Original Research Article

IN VITRO ANTIBACTERIAL EFFICACY OF *Bryophyllum pinnatum* leaf EXTRACTS

ABSTRACT

A study on the antibacterial activities of *Bryophylum pinnatum* against multidrug resistant bacterial pathogens was carried out in this research. Air-dried and powered *Bryophylum pinnatum* leaves was extracted using ethanol and aqueous solvents. Five bacteria strains including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the University of Benin Teaching Hospital and they were preliminarily identified using standard microbiological methods. Antibacterial activity was carried out using agar well diffusion method. Mean zone diameter of inhibition in aqueous extract ranged from 9.20±0.17-10.50±0.50 mm and 9.30±0.33- 10.33±0.89 mm against *Escherichia coli* and *Staphylococcus aureus* respectively at 25-100 mg/ml. In the ethanol extract, mean zone of inhibition ranged from 9.50±0.28-13.33±0.88 mm and 10.67±0.67-19.00±0.58 mm at concentration range of 6.25-100 mg/ml. Minimum inhibitory concentrations of ethanol extract ranged from 6.25-100 mg/ml against bacterial strains. While those of aqueous extract ranged from 25-100 mg/ml against bacterial isolates. Minimum bactericidal/fungicidal concentrations of ethanol extract ranged from 25-50 mg/ml. While in the aqueous extract, value was 50 mg/ml and against bacteria. The test bacterial pathogens were found to possess multiple drug resistance potential with multidrug resistance index ranging from 0.3 – 0.5. This study has shown that multidrug resistant clinical bacterial pathogens are sensitive to aqueous extract of *Bryophyllum pinatum*

Keywords

Antibacterial, antibiotics, inhibition, resistance, pathogen, bacteria.

INTRODUCTION

Bryophyllum pinnatum (*calcynium*) is a medicinal plant belonging to the crassulaceae family. *Bryophyllum pinnatum* has gained extensive recognition for its medicinal properties. The plant *Bryophyllum pinnatum* is frequently known as air plant, love plant, miracle leaf, life plant, Zakham-e-hyat, panfutti and Ghayamari, canterbutury bells, parnabija etc. It is conventionally used as a herbal remedy in approximately all parts of the world (Gupta *et al*., 2010). This plant

Comment [E1]: Use "it" in place of writing the full name of the plant at every line.

widely grows in hot and humid areas, around the dwelling place, along road sides and herbal garden and field. *Bryophyllum pinnatum* plant is widely used in folk medicine and it is easily found in places such as, India, Tropical Africa, Madagascar, China, Australia, Pakistan, Hawaii and Tropical America (Ojewole, 2005). (Okwu, 2006. The active ingredients of most of the commonly used conventional drugs were originally derived from plant part before their pharmaceutical mass production from synthetic chemical (Sofowara, 1993).

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Bryophyllum pinnatum contain appreciable amount of bioactive compounds. Medicinally, the presence of phytochemicals explains the role of this plant leaves in ethnomedicine in Nigeria (Nwali *et al*., 2012). Phytochemical screenings of *Bryophyllum pinnatum* have yielded alkaloids, triterpenes, glycosides, flavonoids, steroids, butadienolides, lipids, and organic acids, Phenol and tannins, free amino acid and terpenoids. Arachidic acid, astragalin, behenic acid, beta amyrin, benzenoids, bersaldegenin, beta-sitsterol, bryophollenone, bryophollone, bryophyllin, caffeic acid, ferulic acid, quercetin, steroids and taraxerol. Despite the progress made in the development of drugs and antimicrobial agents, occurrence of drug resistant microbes and the emergence of unknown disease causing microbes pose an enormous public health concern (Iwu, 1999). This fact has forced scientists to search for new antibiotics/antimicrobial compounds from various sources (Mann *et al*., 2007) such as the medicinal plants to replace those that have become inactive. Traditional medicine uses numerous plants parts for the treatment of respiratory diseases among which is this plant, *Bryophyllum pinnatum* (Okwu, 2005).

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The plant has been found to possess antibacterial activity against several bacterial pathogens including *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* Different solvents such as aqueous, ethanol, methanol and n-hexane have been used for extraction and the respective extracts have shown varying degree of antibacterial actions against selected pathogens (Mudi and Ibrahim, 2008). Irrespective of the researches so far, adequate information on the antibacterial activity of the plant extract is very important. Therefore this study was designed to investigate the antibacterial potency of leaf extract of *Bryophylum pinnatum* against multidrug resistant bacterial pathogens.

MATERIALS AND METHODS

Plant Materials

Bryophylum pinnatum leaves were obtained from Adolor Street in Benin City and identified at the Herbarium, Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State. The leaves were air-dried, ground using sterilized pestle and mortar. The powdered leaf was kept in a sterile bottle container until required.

Preparation of Crude Extracts

Fifty grams (50 g) of the grinded *Bryophylum pinnatum* leaves was soaked in 250 ml each of distilled water and ethanol for 48 hr. The extract was filtered through a sieve with pore size of about 250µm to remove debris. The filtrate was then filtered through membrane filter paper. The final filtrate was evaporated in a water bath at 40°C to get the crude extract. The crude aqueous

Comment [E2]: Is there any report of use of this plant at any "antimicrobial" or related purposes by any community?
If so, you have to add it here.
Every plant may be antimicrobial due to evolutionary reason. That does not mean they are useable medicines.

Comment [E3]: This ic CARDIOTOXIC as per some experiments.

Comment [E4]: It was converted to fine powders by using Pastle and morter?

As the leaves are thick, the air dried leaves cannot be converted to powder by pastle and morter!

Comment [E5]: Without shaking?

Comment [E6]: It is NOT POSSIBLE to evaporate 250 ml of water from the solution at 40^{0}C.
It is even very difficult to do in Vacuum evaporators!

and ethanol extracts were stored at 4°C until required. These were used for antimicrobial analysis (Abdulazeez *et al*., 2014).

Preparation of concentration of plant extract

One gram (1g) each of both ethanol and aqueous extract was added to 10ml of ethanol and distilled water respectively to give a concentration of 100mg/ml. Other concentrations of 50, 25 and 12.5 and 6.25mg/ml were prepared by double dilution method (Aneja, 2003) .

Test microorganisms

Three Gram positive bacteria, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtilis* and two Gram negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*. The microorganisms were obtained from the Microbiology Laboratory stocks in University of Benin Teaching Hospital. The bacteria were then identified in the laboratory based on their cultural, morphological and biochemical characteristics.

Bacteria Inoculum Preparation

The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 hours at 37^0C for the bacteria, After incubation, 0.2 milliliter of the diluted cultures in normal saline was inoculated onto solidified nutrient agar at 45^oC using a Pasteur pipette.

Agar Well Diffusion Technique

The ability of the various extracts to inhibit the growth of the clinical test organisms was determined using the agar well technique. The inoculated nutrient agar plates were allowed to dry. After which, wells were bored on the surface of inoculated agar plates using 4mm cork borer. Zero point two millilitres 0.2ml of the different concentration of each extracts was transferred into the well using Pasteur pipette. The wells were sufficiently spaced to prevent the resulting zones of inhibition from overlapping. The plates were incubated at 37^oC for 24hr. The experiment was performed in triplicate and the resulting zones of inhibition were recorded as mean ± standard error (Jorgensen and Ferraro, 2009).

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

The minimum inhibitory concentration (MIC) of the extracts was determined for each of the test organisms at varying concentrations of 100, 50, 25, 12.5 and 6.25mg/ml. 1ml of various concentrations was added into different test tubes, 1 ml of nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. A tube containing nutrient broth only was seeded with the test organism to serve as control. All the tubes were then incubated at 37^oC for 24 h and then examined for growth by observing for turbidity. The minimum bactericidal concentration (MBC) of the plant extract on the clinical isolates were carried out according to Akinyemi *et al.* (2005). Briefly, 1 ml each of bacterial cultures were pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and subcultured on to nutrient agar. Nutrient agar plates were incubated at 37^oC for 24 h. After incubation the concentration at which there was no single growth of bacteria was taken as MBC (Akinyemi *et al*. (2005)**.**

Antibiotics Susceptibility Testing

Comment [E7]: That means you got the crude extract at SOLID form by only evaporation at 40^0C? Is It possible?

Comment [E8]: How do you come to know that these are "multidrug resistant bacterial pathogens?".

Antimicrobial disc tests of the isolates were performed according to the recommendations of the National Committee Laboratory Standards (NCCLS) using the following antibiotic discs: tetracycline (20µg), ampiclox (30µg), zinnacef (20µg), amoxicillin (30µg), rocephin (25µg), ciprofloxacin (l0µg), Nitrofurantin (20µg), streptomycin (30µg), erythromycin (l0µg), gentamycin (I0µg), septrin (30µg), chloramphenicol (25µg), perfloxacin (l0µg), and ofloxacin (30µg) and antibiotics resistance was interpreted by diameter of inhibition zones around the antibiotic discs (Jorgensen and Ferraro, 2009).

Comment [E9]: ??? Are you using his result?

RESULTS

Table 1: zone of inhibition of aqueous extract of *Bryophyllum pinnatum*(mm) against bacterial isolates

Comment [E10]: Where is the CONTROL?
You are performing the experiment without control?
The susceptibility pattern may be known, but you have to check it in your own system
In such experiment, control should be any antibiotic. Disked impregnated with antibiotics are commercially available. Plain disks may be soaked in liquid antibiotic to prepare control.
But you did nothing!

Test organisms	Concentrations (mg/ml)				
	100	50	25	12.5	6.25
S. pneumonia	9.33±0.33	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B. subtilis	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
P. aeruginosa	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E. coli	10.50±0.50	9.80±0.76	9.2±0.17	0.0±0.0	0.0±0.0
S. aureus	10.33±0.89	10.0±0.29	9.3±0.33	0.0±0.0	0.0±0.0

The zones of inhibition (mm) of aqueous extract of *Brophyllum pinnatum* against bacterial isolates is shown on table 1. No antimicrobial activity of aqueous extract against *Bacillus subtilis* and *Pseudomonas aeruginosa* while a low antibacterial activity was observed against *Streptococcus pneumoniae* (100mg/ml). High antibacterial activity was observed against *Escherichia coli* and *Staphylococcus aureus* at concentration of 25mg/ml.

Table 2: zone of inhibition of ethanolic extract of *Bryophyllum pinnatum* (mm) against bacterial isolates

Comment [E11]: Where is the CONTROL?
The susceptibility pattern may be known, but you have to check it in your own system.
You are performing the experiment without control?
In such experiment, control should be any antibiotic. Disked impregnated with antibiotics are commercially available. Plain disks may be soaked in liquid antibiotic to prepare control.
But you did nothing!

Comment [E12]: The effects may be due to the Ethyl Alcohol present in the solution.
In such experiments, the diluents (here- Ethyl alcohol) is also tested as another control to exclude the effect of the diluents.

Test organisms	Concentration (mg/ml)				
	100	50	25	12.5	6.25
S. pneumoniae	14.33±0.33	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B. subtilis	12.00±0.58	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
P. aeruginosa	19.33±0.33	16.83±0.44	16.5±1.25	0.0±0.0	0.0±0.0
E. coli	19.00±0.58	15.33±0.33	14.33±0.33	11.33±0.33	10.67±0.67
S. aureus	13.33±0.88	12.50±0.29	11.33±0.33	10.83±0.44	9.50±0.28

Antibacterial activity of the ethanolic extract of *B. pinnatum* on the bacteria isolates is shown in table 2 with the lowest activity observed against *Streptococcus pneumoniae* and *Bacillus subtilis* at 100mg/ml. A slightly higher antimicrobial activity was observed on *Pseudomonas aeruginosa* at 25mg/ml while the highest was observed on *Escherichia coli* and *Staphylococcus aureus* at 6.25mg/ml.

Table 3: Minimum inhibitory concentration and Minimum bactericidal concentration of ethanolic and aqueous extract of *Bryophylum pinnatum*

Test organisms	MIC(mg/ml)		MBC (mg/ml)	
	Ethanol	Aqueous	Ethanol	Aqueous
E. coli	6.25	25	25	50
S. aureus	6.25	25	25	50
P. aeruginosa	25	ND	50	ND
B. subtilis	100	ND	ND	ND
S. pneumonia	100	100	ND	ND

Comment [E13]: VERY MUCH DOUBTFUL RESULT!
If any extract fail to show any noticeable effect at agar well test, HOW can it show effects in MIC tests?
It required 100 mg/ml to show a little effect in Well diffusion and having MIC at 25 mg/ml concentration?

KEY
ND- Not determined

The minimum inhibitory concentration (MIC) of ethanolic extract against bacterial isolates is shown in table 3 and they ranged from 6.25-100mg/ml while that of aqueous extract ranged from 25-100mg/ml. Minimum bactericidal concentration (MBC) of ethanolic ranged from 25-50mg/ml and that of aqueous extract was 50mg/ml.

Table 7: Antibiotic susceptibility pattern of bacterial isolates

Comment [E14]: What is the source of these data?
Simple name of the university is not acceptable.
You have to add detail regarding such data.

Gram +ve	**CPX**	**St**	**SXT**	**E**	**PEF**	**CN**	**APX**	**Z**	**AM**	**Ro**	**MDR**
Streptococcus. Pneumoniae	S	S	S	R	S	R	R	S	R	S	0.4
Bacillus subtilis	S	R	S	S	R	S	S	S	R	S	0.3
Staphylococcus aureus	R	S	S	R	S	S	R	S	R	S	0.4
Gram –ve	**CH**	**SP**	**AU**	**OFX**	**SXT**	**PEF**	**AM**	**St**	**CN**	**CPX**	
Pseudomonas aeruginosa	R	S	R	S	R	S	R	S	S	S	0.4
Escherichia coli	S	R	R	R	S	R	S	S	R	S	0.5

KEY: CPX-Ciprofloxacin, Ro-Rocephin, St-Streptomycin, AU-Augmentin, SXT-Septrin, SP-Sparfloxacin, E-Erythromycin, CH-Chloramphenicol, PEF-Pefloxacin, CPX- ciprofloxacin,

CN-Gentamicin, APX-Apmpiclox, AM-Amoxacillin, Z-Zinnace.

DISCUSSION

The antibacterial properties of plants in general have been attributed to the presence of phytochemicals such as flavonoid, alkaloids, tannins, saponins and terpenes, in plants. Flavonoids are known to be synthesized by plants in response to microbial attack. Their activity is probably due to their ability to react with extracellular and soluble proteins and to complex with bacterial cell walls leading to the death of the bacterium (Okwu and Nnamdi, 2011). Tannins are also reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phyto-therapeutically, tannin containing plants are used to treat non-specific diarrhoea, inflammations of mouth and throat and slightly injured skins (Mudi and Ibrahim, 2008)). This study revealed moderate *in vitro* antibacterial activity against test bacterial isolates at higher concentrations while at lower concentrations ranging from 25.0 to 6.25mg/ml, no inhibition zone was observed. The test bacterial isolates exhibited variation in their susceptibility to *B. pinnatum* extract. The lower susceptibility observed at lower concentrations could be due to inability of the extract to permeate the cell wall of the organisms or possession of drug inactivating enzymes mediated by plasmid or chromosomes on the bacterium.

Minimal antibacterial activity was observed against bacterial isolates in the aqueous extract. Mean zone diameter of inhibition ranged from 9.20±0.17-10.50±0.50mm and 9.30±0.33-10.33±0.89mm against *Escherichia coli* and *Staphylococcus aureus* respectively at 25-100mg/ml. At lower concentrations, there were no zones of inhibition recorded.

In the ethanol extract, mean zone of inhibition ranged from 9.50±0.28-13.33±0.88mm and 10.67±0.67-19.00±0.58mm at concentration range of 6.25-100mg/ml. Higher antibacterial activities were observed at higher concentration compared to lower concentrations of the ethanol extract. It was observed that the antibacterial activity of the plant extract was dependent on the solvent used for extraction and also on the concentration of the extract used. Plants have been reported to be vast repertoire of bioactive phytochemical compound. These compounds which include flavonoids, alkaloids, tannins etc., are usually responsible for the various biologic properties of the plant, including antimicrobial and other medicinal properties. It has been reported that organic solvent such as ethanol, usually extract more of the bioactive phytochemical component of the plant compared to aqueous solvent, hence the reason for higher antibacterial activity in the ethanolic fraction of the leaf extract (Ufelle *et al*., 2011).

Minimum inhibitory concentrations of ethanol extract ranged from 6.25- 50mg/ml against bacteria. While those of aqueous extract ranged from 25-100 mg/ml against bacteria. Minimum bactericidal concentrations of ethanol extract ranged from 25-50 mg/ml. While in the aqueous extract, value was 50mg/ml.

Antibiotics sensitivity of the bacterial isolates revealed multidrug resistance of the bacterial pathogens. Escherichia coli had the highest multidrug resistance index (0.5) while Bacillus subtilis had the lowest (0.3).

Conclusion

This work has shown that *Bryophyllum pinnatum* ethanol and aqueous extracts have potent antimicrobial activities against multidrug resistant clinical bacterial isolates. The antibacterial activity was observed to be dependent on the solvent for extraction and concentration of the extract used.

Comment [E15]:

Comment [E16]: Where it is proved? Not in your experiment, I think.

Competing Interests

All authors have declared that no competing interests exist.

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