results presented in this work shows the effects of the plant *Bryophyllum pinnatum* on Methicillin resistant Staphylococus aureus and the possibility of its use in the control of infections caused by them.

2. METHODOLOGY

2.1 Sample Collection

Fresh urine samples were collected aseptically in sterile urine bottles from patients with the help of the laboratory staff at the University of Uyo Teaching hospital. All samples collected were properly labelled and taken to the pharmaceutical microbiology laboratory, Faculty of Pharmacy, University of Uyo for further examinations.

2.2 *Staphylococcus aureus* Isolation and Identification

Mannitol salt agar was prepared according to the manufacturers' instruction, sterilized and allowed to cool to 45℃. It was then poured into a sterile petri dish and allowed to solidify. A loopful of each specimen was inoculated using streak method on the surface of the already solidified mannitol salt agar and incubated at 37°C for 24 hours. The discrete colonies were isolated and further subcultured using mannitol salt to obtain a pure culture. Morphological characteristics of *Staphylococcus aureus* on mannitol salt agar were used to differentiate *Staphylococcus aureus* from other microorganisms. Identified

Staphylococcus aureus were Gram stained and viewed under the microscope to further confirm them. Catalase and coagulase tests as described by [10] were further employed to confirm the presumptive isolates to be *Staphylococcus aureus*.

2.3 Identification of MRSA

Isolates subjected to cefoxitin disc diffusion testing using a 30µg cefoxitin were used. The results obtained during the susceptibility tests were interpreted according to [11] guidelines for the identification of those which are methicillin resistant. An inhibition zone diameter of ≤21mm is considered methicillin resistance while ≥22mm is cosidered methicillin sensitive.

2.4 Detection of mecA gene by PCR Technique

Selected isolates found to be MRSA by specific phenotypic features were further confirmed by the detection of the MecA gene using the Polymerase Chain Reaction(PCR) . The mecA-specific primer pairs used are Forward, 5′- GTT GTA GTT GTC GGG TTT GG-3′, and Reverse, 5′- CTT CCA CAT ACC ATC TTC TTT AAC-3′. The extracted DNA cells were amplified begining with an initial denaturation step at 94°C for 5 min, followed by 33 cycles of amplification at 94°C for 30 sec, annealing at 47°C for 30 sec and extension at 72°C for 30 sec, followed by final extension step at 72°C for 5 min. The amplfied products were visualised by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

2.5 Plant Collection and Authentication

The leaves of *Bryophyllum pinnatum* were obtained from the medicinal plant farm of the Faculty of Pharmacy University Uyo Nigeria. They were authenticated using taxonomic keys provided by the Department of Pharmacognosy, Faculty of Pharmacy, University of Uyo and a voucher with specimen number UUPH27(a) is kept in the Faculty herbarium for further reference.

2.6 Preparation and Extraction of Plant Samples

The leaves were dried in an oven at 45°C, grinded and made into a fine powder using laboratory mortar and pestle.

Methanol (70%) was poured into a container containing the dried leaves and allowed to macerate for 72 hours at room temperature with intermittent shaking. The extract was then filtered and concentrated in a water bath at 40°C.

2.7 Phytochemical Screening

The leaf extract was screened for its phytochemical constituents using the methods described by several authors [12] and [13].

2.8 Fractionation of Extract

The methanol extract was fractionated using petroleum ether, chloroform and water according to the method of [14]. 20 g of dried extract was dissolved in 200 ml of distilled water before shaking vigorously in a separating flask. The mixture obtained was filtered using filter paper to remove debris. Thereafter, 200 ml of petroleum ether was added to the mixture, shaken vigorously and allowed to settle., the petroleum ether layer (on top) was removed and concentrated while a further 200 ml of chloroform was added to the aqueous layer and also shaken vigorously and allowed to settle. The aqueous and the chloroform layers were further separated while the chloroform portion was concentrated to dryness by allowing it to stand on the laboratory bench until all the solvent evaporated. The aqueous layer was concentrated to dryness using mild heat and the resulting fraction was stored in a desiccator until needed.

2.9 Acute Toxicity Testing

Lorke's method [15] was used to determine the lethal dose (LD_{50}) of the crude extract of *Bryophyllum pinnatum* leaf that kills 50% of the test animal population. In the first phase, nine healthy mice were divided into three groups of three animals each. The animals were fasted for 24 hours and each group of animals were administered different doses (2000, 3000 and 5000 mg/kg body weight) of the plant extract. The animals were placed under observation for 24 hours and monitored for mortality. The second phase involved the use of six mice which were distributed into two groups of three animals each. The animals were administered different doses (1000 and 1500 mg/kg body weight) of the plant extract. Then, the third phase involved twelve mice which were distributed into four groups of three animals each. The animals were administered different doses (250, 500, 750 and

1000 mg/kg body weight) of the plant extract. They will then be monitored for 24 hours and mortality taken note of. All experimental protocols were in compliance with the Faculty of Pharmacy University of Uyo ethics on research in animals as well as internationally accepted principles for laboratory animal use and care.

2.10 Susceptibiliy Screening

The agar cup diffusion method was used for this test. Mueller Hinton agar plates were prepared according manufacturer's instructions and with a 4 mm sterile cork borer, wells were bored at equidistant after inoculation on each plate of a 24-hour overnight broth culture of the test organisms. To each of the cups, 0.1 ml each of different concentrations of the crude extract and aqueous fraction ranging from 3.125 -100 mg/ml made using sterile water were introduced. The plates were allowed a pre-diffusion time of 1 hour at room temperature and then incubated at 37°C for 24 hours after which the zones of inhibition were read to the nearest millimeter.

2.11 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration of the crude methanol extract and aqueous fraction were determined using the tube dilution method [16]. 1 ml of the extract solution at concentrations of 25 mg/ml was added to 1ml of nutrient broth and was subsequently transferred thus: 1 ml from the first tube to the next up to the sixth tube. Then, 1 ml of 24 hours culture of test organisms was inoculated into each test tube and mixed thoroughly. The tubes were incubated for 24 hours at 37°C and examined for turbidity as sign of growth. The tube with the lowest concentration of extract with no detectable growth was considered the MIC. A loopful from each tube not showing growth was plated out on nutrient agar and incubated at 37°C for 24 hours. The tube with the lowest concentration that yielded no growth in the plate subculture was considered as the MBC of the extract for each test bacteria isolate.

2.12 Determination of Rate of Kill

Four bottles labelled 1, 2, 3 and 4 were used for each isolate, where bottle 1 served as the control. To each bottle, 9 ml of nutrient broth was added. To bottles 2, 3 and 4, the isolate (1 ml of a standardised overnight culture) and an aliquot of the extract to achieve the MIC of the organism was added. This process was repeated for each isolate being determined. The bottles were then incubated at 37°C and viable counts taken at 30 min interval by withdrawing 0.1 ml of the mixture in the bottle and diluting in normal saline containing 3% Tween 80. The diluted mixtures were plated out on nutrient agar plates and incubated at 37°C for 24 hours. Developed colonies were counted and the colony forming units (cfu/ml) calculated. The process was repeated with an extract concentration of 2×MIC.

3. RESULTS

3.1 Sample Collection and Confirmation of *S. aureus*

Out of a total of 150 fresh urine samples screened, results obtained showed that 89 of the samples were positive for *Staphylococcus aureus.*

3.2 Identification of MRSA

Out of a total of 89 isolates of *S. aureus*, 66 isolates were found to be resistant to cefoxitin (30 µg) confirming them as phenotypic MRSA (74%).

3.3 Detection of mecA gene by PCR Technique

Confirmation of MRSA for some selected isolates was performed by detection of mecA gene using PCR assay. Out of the 8 selected isolates, results revealed that 7 carried mecA gene.The PCR-amplified DNA products of this gene for the 8 selected isolates are shown in Fig. 1.

3.4 Phytochemical Screening

Results of Phytochemical screening showed the presence of a number of secondary metabolites including tannins, flavonoids and cardiac glycosides (Table 1).

3.5 Acute Toxicity Testing

The result of the acute toxicity testing showing concentration of the crude methanol extract of *B. pinnatum* leaf that killed 50% of mice, expressed as LD_{50} is presented in Table 2.

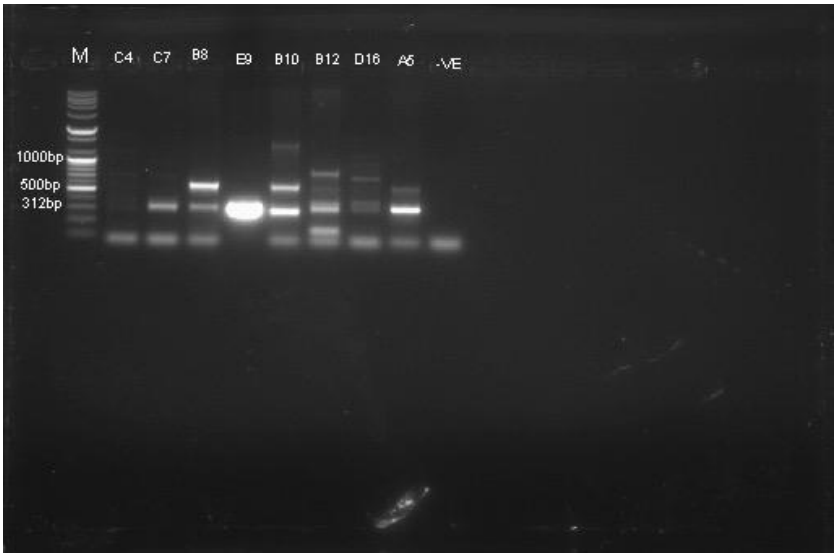

Fig. 1. Amplicon of *mec*A gene: Lanes C7,B8,E9,B10,B12,D16 and A6 are tested isolates with positively amplified *mec*A (indicated by 312 bp PCR amplicon). Lane 4 is *mec*A negative

Table 1. Phytochemical screening of methanol extract of *Bryophyllu pinnatum*

Metabolites	Plant extract
Saponins	-
Alkaloids	-
Tannins	+
Flavonoids	+
Cardiac glycosides	+
Cardenolide	+
Steroids	+

+=positive, - = negative

3.6 Susceptibility Screening

Microbial susceptibility test with the crude methanol extract and aqueous fraction of *B, pinnatum* leaf showed zones of growth inhibitions whose diameters were measured in millimetres (mm) and are presented in Table 3.

3.7 Minimum Inhibitory Concentrations

The MIC and MBC of the methanol and aqueous fraction of the extracts against the test isolates are presented in Table 4. Results showed that the extract had MIC values lower than the MBC for all of the isolates hence showing its effect to be bacteriostatic.

3.8 Rate of Kill

The results that show the relationship between the ability of certain concentrations of the test extracts to control the test organisms and contact time are shown in Tables 4 and 5. They further confirm the activity of the extract against the isolates, its bacteriostatic effect and time dependency.

4. DISCUSSION

Recently, there has been a surge in the report of antibiotic resistant strains of clinically important pathogens. Among the Gram Positive organisms, a pandemic of resistant *Staphylococcus aureus* known as Methicillin resistant *Staphylococcus aureus* (MRSA) currently poses a threat [17]. MRSA, a very important strain of S.*aureus* was first reported in 1961, since then, MRSA infection is increasingly prevailing and continues to pose serious therapeutic challenge. Methicillin acts through competitive inhibition of transpeptidase enzyme by its affinity to penicillin-binding protein 2 (PBP2) used by bacteria to cross-link the peptide (D-alanyl-alanine) mandatory for peptidoglycan synthesis. It was developed to treat staphylococcal infections. Resistance to methicillin is developed due to acquisition of penicillin-binding protein 2A(PBP2A) encoded by the mecA gene from a mobile staphylococcal cassette chromosome (SCC). The current diagnosis for MRSA is basically resistance to either oxacillin or cefoxitin, which indicates non-susceptibility to all other groups of β-lactams. Most MRSA strains are known to be resistant to multiple classes of antibiotics and therefore, cannot be treated with the conventional B-lactams

[18]. The search for the development of novel agents against MRSA has continued and the results presented here are part of our effort to establish the candidacy of *Byophyllum pinnatum* in the formulation of agents that can be used to treat infections due to MRSA.

Results obtained showed that 89(59%) of the 150 urine samples analysed were positive for *Staphylococcus aureus* while 66(74%) of the S. *aureus* isolates were resistant to the cefoxitin (30µg) and were considered MRSA [11]. This shows a high prevalence of MRSA and compares to the work of [19] who reported a prevalence rate of 70% in patients attending clinic in University of Benin Teaching Hospital and [20] who reported a prevalence rate of 75% from the wounds of hospitalised patients of Ahmadu Bello University Teaching Hospital also in Nigeria.

Phytochemical screening confirmed the presence of those secondary metabolites which are known to be responsible for antimicrobial activity namely tannins and flanovoids. Tannins are known to cause death of organisms by depriving them of iron and also forming complexes with polysaccharides while flavonoids form complexes with bacterial cell walls [21]. The presence of these metabolites has been linked to the antibacterial activity of plants [22].

Table 2. Acute toxicity test of methanol extract of *Bryophyllum pinnatum*

Phases	No. of mice	Weight of mice (g)	Dose (mg/kg)	Mortality	LD_{50} (mg/kg)
1	3	20	2000	3/3	$\sqrt{D_0 \times D_{100}}$
		22			$\sqrt{750 \times 1000}$
		24			=866.03 (mg/kg)
	3	25	3000	3/3	
		22			
		23			
	3	22	5000	3/3	
		23			
		23			
2	3	20	1000	3/3	
		21			
		23			
	3	20	1500	3/3	
		22			
		21			
3	3	21	250	0/3	
		22			
		22			
	3	22	500	0/3	
		24			
		23			
	3	22	750	0/3	
		21			
		20			
	3	22	1000	3/3	
		22			
		21			

D_0= highest dose without mortality, 750 mg/kg body weight; D_{100}= lowest dose that produced mortality,1000 mg/kg body weight

Table 3. Antibacterial activity of extracts of *Bryophyllum pinnatum* against selected MRSA isolates

Isolates	CONC (mg/ml)	Zone of inhibition (mm) aqueous fraction	Methanol extract
A5	100	23	13
	50	15	9
	25	NZ	NZ
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ
B8	100	20	13
	50	18	9
	25	10	5
	12.5	9	3
	6.25	5	2
	3.125	NZ	NZ
B10	100	26	16
	50	13	11
	25	9	7
	12.5	4	3
	6.25	NZ	NZ
	3.125	NZ	NZ
B12	100	15	7
	50	13	NZ
	25	NZ	NZ
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ
C4	100	10	8
	50	NZ	NZ
	25	NZ	NZ
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ
C7	100	18	9
	50	15	5
	25	9	3
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ
D16	100	12	7
	50	9	3
	25	NZ	NZ
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ
E9	100	15	10
	50	7	5
	25	NZ	NZ
	12.5	NZ	NZ
	6.25	NZ	NZ
	3.125	NZ	NZ

NZ= No Zone

Table 4. Minimum inhibitory concentration(MIC) and minimum bactericidal concentration (MBC) of extracts against MRSA isolates

Extracts	Isolates	MIC(mg/ml)	MBC(mg/ml)	25	12.5	6.25	3.125	1.56	0.78	0.39
	A5	12.5	25	-	-	+	+	+	+	+
	B8	0.78	1.56	-	-	-	-	-	-	+
Aqueous fraction	B10	0.78	1.56	-	-	-	-	-	-	+
	B12	≥50	≥100	+	+	+	+	+	+	+
	C4	≥50	≥100	+	+	+	+	+	+	+
	C7	≥50	≥100	+	+	+	+	+	+	+
	D16	6.25	12.5	-	-	-	+	+	+	+
	E9	12.5	25	-	-	+	+	+	+	+
	A5	25	50	-	+	+	+	+	+	+
	B8	12.5	25	-	-	+	+	+	+	+
Methanol extract	B10	6.25	12.5	-	-	-	+	+	+	+
	B12	12.5	25	-	-	+	+	+	+	+
	C4	25	50	-	+	+	+	+	+	+
	C7	12.5	25	-	-	+	+	+	+	+
	D16	12.5	25	-	-	+	+	+	+	+
	E9	12.5	25	-	-	+	+	+	+	+

Key: + = Growth; - = No Growth

Table 5a. Reduction pattern of MRSA isolates challenged with aqueous fraction of the methanolic extract of *Bryophyllum pinnatum* at MIC

Time (min)	ISOLATES															
	A5				B8				B10				B12			
	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)
0	3.4×10^4	4.531			7.3×10^4	4.863			7.5×10^4	4.875			3.6×10^4	4.556		
30	2.6×10^4	4.415	0.116	2.56	6.2×10^4	4.792	0.071	1.45	6.2×10^4	4.793	0.082	1.68	3.1×10^4	4.491	0.065	1.42
60	2.4×10^4	4.380	0.151	3.33	5.5×10^4	4.740	0.123	2.52	5.8×10^4	4.763	0.112	2.29	2.6×10^4	4.415	0.141	3.09
90	1.7×10^4	4.230	0.301	6.64	4.4×10^4	4.643	0.22	4.52	4.7×10^4	4.672	0.203	4.16	1.7×10^4	4.230	0.326	7.15
120	1.4×10^4	4.146	0.385	8.49	3.6×10^4	4.556	0.307	6.3	3.5×10^4	4.544	0.331	6.7	1.3×10^4	4.114	0.442	9.7
150	5.0×10^3	3.699	0.832	18.36	2.6×10^4	4.415	0.448	9.2	2.7×10^4	4.431	0.444	9.1	6.0×10^3	3.778	0.778	17.02

Key: A= Cfu/ml; B= Log_{10}Cfu/ml; C= Log_{10}Cfu/ml reduction = Log_{10} (Initial count) – Log_{10} (count at time interval) D = percentage reduction

Though the detection of the Mec A gene is generally accepted as the gold standard for the detection of MRSA, the cefoxitin (30 µg) disc diffusion test has been reported to be in concordance with the detection of Mec A gene by PCR. It is therefore widely accepted as a genuine method for the detection of MRSA [23]. This method was used in this work for the identification of MRSA. The PCR assay technique for Mec A gene detection was however used in further confirmation of MRSA species of a few isolates which were particularly interesting as they showed resistance to a wide range of antibiotics than others. Results obtained confirm that 7 out of 8 isolates tested were Mec A gene positive (Fig. 1). This appears good enough to confirm that the PCR assay technique and the disc diffusion test are comparable since we did not have the capacity to run all the samples identified by the disc diffusion method.

How useful a plant product will be in the formulation of a medicament will be determined to a great extent by its toxicity. Any extract whose LD_{50} is greater than 500mg/kg is considered not toxic [24]. Result obtained shows the LD_{50} of our extract is 866.03 (Table 2) confirming it only moderately toxic.

Susceptibility results obtained confirmed the potential of the plant extract in inhibiting the organisms used in the study (Table 3). The aqueous fraction showed a relatively better activity possibly because the active metabolites

identified which are known to be polar must have been concentrated into the aqueous fraction since water which is polar will attract polar compounds. The results showed the activity of the extract to be concentration dependent. It is clear that the purer the extract is, the better the activity will be. The result of isolate C4 seems to be of interest. It is one of the original 66 isolates confirmed to be cefoxitin (30 µg) resistant hence considered an MRSA. The PCR assay however showed it to be Mec A gene negative (Fig. 1). When susceptibility tests were done, it showed very poor susceptibility and high MIC with the concentration of the extract employed. This points to a higher resistant state compared to the other isolates which were Mec A gene positive and confirmed MRSA by the golden rule. Is it possible that the resistance in this isolate is due to a possible alternative genetic possibility other than Mec A gene acquisition?

The result of the rate of kill of the test fraction is shown in Tables 5 and 6. It can be observed that the extract exhibited some reduction in the viable cell count of the 8 MRSA isolates tested. The results showed a reduction in viable cells of between 0.44Log_{10}cfu/ml and 1.20Log_{10} cfu/ml which represents a percentage reduction of between 9.1 and 26.72 after 150 minutes of contact with the isolates. Initially, after 30 minutes of interaction, the reduction in viable cell count was only between 0.023Log_{10} cfu/ml and0.116Log_{10} cfu/ml representing a percentage reduction of between 0.48 and 2.56. This confirms the activity of the test fraction to be time dependent since its effectiveness in reducing the number of viable cell is better after longer time of contact with the cells. At 2×MIC, results of rate of kill obtained showed that the reduction in viable cells after 30 minutes of interaction was between 0.068 Log_{10}cfu/ml and 0.146 Log_{10}cfu/ml

Table 5b. Reduction pattern of MRSA isolates challenged with aqueous fraction of the methanolic extract of *Bryophyllum pinnatum* at MIC

Time (min)	ISOLATES															
	C4				C7				D16				E9			
	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)
0	3.1×10^4	4.491			3.2×10^4	4.505			5.9×10^4	4.771			5.6×10^4	4.748		
30	2.5×10^4	4.398	0.093	2.07	2.5×10^4	4.398	0.107	2.37	5.6×10^4	4.748	0.023	0.48	4.7×10^4	4.672	0.076	1.6
60	1.8×10^4	4.255	0.236	5.25	1.6×10^4	4.204	0.301	6.68	3.9×10^4	4.591	0.18	3.77	3.4×10^4	4.531	0.217	4.57
90	8.0×10^3	3.903	0.588	13.09	1.5×10^4	4.176	0.329	7.30	3.3×10^4	4.519	0.252	5.28	2.5×10^4	4.398	0.35	7.37
120	6.0×10^3	3.778	0.713	15.87	6.0×10^3	3.778	0.727	16.13	2.2×10^4	4.342	0.429	8.99	1.6×10^4	4.204	0.544	11.45
150	-	-	-		2.0×10^3	3.301	1.204	26.72	1.2×10^4	4.079	0.692	14.5	1.2×10^3	4.079	0.699	14.09

Key: A= Cfu/ml; B= Log_{10}Cfu/ml; C= Log_{10}Cfu/ml reduction = Log_{10} (Initial count) – Log_{10} (count at time interval); D = percentage reduction

Table 6a. Reduction pattern of MRSA isolates challenged with aqueous fraction of the methanolic extract of *Bryophyllum pinnatum* at 2x MIC

Time (min)	ISOLATES															
	A5				B8				B10				B12			
	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)
0	1.4×10^4	4.146			6.2×10^4	4.792			5.6×10^4	4.748						
30	1.1×10^4	4.041	0.105	2.53	5.3×10^4	4.724	0.068	1.41	4.6×10^4	4.663	0.085	1.7	-	-	-	-
60	7.0×10^3	3.845	0.301	7.26	3.5×10^4	4.544	0.248	5.17	3.5×10^4	4.544	0.204	4.29	-	-	-	-
90	5.0×10^3	3.699	0.447	10.78	2.7×10^4	4.431	0.361	7.53	2.8×10^4	4.447	0.301	6.33	-	-	-	-
120	3.0×10^3	3.477	0.669	16.85	1.6×10^4	4.204	0.588	12.27	1.3×10^4	4.114	0.634	13.35	-	-	-	-
150	1.0×10^3	3.000	1.146	27.64	7.0×10^3	3.845	0.947	19.76	8.0×10^3	3.903	0.845	17.79	-	-	-	-

Key: A= Cfu/ml, B= Log_{10}Cfu/ml, C= Log_{10}Cfu/ml reduction = Log_{10} (Initial count) – Log_{10} (count at time interval), D = percentage reduction

Table 6b. Reduction pattern of MRSA isolates challenged with aqueous fraction of the methanolic extract of *Bryophyllum pinnatum* at 2x MIC

Time (min)	ISOLATES															
	C4				C7				D16				E9			
	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)	A	B	C	D (%)
0	-	-	-	-	-	-	-	-	1.9×10^4	4.279			1.4×10^4	4.146		
30	-	-	-	-	-	-	-	-	1.7×10^4	4.230	0.085	1.98	1.0×10^4	4.000	0.146	3.52
60	-	-	-	-	-	-	-	-	1.2×10^4	4.079	0.204	4.76	5.0×10^4	3.699	0.447	10.78
90	-	-	-	-	-	--	-	-	1.0×10^4	4.000	0.301	7.03	2.0×10^4	3.301	0.845	20.38
120	-	-	-	-	-	-	-	-	5.0×10^3	3.699	0.634	14.81	1.0×10^4	3.000	1.146	27.64
150	-	-	-	-	-		-	-	3.0×10^3	3.477	0.845	19.74	-	-	-	-

Key: A= Cfu/ml, B= Log_{10}Cfu/ml, C= Log_{10}Cfu/ml reduction = Log_{10} (Initial count) – Log_{10} (count at time interval), D = percentage reduction

representing a percentage reduction of 1.41% and 3.52% while after 120 minutes it was between 0.588Log_{10}cfu/ml (12.27%) and 1.146 Log_{10}cfu/ml (27.64%) confirming that the activity of the test fraction is also concentration dependent when compared with the result obtained using the MIC. For time- kill endpoint determinations, bacteriostatic activity is a reduction of between 0 and 3 Log_{10} cfu/ml while bactericidal activity is a reduction of 3 Log_{10} cfu/ml and above at different time intervals (30,60,90,120,150) from the original population at 0 minutes [25]. Results of the time-kill assay obtained, therefore confirms a bacteristatic activity at the MIC and 2×MIC concentrations used in the study. It is not impossible that at higher concentration of extract like 4xMIC, 8xMIC, I6xMIC a bacteriocidal effect can be obtained but this was not studied.

5. CONCLUSION

As the menace of bacterial resistance continues to pose serious problems and the search for alternative anti-MRSA agents from natural sources continues, results obtained in this study showed that *Bryophyllum pinnatum* will be a possible candidate for further investigation for use in the formulation of new anti-MRSA agent.

ETHICAL APPROVAL

All experimental protocols were in compliance with the Faculty of Pharmacy University of Uyo ethics on research in animals as well as internationally accepted principles for laboratory animal use and care.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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