

Antibacterial Activities of the Leaf Extracts of *Bryophyllum Pinnatum* (African Never Die Flower) on Pathogenic Bacteria Isolated from Cow Dung

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Abstract- The cost of orthodox drugs and incidence of antibiotic resistance among bacteria has inspired scientists to search for natural alternatives like plants extracts as they are safer in biological system. The leaf extracts of *Bryophyllum pinnatum* has been used in folklore medicine in the treatment of varieties of diseases in Nigeria, India and China. Ethanol and methanol extracts of leaf of *B.pinnatum* were analyzed and their antibacterial activities were also tested against pathogenic bacteria isolated from cow dung. The six pathogenic bacteria isolated were identified as *Salmonella entrica*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibro cholerae* and *E.coli*. The result showed that the ethanol and methanol extracts have antibacterial properties. The pathogenicity of the isolates was studied by infecting the mice with them. There were death of two mice infected with *Pseudomonas aeruginosa* and *Vibro cholerae*. Other mice in the same group with them were asymptomatic carriers. For the mice infected with *Salmonella entrica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *E.coli*, *Staphylococcus aureus* and *Vibro cholerae*, 50×10^8 , 20×10^8 , 25×10^8 , 10×10^8 , 10×10^8 , 2×10^8 cfu/ml of the infected organisms were recovered from the intestine respectively. The bacterial load in the intestine reduced drastically after the ethanol and methanol treatment.

Index Terms- Antibacterial , *Bryophyllum pinnatum*, cow dung, pathogenic bacteria

I. INTRODUCTION

Presence of chemical and medicinal contents in natural form, plants and herbal medicines have important position in modern medicine. They contain various secondary metabolites which work together and show wide range of antibacterial activities. Microorganisms may get mutated and become resistant to many antibiotics and so generate a global health problem. These inspired scientists to search for new natural alternative to treat diseases (Kamboj, 2009). Infectious diseases caused by resistant microorganisms are associated with prolonged hospitalization, increased cost and greater risk of morbidity and mortality. Resistance is an especially vexing problem for people with impaired immune systems, such as AIDS, cancer patients and recipients of organ transplants.

The resistance problem demands that a renewed effort be made to screen various medicinal plants for their potential antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body. Medicinal plants are relied upon

by 80% of the world's population and in India there is a rich tradition of using herbal medicine for the treatment of various infectious diseases, inflammations and injuries. Many of the plant materials used in traditional medicine have proven more effective and relatively cheaper than modern medicine (Mann,2008) against certain ailments while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu,1999).

Bryophyllum pinnatum is an environmental weed from the family Crassulaceae that is most often used to treat urinary stones, hypertension, cold, abscesses, asthma, insect bite, skin disorders and other ailments. It is a succulent glabrous herb that is 0.3 m-1.2 m high and is native to Madagascar and Southern Africa and grows mainly in the tropics (Nagaratna, 2015). It is a widely distributed perennial medicinal herb and a popular houseplant.

The plant is locally called "African Never Die" in Nigeria and is very popular in folklore medicine. It has been used for the treatment of a variety of conditions in tropical America, India, China, Australia and Africa, including rheumatism, body pain, arthritis, heartburn, skin ulcers, peptic ulcer, diabetes mellitus, microbial infections and hypertension (Chopra,2002).

Studies have also reported a wide range of active phytochemicals such as alkaloids, triterpenes, glycosides, flavonoids, steroids, bufadienolides, lipids and organic acids (Ojewole,2005). These compounds have been considered to be responsible for the plant's diverse pharmacological activities.

Due to the increase in the incidence of antibiotic resistance which has become a public concern, newer and safer antibiotics need to be introduced of which some plants have been found to be effective, therefore this study was to determine the antibacterial activities of extracts of *Bryophyllum pinnatum* on some pathogenic bacteria isolated from cow dung.

II. MATERIALS AND METHODS

1. Collection of Plant Materials and Cow Dung Sample



Fig 1: *Bryophyllum pinnatum* (Lam.) bred at Omagba in Onitsha, Anambra State

The plant specimen (*Bryophyllum pinnatum*) was collected from Ogidi in Idemili North Local Government Area, Anambra State and transplanted in Omagba in Onitsha North Local Government Area, Anambra State. The plant Specimen was identified by Dr. B. O. Aziagba a Plant Taxonomist in the Department of Botany, Faculty of Biosciences, Nnamdi Azikiwe University Awka. A voucher number NO NAUH-172A was given to the specimen and thereafter deposited at the Herbarium in the Botany Department. The leaves samples were collected on 1st December 2019. The fresh cow dung samples were collected from twenty (20) different cows in Umunya Abattoir at Anambra State using sterile spoons and bottles and stored inside refrigerator for future use.

1. Sample Processing

Leaves of the plant were washed in running water and dried in shade at room temperature for a period of two weeks. The

dried leaves were blended differently using a sterile electric blender (rinsed with absolute alcohol) to coarse powder and stored in air- tight bottle at room temperature.

2. Preparation of Extracts

The methanol and ethanol extracts were prepared according to the method described by Karabi (2015) and Ekpe (1990). It was prepared in a ratio of 1:2 (10 g of the fine powder of each plant added to 20 ml of methanol and ethanol each). The mixture was allowed to stand at room temperature for 4 hours with occasional stirring.

The mixture was filtered separately using Whatman No 1 filter paper to remove the residues. The filtrate was allowed to settle for 30 minutes and left for two days for the ethanol and methanol content to evaporate at room temperature. The concentrated aqueous, ethanol and methanol extracts were stored at 5°C in the refrigerator for use.

3. Isolation of Bacterial Species

Escherichia spp

The isolation of *Escherichia* spp was done as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of peptone water and allowed to stand for 18hrs of room temperature. The broth culture was then plated out on Eosine Methylene Blue agar using pour plate method and incubated for 24hrs. Green metallic sheen colonies were isolated and sub cultured into MacConkey agar to get pure culture. Discrete pinkish colonies, that developed after incubation for 24hrs at room temperature were isolated and identified.

Salmonella spp

This was done as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of peptone water and allowed to stand for 18hrs of room temperature. The broth culture was than plated out on SS agar using pour plate method and incubated for 24hrs. Blackish colonies that developed after 24hrs of incubation at room temperature were isolated and purified by repeated streaking on fresh *Salmonella-Shigella* agar plates and incubated at room temperature for 24hrs. The isolates were thereafter identified.

Proteus spp

The isolation of *Proteus* spp was carried out as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of peptone water and allowed to stand for 18h of room temperature. The broth culture was than plated out on Nutrient agar using pour plate method and incubated for 24hrs. Greyish white swarming colonies that developed after 24hrs of incubation at room temperature were isolated and purified by repeated streaking on fresh Nutrient agar plates and incubation at room temperature for 24hrs. The isolates were thereafter identified.

Staphylococcus spp

The isolation of *Staphylococcus* spp was carried out as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of sterile peptone water, and allowed to stand for 18hrs at room temperature. The broth culture was plated out on Mannitol salt agar using pour plate method and incubated for 24hrs. The plates were incubated at room temperature for 24hrs. White to deep yellow colonies that developed on the plates were isolated and sub-cultured to obtain pure colonies which were later identified.

Vibrio Cholerae

The isolation of *Vibrio* spp was done as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of sterile peptone water and allowed to stand for 18h at room temperature. The broth culture was thereafter plated out on Thiosulphate-Citrate-Bile salt-Sucrose agar using pour plate method and incubated for 24hrs. The plates were incubated aerobically at room temperature for 24hrs. Yellow colonies that developed were isolated and sub cultured to Twenty-five grammes obtained pure culture. The isolates were later identified.

Pseudomonas spp

The isolation of *Pseudomonas* spp was performed as described by Ogbo (2005). Twenty-five grams of the cow dung was dissolved in 225ml of sterile peptone water and allowed to stand for 18h at room temperature. The broth culture was then plated out on Cetrimide agar using pour plate method and incubated for 24hrs. The plates were incubated aerobically at room temperature for 24hrs. The creamy to yellow colonies that developed were isolated and the isolates were there after identified.

Determination of in vivo Pathogenicity of the Bacterial Isolates

Laboratory Animal

Twenty-eight (28) albino mice (male) weighing between 30 and 33g, bred in Chris poultry farm Awka were used. They were housed in seven (7) different metal cages and fed prior to infection. The animals were housed in seven groups of four (4) each. The experimental groups were labelled A, B, C, D, E, F, and control group G.

The animals were allowed to acclimatize for seven (7) days before the experiment and were fed with a standardized balance of commercial pellet diet and potable water as described by OECD (2001). The inoculum from each isolate was prepared by inoculating each isolate in the 50ml of sterile Peptone water in the 100ml of flask and incubated at 37°C in the shaker for 48hrs to get their optimum growth. Then after, the broth cultures were centrifuged at r.p.m for 10mins to obtain their pellets. The pellets were washed with 2ml of sterile saline. 0.1ml of the inoculum from each isolate was given to each mouse in each group orally and respectively as

done by Zwijnenburg et al. (2001). That is, each group were given *E.coli*, *V.cholerae*, *Salmonella enterica*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and potable water respectively.

The four mice at control group G were given potable water. The mice were fed and observed for pathological signs for 14days. At the end of 14 days, the survived mice were sacrificed and their intestine, lungs, livers were harvested. 2g of each intestine was weighed and ground in 2ml of saline with mortar and pestle. The numbers of the infecting organisms in the intestine were determined by plating after serial dilution.

The organs were also homogenized in 1ml of sterile distilled water. The homogenated organs were serially diluted using ten-fold dilution with sterile water and the numbers of organisms were determined by plating. The biochemical test results of the isolates were used as the marker to note whether the organisms injected were the ones isolated from the organs. All animal experiments were approved by the Animal Research Ethics Committee of the Nnamdi Azikiwe University, Awka in accordance to the guide for care and use of laboratory animals

Determination of in Vivo Effectiveness of the Plant Extracts

This was carried out as described by Mabeku et al. (2007). Two mice from each of the seven (7) groups namely A, B, C, D, E, F and G that were not sacrificed were used for this analysis. The two (2) most effective extracts (ethanol and methanol extracts) obtained after the in vitro antibacterial determination of plants extracts was used.

The two mice from each group were given 0.2ml of the extracts daily. That is, one (1) mouse from each group was given 0.2ml of the ethanol extract and the other 0.2ml of the methanol extract. This was done in the same way to all the other mice in the other groups. The mice were fed and observed for 7days. At the end of 7days, the mice were sacrificed and their intestines were harvested. 2g of each intestine was weighed and ground in 2ml of saline with mortar and pestle. The number of the organisms that survived in the intestine if any was determined by plating after serial dilution.

Results

Isolation, characterization and identification of Pathogens in cow dung Potentially pathogenic bacteria were isolated from the cow dung and they were Gram-positive and Gram-negative bacteria. The isolates were characterized and identified using cultural, morphological, biochemical and molecular tests as members of the genera *Salmonella*, *Proteus*, *Vibrio*, *Staphylococcus*, *Escherichia* and *Pseudomonas* as shown in Table 1 The Pathogenicity test of the Isolates

The invivo pathogenicity of the isolates that was carried out using mice. After the oral inoculation of mice, they were fed and observed for pathological sign for 14days. There was death of two mice which were infected with Pseudomonas aeruginosa and Vibrio cholerae.

Others in the same group with them were asymptomatic carriers because they showed no symptoms of disease, but they shed the organisms in their faeces. Bacterial numbers recovered from the liver, kidney, lung and intestine of the infected mice are shown in Table 4. More number of the organisms was recovered from the intestine.

Table 1: Morphological and biochemical characteristics of the isolates

Colour of the colonies	Pinkish on MA & green metallic		Black on SS agaro		Black with pink end on SS		Organisms	
	Short rod	-	Short rod	-	Short rod	-		
Shane	-	-	-	-	-	-	<i>Escherichia coli</i>	
Gram stain	+	-	-	-	+	+		
Indole	-	-	-	-	-	-		
Voges-Proskaver test	-	-	-	-	+	-		
Methyl red test	+	+	+	+	+	+		
Citrate	-	-	-	-	+	-		
Motility	+	+	+	+	+	+		
I rease	-	-	-	-	-	-		
Coagulase test	-	-	-	-	-	-		
Oxidase test	-	-	-	-	-	-		
Catalase test	+	+	+	+	+	+		
Snoore stain	-	-	-	-	-	-		
Glucose	+	+	+	+	+	+		
Sorbitol	+	+	+	+	+	+		
Mannitol	+	+	+	+	+	+		
Sucrose	+	+	+	+	+	+		
Lactose	+	+	+	+	+	+		
Maltose	+	+	+	+	+	+		
Raffinose	+	+	+	+	+	+	<i>Salmonella enterica</i>	
Sugar fermentation								
Glucose	+	+	+	+	+	+		
Sorbitol	+	+	+	+	+	+		
Mannitol	+	+	+	+	+	+		
Sucrose	+	+	+	+	+	+		
Lactose	+	+	+	+	+	+		
Maltose	+	+	+	+	+	+		
Raffinose	+	+	+	+	+	+		
Organisms								
Shane	-	-	-	-	-	-		<i>Proteus mirabilis</i>
Gram stain	-	-	-	-	-	-		
Indole	-	-	-	-	-	-		
Voges-Proskaver test	-	-	-	-	-	-		
Methyl red test	+	+	+	+	+	+		
Citrate	-	-	-	-	-	-		
Motility	+	+	+	+	+	+		
I rease	-	-	-	-	-	-		
Coagulase test	-	-	-	-	-	-		
Oxidase test	-	-	-	-	-	-		
Catalase test	+	+	+	+	+	+		
Snoore stain	-	-	-	-	-	-		
Glucose	+	+	+	+	+	+		
Sorbitol	+	+	+	+	+	+		
Mannitol	+	+	+	+	+	+		
Sucrose	+	+	+	+	+	+		
Lactose	+	+	+	+	+	+		
Maltose	+	+	+	+	+	+		
Raffinose	+	+	+	+	+	+		

Colour of the colonies	Yellow on TCBS	Pinkish on EMB	White to deep yellow on MSA	Sugar fermentation							Organisms	
				Glucose	Sorbitol	Mannitol	Sucrose	Lactose	Maltose	Raffinose		
Shane	Curved rod	Rod	Cocci	+	-	-	-	-	-	-	-	<i>Staphylococcus aureus</i>
Gram stain	-	-	-	-	-	-	-	-	-	-	-	
Indole	+	-	-	-	-	-	-	-	-	-	-	
Voges-Proskaver test	+	-	-	-	-	-	-	-	-	-	-	
Methyl red test	-	+	+	+	+	+	+	+	+	+	+	
Citrate	+	+	+	+	+	+	+	+	+	+	+	
Motility	+	+	+	+	+	+	+	+	+	+	+	
I rease	-	-	-	-	-	-	-	-	-	-	-	
Coagulase test	-	-	-	-	-	-	-	-	-	-	-	
Oxidase test	+	+	+	+	+	+	+	+	+	+	+	
Catalase test	+	+	+	+	+	+	+	+	+	+	+	
Snoore stain	-	-	-	-	-	-	-	-	-	-	-	
Glucose	+	+	+	+	+	+	+	+	+	+	+	
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	
Mannitol	+	+	+	+	+	+	+	+	+	+	+	
Sucrose	-	-	-	-	-	-	-	-	-	-	-	
Lactose	-	-	-	-	-	-	-	-	-	-	-	
Maltose	-	-	-	-	-	-	-	-	-	-	-	
Raffinose	-	-	-	-	-	-	-	-	-	-	-	
Organisms											<i>V. cholerae</i>	
Organisms											<i>Pseudomonas</i>	

Key to the biochemical results

- + = positive
- = Negative
- ++ = Positive with gas production

Graphical Representation of Bacterial Load Of The Intestine, Lung, Liver And Kidney After Oral Infected Mice With The Bacterial Isolates

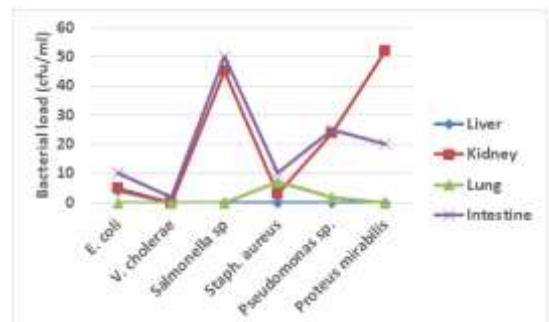


Figure 2: Bacterial load of parts of the mice after oral infected mice with the Bacterial Isolates

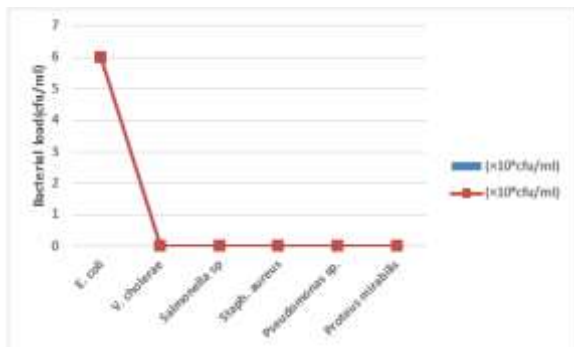


Figure 3: Bacterial load of the liver of the mice after treatment with methanol

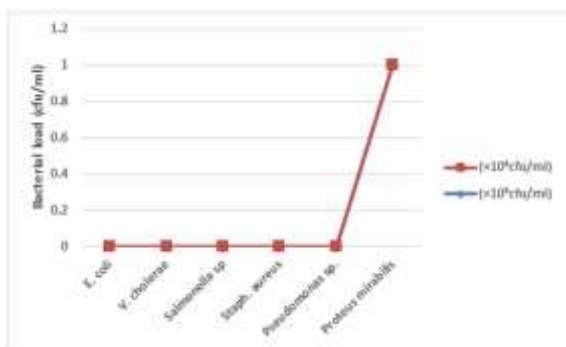


Figure 4: Bacterial load of the kidney of the mice after treatment with ethanol extracts

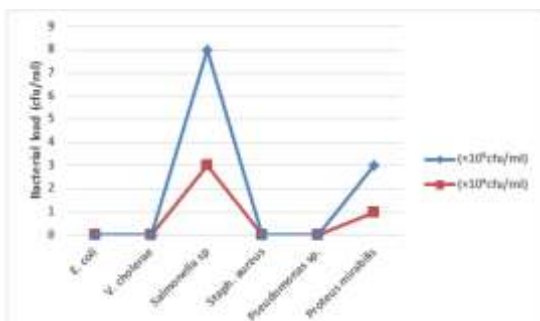


Figure 5: Bacterial load of the intestine of the mice after treatment with ethanol extracts

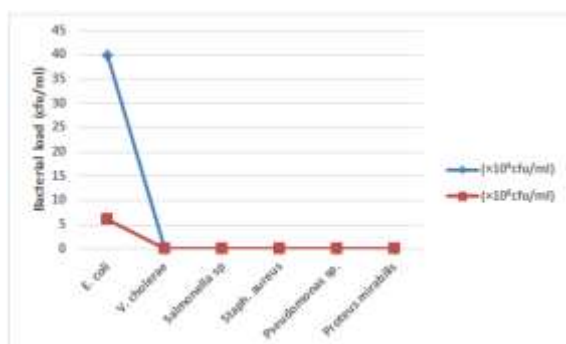


Figure 6: Bacterial load of the intestine of the mice after treatment with methanol extracts

III. DISCUSSION

Six human pathogens were isolated in cow dung collected from twenty different cows at Umunya abattoirs in Anambra State. The isolates were characterized and identified as *Salmonella enterica*, *Proteus mirabilis*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Table 1). The plant which was named by a Botanist as *Bryophyllum pinnatum* (Lam.) was collected from Ogidi in Idemili North Local Government Area and bred in Omagba in Onitsha North Local Government Area, both in Anambra State (Figure 1). Ethanol and methanol extracts of leaves of *B. pinnatum* were analyzed for antibacterial activities against the isolates obtained from the cow dung samples. Croxen (2010) reported that fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination. Most available information on STEC relates to serotype O157:H7, since it is easily differentiated biochemically from other *E. coli* strains. The reservoir of this pathogen appears to be mainly cattle. In addition, other ruminants such as sheep, goats, and deer are considered significant reservoirs, while other mammals (such as pigs, horses, rabbits, dogs, and cats) and birds (such as chickens and turkeys) have been found infected.

The antibacterial effectiveness of the extracts of *B. pinnatum* was evaluated using in vivo analysis. The result showed that the extracts of *B. pinnatum* have antimicrobial properties. The infected mice showed high number of the organisms in their intestine. The mice infected with *E. coli*, *Vibrio cholerae*, *Salmonella enterica*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* showed the bacterial load of 10×10^8 cfu/ml, 2×10^8 cfu/ml, 50×10^8 cfu/ml, 10×10^8 cfu/ml, 25×10^8 cfu/ml and 20×10^8 cfu/ml respectively in their intestine (Fig 2). When treated with ethanol and methanol extracts, the numbers of the organisms in the intestine reduce drastically to 0×10^6 cfu/ml, 0×10^6 cfu/ml, 3×10^6 cfu/ml, 0×10^6 cfu/ml, 0×10^6 cfu/ml and 1×10^6 cfu/ml respectively for ethanol treatment. And the 6×10^6 cfu/ml, 0×10^6 cfu/ml, 0×10^6 cfu/ml, 0×10^6 cfu/ml, 0×10^6 cfu/ml and 0×10^6 cfu/ml respectively for methanol treatment (Fig 3-6). These reduction in the bacterial load showed that the leaves extracts (ethanol and methanol extracts) are effective against the isolates. Akinsulire et al. (2007) reported that alcoholic extracts of *B. pinnatum* showed antimicrobial activity against a number of Gram (+ve) and (-ve) bacterial strains. Ofokansi et al. (2005) reported that plant is effective in the treatment of typhoid fever and other bacterial infections, particularly those caused by *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, *K. aerogenes*, *K. pneumoniae* and *S. typhi*. In his study antibacterial activities of the infusion and methanolic extracts against *S. aureus* ATCC 13709, *E. coli* ATCC 9637, *Bacillus*,

P. aeruginosa, *K. pneumonia* and *S. typhi* using the agar diffusion method; also against *S. aureus*, *E. coli*, *S. typhi*, *Klebsiella* spp and *P. aeruginosa* using a modification of checkerboard method. These findings supported its use in treating the placenta and navel of newborn baby, which not only heals fast but also prevent the formation of infections. Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects. Also, Obaseiki-Ebor et al investigated the invitro antibacterial activity of the leaf juice. They reported that the extract at 5% v/v was found to be bactericidal to a wide spectrum of gram-positive and gram-negative bacteria such as *B. subtilis*, *S. aureus*, *S. pyogenes*, *S. faecalis*, *E. coli*; *Proteus* spp *Klebsiella* spp; *Shigella* spp; *Salmonella* spp; *S. marcescens*; and *P. aeruginosa* including the clinical isolates of these organisms possessing multiple antibiotic resistance.

Akinsulire (2007) reported that the alcoholic extracts of *B. pinnatum* showed antimicrobial activity against a number of Gram (+ve) and (-ve) bacterial strains. Gibbons (2004) noted that *B. pinnatum* has been proven to have antimicrobial importance both in vivo and in vitro. Karabi and Sankar (2015) recorded that methanolic extract show good antibacterial activity against highly resistant UTI isolates and *Pseudomonas aeruginosa*. Praveen (2012) recorded that methanol extracts of *B. pinnatum* showed pronounced antibacterial activity against multidrug resistance *E. coli* with zones of inhibition varying between 8mm to 22mm. Akinpelu (2000) reported that the extracts of *B. pinnatum* possess antimicrobial properties. The antibacterial activity might be due to presence of bioactive compounds in plant extracts. According to Dholaria and Desai (2014) methanol extract showed maximum activity against all selected isolates except *Pseudomonas aeruginosa* while maximum activity against *Pseudomonas aeruginosa* was shown by ethanol extract. Nguelefack (2006) reported that alkaloids and saponins are present in the aqueous and alcoholic extracts of leaves of *B. pinnatum*. Also, Ojwole (2005) reported presence of flavonoids, polyphenols and terpenoids in the leaves of *B. pinnatum*.

B. pinnatum is widely used in traditional medicine in the treatment of many ailments in Nigeria, China and India. They are eaten for diabetes, diuresis, dissolving kidney stones, respiratory tract infections, as well as applied to wounds, boils, and insect bites. The aqueous extracts of this plant have shown anti-inflammatory, anti-diabetic, anti-tumour and cutaneous leishmanicidal activities (Akinsulire et al; 2007). Swai and Schoonman (2012) noted that in the livestock sector, different types of farm animals are capable of carrying a wide range of zoonotic pathogen. The cow and the mice often act as non-symptomatic carrier of human pathogens such as *E. coli* and *Salmonella enteritidis* which are rarely detected during routine anti-mortem examination.

This study shows that animals are carriers of human pathogens and *B. pinnatum* leaves have antibacterial properties. *B. pinnatum* leaves can be used to treat many diseases. It can also be used to get rid of some multidrug resistant pathogens and infections. No doubt, the use of the leaves of *B. pinnatum* has been helpful in the treatment of wounds, cough and many other illnesses in our rural communities by our great grandfathers and ancestors. It is strongly believed that, the promotion of the use of leaves of *B. pinnatum* would help to reduce and fight the incidence of antibiotic resistance crises in our various hospitals and health centres globally.

IV. CONCLUSION

This study showed the in vivo antibacterial effects of medicinal plant (*B. pinnatum*) used in traditional Nigeria medicine. The leaves of *Bryophyllum pinnatum* possess antibacterial properties and as such can be harnessed for the production of antimicrobial drugs. The findings also showed that the methanol and ethanol extracts of leaves of *B. pinnatum* are effective against some human pathogen isolated from cow dung. No doubt, the use of medicinal plants has been helpful in the treatment of wounds, cough and many other illnesses in some Nigeria rural communities by our great grandfathers and ancestors. This study also showed that *B. pinnatum* has good antibacterial properties.

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