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Antimicrobial Activities of *Bryophyllum pinnatum* on Some Selected Clinical Isolates.

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Abstract

Ethanol, methanol and aqueous extracts of the leaves of *Bryophyllum pinnatum* obtained through cold maceration, were screened for their antibacterial activities against selected multi-drug resistant bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi Staphylococcus aureus*) using the agar well diffusion method. Broth dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts at concentrations ranging from 100mg/ml to 25mg/ml. The ethanol extract was the most reactive while the aqueous extract showed lesser antibacterial activity. Ofloxacin was the most effective antibiotic in the antibiotic susceptibility profiling of the test organisms. It was however evident that the ethanol extract of *Bryophyllum pinnatum* has higher antibacterial efficacy on the test organisms than Ofloxacin. Bacteriostatic and -cidal activities were exhibited by the plant extracts against the organisms ranging from 25 – 100 × 10^3 µg/ml. Succinctly, *Bryophyllum pinnatum* possesses biologically active constituents with explorable pharmacological potentials.

Keywords: Antimicrobial, *Bryophyllum pinnatum*, Extraction solvents, minimum inhibitory concentration (MIC), minimum bactericidal concentration.

Introduction

In recent times, there has been increase in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains. (Aibinu *et al.*, 2004). The non-availability and high cost of new generation antibiotics with limited effective lifespan have resulted in increase in morbidity and mortality (Williams, 2000). Therefore, there is a need to look for substances from other sources with proven antimicrobial activity. Consequently, this has led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (Anibijuwon *et al.*, 2017).

About 80% of Africans rely on traditional medicine for their health care needs (Calixto, 2000). According to the United Nations Conference on Trade and Development, 33% of total modern drugs produced by industrialized Countries are plant based (Raskin *et al.*, 2002). The use of plants for medicinal purposes predates the introduction of antibiotics and other modern

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drugs. The potency of herbal remedies soon became an issue of dispute due to lack of qualitative identification of their bioactive components (Sofowora, 1986).

Bryophyllum pinnatum is used in ethnomedicine generally for the treatment of ear-ache, cough, diarrhoea, dysentery, abscesses, ulcers, insect bites, heart-troubles, epilepsy, arthritis, dysorrhea and whitlow (Gill, 1992). In southern Nigeria, it is used to facilitate the dropping and healing placenta wound of newly born babies. The plant leaf is mildly exposed to heat and the juice is squeezed out and applied as poultice to the baby's placenta on daily basis. Also, the crushed leaves as well as the extracted juice are mixed with shear butter or palm oil and rubbed on abscesses or other swellings. This is also applied on ulcers, burns and on the bodies of young children when they are ill.

The leaves of this plant contain bryophyllin, potassium, malate, ascorbic, malic, and citric acids (Oliver, 1989). The plant is rich in both macro and micro elements, vitamins, calcium, phosphorus, ascorbic acid, inulin (Okwu and Josiah, 2006) and other compounds like saponins, flavonoids, anthraquinones, xanthones, bryophyllin A and B (Iwu, 1993). Anti-inflammatory, hypoglycaemic, anti diabetic and anticancer properties have been reported (Gill, 1992).

Alkaloids and saponins are present in the aqueous and alcoholic extracts of leaves and lectins in the juice from the fresh leaves (Nguelefack *et al.*, 2006). The green callus of the plant contains malic acid, quinones and tocopherol (Sofowora, 1993). The antibacterial activity of the leaf juice of *B. pinnatum* was reported by (Obaseiki-Ebor, 1985). Flavonoids, polyphenols, and triterpenoids have been identified from the leaves of *B. pinnatum* (Ojewole, 2005).

Other works have also shown that this plant possesses analgesic, anticonvulsion, anti-inflammatory, antiarthritic and antispasmolytic properties (Theophil *et al.*, 2006). The conventional method to extract plant materials is to use methanol, ethanol, distilled water, acetone and so on as extracting solvents. The type of solvents and methods of preparation affect antimicrobial activity of plants (Adesanya, 2005). On the basis of this background, in-vitro antimicrobial activities of the extracts *Bryophyllum pinnatum* from various solvents was tested against clinically important pathogens

Therefore, the aim of this work to determine the bio effects of *B. pinnatum* against respiratory tract pathogenic bacteria (i.e. *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia coli*).

Materials and Methods

Collection and Identification of Plant Materials.

The leaves of *Bryophyllum pinnatum* were collected from Oke-igbo in Ondo State. These plants can be collected during rainy season particularly between March and July. The samples were identified at the Herbarium Section Department of Plant biology, University of Ilorin, Ilorin, Nigeria.

Sample Preparation and Extraction Procedure

Fresh leaves of *Bryophyllum pinnatum* were air-dried for a period of about two weeks, and crushed into powder form using a mechanical grinder. Methanol, Ethanol and distilled water extraction were done by percolation of the powdered crude sample in these solvents, and left on an orbital shaker for 48 hours; after which it was filtered using Whatman number 1 filter paper and later concentrated to dryness under reduced pressure with a vacuum evaporator at 40°C (Model type 349/2, Corning Limited). The residual extract was stored at refrigerator temperature of 4°C in sterile plastic bottles until required for use. Before use, each extract was re-suspended in their respective extractant to yield 50mg extract residue per ml solvent. Both aqueous and methanol extraction were carried out by using Soxhlet extractor (Quickfit U.K). Powdered dried leaves (50 g) were extracted with 250 ml of solvent (Akinyemi *et al.*, 2005).

Source and Maintenance of Bacteria Isolates.

Gram-positive (*Staphylococcus aureus*) and Gram-Negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi*) were obtained and confirmed at the Department of Medical Microbiology and Parasitology, College of Medicine, University of Ilorin, Ilorin. They were maintained on double-strength nutrient Agar

Slant. Twenty-four hour old pure cultures were prepared for use each time.

Reconstitution and Sterilization of Extract

After using Soxhlet extractor to concentrate the extract, the powder obtained then was weighed and then dissolved into extractant (distilled water, ethanol and methanol respectively). This makes the stock solution for each extract (Akinyemi *et al.,* 2005).

Standardization of Organism

A sterile loop was used to pick colonies of the test organism on nutrient agar plates and the growth was inoculated into 5ml nutrient broth. The culture was incubated over night at 37°C and its turbidity was compared with MacFarland standard ($1.5x10^8$) cells per ml.

Antimicrobial Bioassay

Suspension of micro-organisms was made in sterile nutrient broth and adjusted to 0.5 Macfarland standards (10^8 Cfu/ml). From the stock, serial dilutions were made to 100, 75, 50, 25 mg/ml of distilled water, methanol and ethanol. Agar well diffusion method was used. Each labeled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed this method is called lawn method. A sterile cork borer of 6mm diameter was used to make five (5) wells on each plate. 0.2ml of the various extract concentration were dropped into each, appropriate labeled well. Solvents used for extraction were tested neat for each organism (Akinyemi *et al.,* 2005).

The inoculated plates were kept on the working bench for 1 hour to allow the extracts to diffuse into the agar. The Sensitivity test Agar plates were incubated at 37°C for 48 hours. Antimicrobial activity was determined by measuring the diameter of zones of inhibition in millimeter (mm) produced after incubation. (From the edge of the ditch to the line was growth of organism started). This serve as the preliminary evidence of antibacterial activities and the control were interpreted also.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of distilled water, ethanol and methanol extract of the plant were determined by using the tube dilution method. Nine (9) ml of sterile nutrient broth was dispensed into each test tube after which 1ml of the extract of different concentrations of the plant extract were prepared in the arithmetic progression 100, 75, 50, 25 mg/ml were introduced. 0.1ml of the test organism was introduced and the tubes were shaken. They were incubated at 37°C for 24hours. The tubes were observed for growth by observing the turbidity of the tubes this was compared with the control and the tube that produce no growth were selected (Akinyemi *et al.,* 2005). The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by sub culturing the MIC assay which did not show any visible growth after 24 hours of incubation on sensitivity test agar and further incubated for 24 hours. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration (Akinyemi *et al.,* 2005). This was carried out on some of the extracts with high antimicrobial activity and some of the highly sensitive organisms

Results

Antimicrobial activities of the extracts of *Bryophyllum pinnatum* leaves against the test organisms indicated that the ethanolic extract was the most reactive at all concentrations (Figs 1-3). The values of the zones of inhibition also showed that *E. coli* was the most susceptible organism ranging from 6mm - 28mm. *K. pneumoniae* and *S. aureus* both exhibited resistance to the methanolic and aqueous extracts as well as lower concentrations of the ethanolic extract (Figs 1-3). The result of the antibiotic susceptibility test also affirmed that the test organisms are multi-drug resistant (Table 1). Ofloxacin was the most

effective antibiotic on all the test organisms, however, the extracts showed higher antibacterial activity (Fig 5). The zones of inhibition were also compared to the standard antibiotic susceptibility test guidelines of CLSI (2016) (Table 1).The MIC of the ethanolic extract of *Bryophyllum pinnatum* for *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, and Staphylococcus aureus* were 25 × 10^3 µg/ml, 50 × 10^3 µg/ml, 50 × 10^3 µg/ml, 75 × 10^3 µg/ml and 50 × 10^3 µg/ml respectively. And the MIC for methanolic extract of *Bryophyllum pinnatum* for *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* were 50 × 10^3 µg/ml, 50 × 10^3 µg/ml, 50 × 10^3 µg/ml, respectively; *Klebsiella pneumoniae and Staphylococcus aureus* had no MIC value. The MIC of the distilled water extract of *Bryophyllum pinnatum* for *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* were 100 × 10^3 µg/ml, 75 × 10^3 µg/ml and 100 × 10^3 µg/ml respectively. *Klebsiella pneumoniae* and *Salmonella typhi* had no MIC values (Table 2). The aqueous extract had no bactericidal activity on the test organisms; methanol extract had no cidal effect on all organisms but *E. coli* at a concentration 100 × 10^3 µg/ml. However, the ethanolic extract exhibited higher bactericidal effect on the test organims except *P. aeruginosa* and *S. typhi* (Table 2)

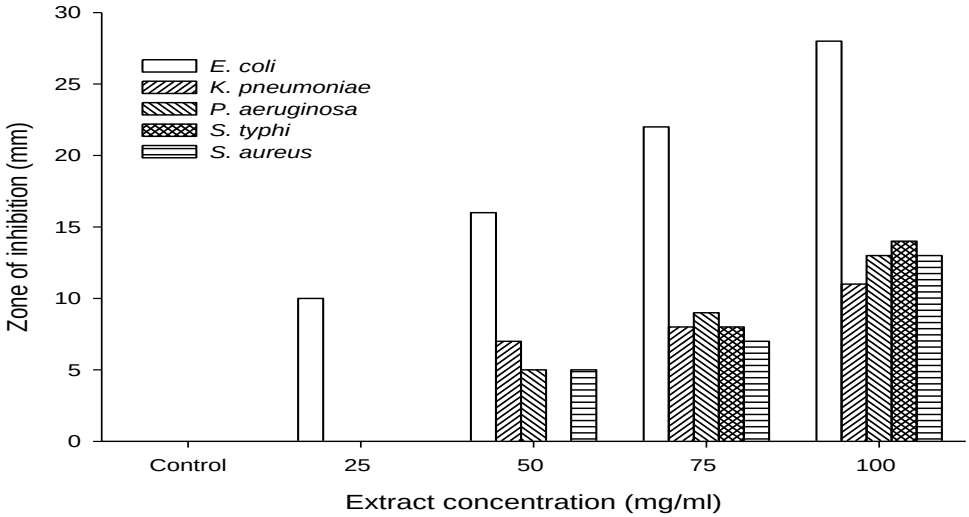

Fig 1: Antibacterial activities of Ethanol extract of *Bryophyllum pinnatum*

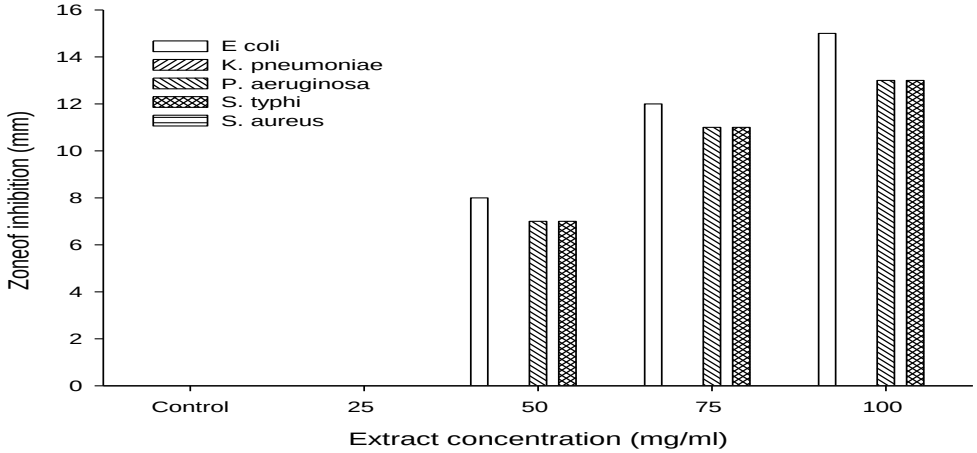

Fig 2: Antibacterial activities of Methanol extract of *Bryophyllum pinnatum*

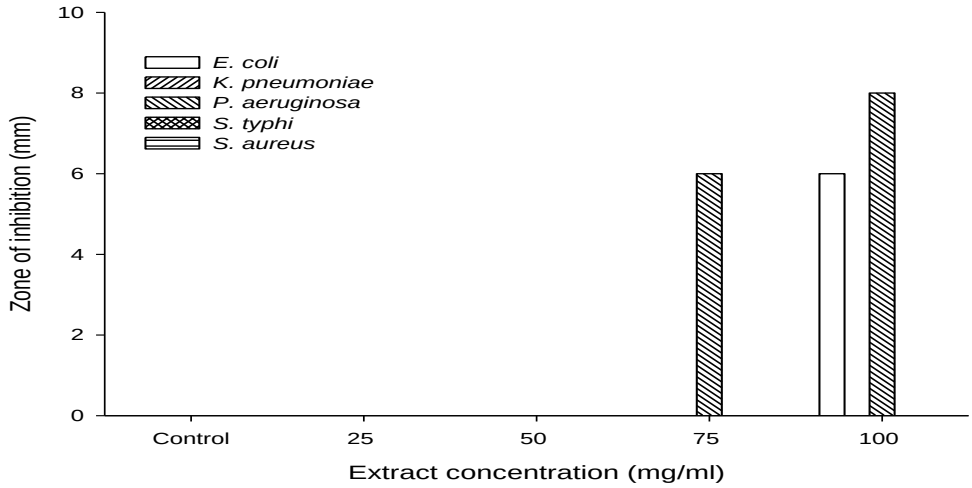

Fig 3: Antibacterial activities of aqueous extract of *Bryophyllum pinnatum*

TABLE 1: Antibiotic susceptibility test

Test organisms	Antibiotics								
	Zone of inhibition (mm)								
	OFL	AMX	CPX	TET	PFX	GEN	COT	ERY	CHL
E. coli	10	N	N	N	6	NA	NA	NA	NA
K. pneumoniae	10	N	8	N	N	NA	NA	NA	NA
Sal. typhi	11	N	8	N	N	NA	NA	NA	NA
R≤I≤S#	12≤13-15≤16	13≤14-16≤17	15≤16-20≤21	11≤12-14≤15	23≤x≤24	-	-	-	-
P. aeruginosa	9	N	12	6	8	NA	NA	NA	NA
R≤I≤S#	12≤13-15≤16	14≤15-20≤21	15≤16-20≤21	14≤15-20≤21	15≤16-23≤24	-	-	-	-
S. aureus	8	NA	NA	NA	NA	5	6	8	7
R≤I≤S#	14≤15-17≤18	-	-	-	-	12≤13-15≤16	12≤13-16≤17	13≤14-17≤18	12≤13-17≤18

N- No zone of inhibition NA- Not applicable
OFL- Ofloxacin AMX- Amoxicillin CPX-Ciprofloxacin TET-Tetracycline PFX-Pefloxacin GEN-Gentamicin COT-Cotrimoxazole ERY-Erythromycin CHL-Chloramphenicol #Resistant≤Intermediate≤Sensitive (CLSI guidelines, 2016)

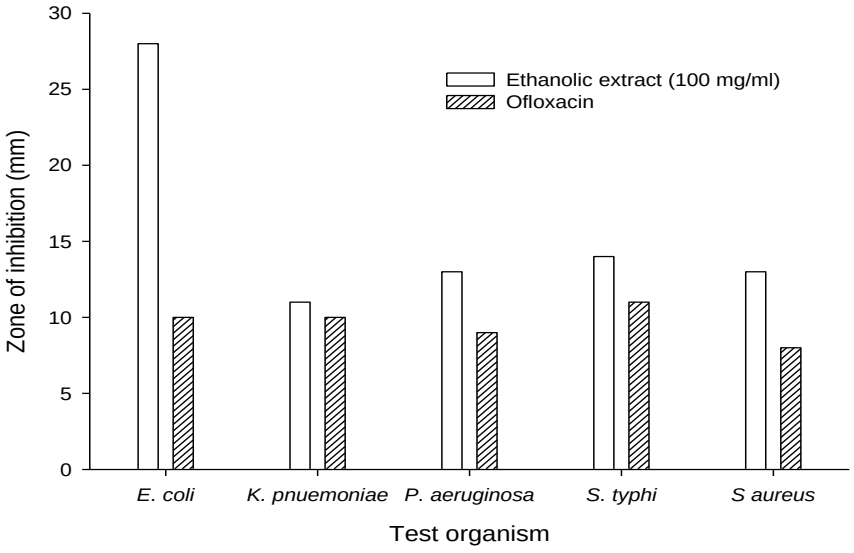

Fig 4: Comparison of the efficacy of the most effective plant extract and antibiotic.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Extracts on Test Organisms.

Test organisms	Minimum Inhibitory Concentration (× 10^3 µg/ml)			Minimum Bactericidal Concentration (× 10^3 µg/ml)		
	Extracts			Extracts		
	Ethanol	Methanol	Aqueous	Ethanol	Methanol	Aqueous
E. coli	25	50	100	75	100	N
K. pneumoniae	50	N	N	100	N	N
P. aeruginosa	75	50	75	N	N	N
S. typhi	50	50	N	N	N	N
S. aureus	50	N	100	100	N	N

N- No MIC/MBC

Discussion and Conclusion

The effective use of plants for therapy has been attributed to the presence of pharmacologically active substances known as phytochemicals. Previous researches on the phytochemical content of the leaves of *Bryophyllum pinnatum* showed the presence of alkaloids, saponins, malic acid, quinones, tocopherol, flavonoids, polyphenols and triterpenoids ((Sofowora, 1993; Ojewole, 2005; Nguelefack *et al.*, 2006). These biologically active substances have been revered for their therapeutic efficacy. The test organisms used in this study were multi-drug resistant bacteria except *P. aeruginosa* and *S. aureus*. The multiple antibiotic resistance index (MARI) of the bacteria ranged from 0.2-0.6 ≤ 1.0. The increase in antimicrobial resistance especially among bacteria is currently one of the highest global public health threats (CDC, 2014). The difficulties experienced in the treatment of common infections coupled with increase in mortality/fatality rate of infections have all been linked to antimicrobial resistance.

The ethanol extract of the leaves was the most potent showing antibacterial activity on one or all of the test organisms at low to higher concentrations (25- 100 mg/ml). Several researches have reported the efficiency of ethanol as an extraction solvent due to its polarity and concentration. Also, more phytochemicals tend to dissolve more in compounds with high polarity and potency. The methanol and aqueous extracts also exhibited antibacterial effects, though lesser than ethanol. This is in accordance with the report of Aquil and Ahmad (2003), that the ethanol extract of *Bryophyllum pinnatum* has broad antibacterial spectrum. The zones of inhibition observed in the Gram-positive organism can be compared with the work of Akinpelu (2000) and Ofokansi (2005) that showed strong activities of ethanol extract of *Bryophyllum pinnatum* against some Gram-positive organisms. The extracts were effective on both Gram positive and negative bacteria. *E. coli* showed significant susceptibility to all concentrations of the ethanol extract with inhibition values ranging from 10-28 mm. In contrast to the commonly reported resistance of Gram negative bacteria to antimicrobial agents, the extracts showed better activity against them than the Gram positive bacterium used.

According to the CLSI (2016) guidelines, all the antibiotics used failed to attain the required inhibition value for reactivity against the test organisms (Table 1). Hence, all the test organisms were completely resistant to the antibiotics used in this study. This further appraises the potency of the extracts of the leaves of *Bryophyllum pinnatum*. The MIC of the ethanol extract ranged from 25- 75 × 10^3 µg/ml for all the test organisms, with *E. coli* and *P. aeruginosa* having the highest and lowest respectively. The MIC values of the methanol and aqueous extracts ranged from 50- 100 × 10^3 µg/ml for some of the test organims, while other organisms showed no inhibitory effect. *K. pneumoniae* had no MIC for methanol and aqueous extracts. The ethanol extract showed bactericidal effects on *E. coli, K. pneumoniae and S. aureus* (75-100 × 10^3 µg/ml). A MBC value of 100 × 10^3 µg/ml was obtained for the methanol extract on *E. coli*, but no cidal activity was observed for the remaining organisms. The aqueous extract

exerted no bactericidal effect on any of the organisms. The MIC and MBC values are considerably high compared to the MIC interpretative criteria of the conventional antibiotics (CLSI, 2016). These can be attributed to the crude form of the extracts. Further analysis on the isolation of the main bioactive components will give a significantly lowered MIC. In conclusion, the antibacterial; activity of the extract from *B. pinnatum* leaves extracts on the tested pathogenic bacteria confirms the historical use of the plant in the treatment of several infections.

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