

IN VITRO ANTIBACTERIAL EFFICACY OF *Bryophyllum pinnatum* leaf EXTRACTS

ABSTRACT

A study on the antibacterial activities of *Bryophyllum pinnatum* against multidrug resistant bacterial pathogens was carried out in this research. Air-dried and powdered *Bryophyllum 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 (*calcyonium*) is a medicinal plant belonging to the crassulaceae family. It has gained extensive recognition for its medicinal properties. It is frequently known as air plant, love plant, miracle leaf, life plant, Zakhm-e-hyat, panfutti and Ghayamari, canterbutury bells, parnabija etc. It is conventionally used as an herbal remedy in approximately all parts of the world (Gupta *et al.*, 2010). This plant widely grows in hot and humid areas, around the dwelling

32 place, along road sides and herbal garden and field. *Bryophyllum pinnatum* plant is widely used
33 in folk medicine and it is easily found in places such as, India, Tropical Africa, Madagascar,
34 China, Australia, Pakistan, Hawaii and Tropical America (Ojewole, 2005). (Okwu, 2006. The
35 active ingredients of most of the commonly used conventional drugs were originally derived
36 from plant part before their pharmaceutical mass production from synthetic chemical (Sofowara,
37 1993). The plant is has been used as an herbal remedy to treat infections by many people in
38 different parts of the world including many African countries (Gupta *et al.*, 2010).
39 *Bryophyllum pinnatum* contain appreciable amount of bioactive compounds. Medicinally, the
40 presence of phytochemicals explains the role of this plant leaves in ethnomedicine in Nigeria
41 (Nwali *et al.*, 2012). Phytochemical screenings of *Bryophyllum pinnatum* have yielded alkaloids,
42 triterpenes, glycosides, flavonoids, steroids, butadienolides, lipids, and organic acids, Phenol and
43 tannins, free amino acid and terpenoids. Arachidic acid, astragalin, behenic acid, beta amyirin,
44 benzenoids, bersaldegenin, beta-sitsterol, bryophollenone, bryophollone, bryophyllin, caffeic
45 acid, ferulic acid, quercetin, steroids and taraxerol. Despite the progress made in the
46 development of drugs and antimicrobial agents, occurrence of drug resistant microbes and the
47 emergence of unknown disease causing microbes pose an enormous public health concern (Iwu,
48 1999). This fact has forced scientists to search for new antibiotics/antimicrobial compounds from
49 various sources (Mann *et al.*, 2007) such as the medicinal plants to replace those that have
50 become inactive. Traditional medicine uses numerous plants parts for the treatment of respiratory
51 diseases among which is this plant, *Bryophyllum pinnatum* (Okwu, 2005).
52 The plant has been found to possess antibacterial activity against several bacterial pathogens
53 including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Different
54 solvents such as aqueous, ethanol, methanol and n-hexane have been used for extraction and the
55 respective extracts have shown varying degree of antibacterial actions against selected pathogens
56 (Mudi and Ibrahim, 2008). Irrespective of the researches so far, adequate information on the
57 antibacterial activity of the plant extract is very important. Therefore this study was designed to
58 investigate the antibacterial potency of leaf extract of *Bryophyllum pinnatum* against multidrug
59 resistant bacterial pathogens.

60 MATERIALS AND METHODS

61 Plant Materials

62 *Bryophyllum pinnatum* leaves were obtained from Adolor Street in Benin City and identified at
63 the Herbarium, Department of Plant Biology and Biotechnology, University of Benin, Benin
64 City, Edo State. The leaves were air-dried, macerated using sterilized laboratory blender. The
65 powdered plant material was kept in a sterile bottle container until required.

66 Preparation of Crude Extracts

67 Fifty grams (50 g) of the grinded *Bryophyllum pinnatum* leaves was soaked in 250 ml each of
68 distilled water and ethanol (95%) for 48 hr with shaking. The extract was filtered through a sieve
69 with pore size of about 250 μ m to remove debris. The filtrate was then filtered through membrane
70 filter paper. The final filtrate was evaporated in a water bath at 40°C to get the crude extract.
71 During evaporation, batch evaporation was carried out, with small volume of the filtrate added to
72 evaporation dish. This made possible effective evaporation. The crude aqueous and ethanol

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

75 **Preparation of concentration of plant extract**

76 One gram (1g) each of both ethanol and aqueous extract (that of aqueous extract was jelly-like)
77 was separately added to 10ml distilled water in different sterile test tubes to give a concentration
78 of 100mg/ml. Other concentrations of 50, 25 and 12.5 and 6.25mg/ml were prepared by double
79 dilution method (Aneja, 2003).

80 **Test microorganisms**

81 Three Gram positive bacteria, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus*
82 *subtilis* and two Gram negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*. The
83 microorganisms were obtained from the Microbiology Laboratory stocks in University of Benin
84 Teaching Hospital. The bacteria were then identified in the Microbiology laboratory, University
85 of Benin, Benin City, based on their cultural, morphological and biochemical characteristics. The
86 multidrug resistance ability of the bacteria was also assayed. The reagents and chemicals used
87 were sourced from the school laboratory.

88 **Bacteria Inoculum Preparation**

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

92 **Agar Well Diffusion Technique**

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

101 **Determination of Minimum Inhibitory Concentration (MIC) and Minimum 102 Bacteriocidal Concentration (MBC)**

103 The minimum inhibitory concentration (MIC) of the extracts was determined for each of the test
104 organisms at varying concentrations of 100, 50, 25, 12.5 and 6.25mg/ml. 1ml of various
105 concentrations was added into different test tubes, 1 ml of nutrient broth was added and then a
106 loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was
107 introduced to the tubes. A tube containing nutrient broth only was seeded with the test organism
108 to serve as control. All the tubes were then incubated at 37°C for 24 h and then examined for
109 growth by observing for turbidity. The minimum bacteriocidal concentration (MBC) of the plant
110 extract on the clinical isolates were carried out according to Akinyemi *et al.* (2005). Briefly, 1 ml
111 each of bacterial cultures were pipetted from the mixture obtained in the determination of MIC
112 tubes which did not show any growth and subcultured on to nutrient agar. Nutrient agar plates

113 were incubated at 37°C for 24 h. After incubation the concentration at which there was no single
 114 growth of bacteria was taken as MBC (Akinyemi *et al.* (2005).

115 **Antibiotics Susceptibility Testing**

116 Antimicrobial disc tests of the isolates were performed according to the recommendations of
 117 the National Committee Laboratory Standards (NCCLS) using the following antibiotic discs:
 118 tetracycline (20µg), ampiclox (30µg), zinnacef (20µg), amoxicillin (30µg), rocephin (25µg),
 119 ciprofloxacin (10µg), Nitrofurantin (20µg), streptomycin (30µg), erythromycin (10µg),
 120 gentamycin (10µg), septrin (30µg), chloramphenicol (25µg), perfloxacin (10µg), and ofloxacin
 121 (30µg) and antibiotics resistance was interpreted by diameter of inhibition zones around
 122 the antibiotic discs (Jorgensen and Ferraro, 2009).

123 **RESULTS**

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

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

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

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

Test organisms	Concentration (mg/ml)				
	100	50	25	12.5	6.25
<i>S. pneumoniae</i>	14.33±0.33	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>B. subtilis</i>	12.00±0.58	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
<i>P. aeruginosa</i>	19.33±0.33	16.83±0.44	16.5±1.25	0.0±0.0	0.0±0.0
<i>E. coli</i>	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

133 Antibacterial activity of the ethanolic extract of *B. pinnatum* on the bacteria isolates is shown in
 134 table 2 with the lowest activity observed against *Streptococcus pneumoniae* and *Bacillus subtilis*
 135 at 100mg/ml. A slightly higher antimicrobial activity was observed on *Pseudomonas aeruginosa*
 136 at 25mg/ml while the highest was observed on *Escherichia coli* and *Staphylococcus aureus* at
 137 6.25mg/ml.
 138

139 Table 3: Minimum inhibitory concentration and Minimum bactericidal concentration of ethanolic
 140 and aqueous extract of *Bryophyllum pinnatum*

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

141 KEY

142 ND- Not determined

143 The minimum inhibitory concentration (MIC) of ethanolic extract against bacterial isolates is
 144 shown in table 3 and they ranged from 6.25-100mg/ml while that of aqueous extract ranged from
 145 25-100mg/ml. In the aqueous extract, there were no MIC determined against *P. aeruginosa* and
 146 *B. subtilis*. Minimum bactericidal concentration (MBC) of ethanolic ranged from 25-50mg/ml
 147 and that of aqueous extract was 50mg/ml. There were no MBC determined against *P.*
 148 *aeruginosa*, *B. subtilis* and *S. pneumonia* in the aqueous extract while for the ethanol extract, no
 149 MBC was determined against *B. subtilis* and *S. pneumonia*

150

151

152 Table 7: Antibiotic susceptibility pattern of bacterial isolates

Gram +ve	CPX	St	SXT	E	PEF	CN	APX	Z	AM	Ro	MDR
<i>Streptococcus pneumoniae</i>	S	S	S	R	S	R	R	S	R	S	0.4
<i>Bacillus subtilis</i>	S	R	S	S	R	S	S	S	R	S	0.3
<i>Staphylococcus aureus</i>	R	S	S	R	S	S	R	S	R	S	0.4
Gram -ve	CH	SP	AU	OFX	SXT	PEF	AM	St	CN	CPX	

<i>Pseudomonas aeruginosa</i>	R	S	R	S	R	S	R	S	S	S	0.4
<i>Escherichia coli</i>	S	R	R	R	S	R	S	S	R	S	0.5

153 **KEY:** CPX-Ciprofloxacin, Ro-Rocephin, St-Streptomycin, AU-Augmentin, SXT-Septin, SP-
 154 Sparfloxacin, E-Erythromycin, CH-Chloramphenicol, PEF-Pefloxacin, CPX- ciprofloxacin,
 155 CN-Gentamicin, APX-Apmpiclox, AM-Amoxacillin, Z-Zinnace.

156 DISCUSSION

157 The antibacterial properties of plants in general have been attributed to the presence of
 158 phytochemicals such as flavonoid, alkaloids, tannins, saponins and terpenes, in plants.
 159 Flavonoids are known to be synthesized by plants in response to microbial attack. Their activity
 160 is probably due to their ability to react with extracellular and soluble proteins and to complex
 161 with bacterial cell walls leading to the death of the bacterium (Okwu and Nnamdi, 2011).
 162 Tannins are also reported to have various physiological effects like anti-irritant, antisecretolytic,
 163 antiphlogistic, antimicrobial and antiparasitic effects. Phyto-therapeutically, tannin containing
 164 plants are used to treat non-specific diarrhoea, inflammations of mouth and throat and slightly
 165 injured skins (Mudi and Ibrahim, 2008)). This study revealed moderate *in vitro* antibacterial
 166 activity against test bacterial isolates at higher concentrations while at lower concentrations
 167 ranging from 25.0 to 6.25mg/ml, no inhibition zone was observed. The test bacterial isolates
 168 exhibited variation in their susceptibility to *B. pinnatum* extract. The lower susceptibility
 169 observed at lower concentrations could be due to inability of the extract to permeate the cell wall
 170 of the organisms or possession of drug inactivating enzymes mediated by plasmid or
 171 chromosomes on the bacterium.
 172 Minimal antibacterial activity was observed against bacterial isolates in the aqueous extract.
 173 Mean zone diameter of inhibition ranged from 9.20±0.17-10.50±0.50mm and 9.30±0.33-
 174 10.33±0.89mm against *Escherichia coli* and *Staphylococcus aureus* respectively at 25-
 175 100mg/ml. At lower concentrations, there were no zones of inhibition recorded.

176 In the ethanol extract, mean zone of inhibition ranged from 9.50±0.28-13.33±0.88mm and
 177 10.67±0.67-19.00±0.58mm at concentration range of 6.25-100mg/ml. Higher antibacterial
 178 activities were observed at higher concentration compared to lower concentrations of the ethanol
 179 extract. It was observed that the antibacterial activity of the plant extract was dependent on the
 180 solvent used for extraction and also on the concentration of the extract used. Plants have been
 181 reported to be vast repertoire of bioactive phytochemical compound. These compounds which
 182 include flavonoids, alkaloids, tannins etc., are usually responsible for the various biologic
 183 properties of the plant, including antimicrobial and other medicinal properties. It has been
 184 reported that organic solvent such as ethanol, usually extract more of the bioactive
 185 phytochemical component of the plant compared to aqueous solvent, hence the reason for higher
 186 antibacterial activity in the ethanolic fraction of the leaf extract (Ufelle *et al.*, 2011).

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

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

194 **Conclusion**

195 This work has shown that *Bryophyllum pinnatum* ethanol extract has potent antibacterial
196 activities against multidrug resistant clinical bacterial isolates while the aqueous extract has low
197 to moderate activity. The antibacterial activity was observed to be dependent on the solvent for
198 extraction and concentration of the extract used. Therefore this plant can be incorporated into
199 medicine for phyto-therapeutic purposes

200

201 **Competing Interests**

202 All authors have declared that no competing interests exist.

203

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