Phytochemical Analysis and Evaluation of Antibacterial Activity of *Bryophyllum pinnatum* Leaf Extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from Patients with Otitis Media

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Abstract: *Bryphyllum pinnatum* is used in traditional medicine in various parts of the world as a remedy against numerous conditions like hypertension, skin disorders, cancer, diabetes, hepatitis, abscesses and ear infections. This study was carried out to evaluate the antibacterial potentials of the plant leaves against some bacteria isolated from patients with otitis media. Dried leaves of the plant were collected and subjected to cold maceration and Soxhlet extraction processes using water and ethanol to obtain aqueous and ethanolic extracts respectively. The extracts obtained were subjected to phytochemical screening and antibacterial activity against two clinical isolates; *Staphylococcus aureus* and *Pseudomonas aeruginosa* associated with Otitis Media. The result of the phytochemical screening revealed the presence of Alkaloids, Flavonoids, Saponins, and Glycosides in both extracts whereas ethanolic extract has in addition Steroids and Tannins. The result of antibacterial activity showed that aqueous extract has activity against *Pseudomonas aeruginosa* only at 500mg/ml concentration(zone of inhibition15.5mm) and against *Staphylococcus aureus* at 500, 250 and 125mg/ml with zone of inhibition 18, 14 and 11mm respectively. Similarly, ethanolic extract showed activity against *Pseudomonas aeruginosa* at 500, 250 and 125mg/ml with inhibition zone 17, 12, and 11mm respectively. It shows significant activity on *Staphylococcus aureus* at concentrations 500, 250, 125 and 62.5mg/ml with inhibition zones 19, 15, 13, 12mm respectively. The minimum inhibitory concentration (MIC) recorded for aqueous extract is 500 mg/ml and 250mg/ml against *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively while for ethanolic extract, are 250 and 125mg/ml respectively. The minimum bactericidal concentration (MBC) of the aqueous extract against *Staphylococcus aureus* is 250mg/ml and ethanolic extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus* are 250 and 125mg/ml respectively. The result suggests that *Bryophyllum pinnatum* leaf could have potential application in the management of otitis media.
Key words: Otitis media, phytochemical, antibacterial, *Bryophyllum pinnatum*, leaf

INTRODUCTION

Bryophyllum pinnatum is commonly known as miracle leaf, life plant (English) (Musa *et al*., 2016) '*Sutura'* or '*Shuka halinka'* (Hausa) (Mudi and Ibrahim 2008), '*panfuti*' in Hindi (Prasad *et al.,* 2012). It is widely used in traditional medicine in many regions like Madagascar, Tropical Africa, India, China, Australia, Hawaii, and Tropical America where the plant is indigenous (Nisha *et al*., 2016). The plant is being used by several traditional practitioners in various part of the world as remedy against numerous conditions like hypertension, skin disorders, cancer, fever, diabetes, hepatitis, abscesses and wound healing as well as ear infections (Rajsekhar *et al*., 2016).

Otitis media (OM) is a spectrum form of ear infection associated with inflammatory disease of the middle ear. It is classified into four domains namely; acute otitis media (AOM), otitis media with effusion (OME), mucoid otitis media (MOM) and chronic suppurative otitis media (CSOM) (Cunningham *et al*., 2012). OM lead to several complications which can be fatal (rare) and spread to other parts of the ear leading to abscess formation (Wallis *et al.,* 2015). Age has been identified as a risk factor of the disease which explained why it is common in infants and children especially from developing countries (specifically those in the tropical region) like Nigeria or remote areas (Uju and Nosa 2013; Afolabi *et al*., 2012; Morris *et al.,* 2005; Minja and Machemba., 1996). Generally, Studies have shown that 80% of children should have experienced at least an episode of otitis media by their third birthday (Venekamp *et al.,* 2015). The World Health Organization estimated that about 42 million people (age

> 3 years) have hearing loss with OM recording 18.25%, as the major cause (Kishve *et al.,* 2010).

Bioactive components of *B. pinnatum* leaf extract have medicinal value against several infections including ear infection (Akinpelu, 2000). It is a common practice in many rural areas in Northern Nigeria to use the leaves of the plant to cure ear infections. The leaves of *Bryophyllum pinnatum* are mostly used by several traditional practitioners as a remedy for different illnesses in different parts of the world (Al-Snafi, 2013). This has encouraged the search for the pharmacologic properties of the plant endowed with vast assortments of phytochemical constituents such as flavonoids, alkaloids, tannins among others (Sharma *et al*., 2014).

It is therefore important to assess the antimicrobial activity of *B. pinnatum* leaf extract against otitis media causing pathogenic bacteria as quite a number of researches confirmed the traditional use of the plant for curing middle ear infections. This study was carried out to evaluate the therapeutic potential of *B. pinnatum* leaves in the treatment of otitis media infections.

MATERIALS AND METHODS

Collection and identification of plant material

Bryophyllum pinnatum plant was collected from the outskirts of Kaduna North, Kaduna state, Nigeria. It was identified and authenticated by a taxonomist at the Department of Biological Sciences, Kaduna State University, Kaduna. Voucher specimen 01517 of the plant was deposited at the herbarium of the Department. The plant was washed thoroughly using water, dried and the leaves were carefully selected.

Collection of test organisms

Pure isolates each of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from OM cases were obtained from National Ear Care Centre (NECC) Kaduna, Nigeria and transported immediately to Microbiology laboratory, Kaduna State University, Kaduna, Nigeria for confirmation.

Preparation of plant extract

The leaves of the *B. pinnatum* were washed, dried under shade at ambient temperature, grinded into powder and stored in an airtight bag in low temperature and dry condition. Simple cold maceration and Soxhlet extraction method were used for the extraction. For the former, 100g of *B. pinnatum* powdered leaf was macerated and soaked in 300ml of distilled water contained in 1000ml capacity conical flask and allowed to stand on a bench with occasional shaking for 72 hours and finally filtered through Whatman filter paper No. 1. The filtrate was evaporated to dryness in a water bath and extract was finally obtained. For the latter, 100g of *B. pinnatum* powdered leaf was also weighed and parked into a filter thimble and then placed into a Soxhlet apparatus set up for extraction. After the extraction, the ethanolic and aqueous extracts obtained were then evaporated to dryness on a water bath to obtain pure crude extracts (Biswas and Sinha, 2015). Stock solution was prepared by dissolving the extracts (1 g) in 2 ml dimethyl sulfoxide (DMSO) to prepare a concentration of 500 mg/ml which was further diluted to prepare lower concentrations (250, 125, 62.5 mg/ml).

Phytochemical screening

Phytochemical screening of *Bryophyllum pinnatum* aqueous and ethanolic leaf extract was carried out using standard qualitative methods (Bankole and Shuaibu, 2008). Tests were carried out to detect the presence of Alkaloids, Flavonoids, Tannins, Saponins, Steroids and Glycosides.

Antibacterial susceptibility assay

The susceptibility test of the plant extracts was carried out using agar well diffusion method according to Singh *et al*. (2012) method with little modification. Briefly, standardized bacterial inocula (McFarland's standard) equivalent to 10^5cfu/ml made from 24hrs culture was prepared by suspending the organisms in sterile normal saline.

The organisms were inoculated uniformly on the surface of prepared Muller Hinton agar plates by surface spreading technique. Six (6) equidistant wells of 7mm diameter were aseptically prepared in each of the seeded plates using sterile cork-borer. Using a micropipette, 200 µl each of 500, 250, 125 and 62.5 mg/ml concentration of the extracts was dispensed into each of the corresponding wells, made in the plates and 200µl of 500mg ciprofloxacin was added as positive control and 200µl dimethyl sulfoxide (DMSO) was used as negative control. The plates were kept in a refrigerator for 30min at 4^{o}C for pre-diffusion and were then finally incubated at 37^{o}C for 24hours. Following the incubation, the diameters of the zones of inhibition were measured in mm using transparent meter rule.

Determination of Minimum inhibitory concentration (MIC)

The MIC of the aqueous and ethanolic extracts of *B. pinnatum* were determined against the selected bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) separately by broth dilution method as described by Biswas and Sinha (2015) with little modification. For each extract, five concentrations (500, 250, 125, 62.5, 31.25 mg/ml) were prepared by doubling dilutions in DMSO and then 500 µl was transferred to tubes containing 5 ml of Nutrient Broth and 0.1 ml of bacterial suspension (equivalent to 10^5cfu/ml), incubated at 37ºC for 24 hours. After incubation the tubes were examined for bacterial growth by observing turbidity. The MIC was determined as the minimum concentration that inhibits the growth of a specified organism (i.e. showing no visible growth indicated by absence of turbidity).

Determination of Minimum bactericidal concentration (MBC)

The MBC of the extracts was determined by selecting the tubes that showed no visible growth in the MIC assay. A loopful from each of the tubes was subcultured onto a freshly prepared Mueller Hinton agar medium and later incubated at 37ºC for 24 hours. The MBCs were taken as the lowest concentration of extract that did not allow any bacterial growth on the surface of the agar plates.

RESULTS

Result for the phytochemicals screening of *Bryophyllum pinnatum* aqueous and ethanolic leaf extract is shown in Table 1. The extracts were found to contain various bioactive compounds making them a potential source of therapeutic activities. The results of the antibacterial activities of ethanolic and aqueous extracts is shown in Table 2 which reveal strong antibacterial activity of the extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. MIC and MBC results is presented in Table 3 and 4 respectively.

Table 1: Phytochemical Constituents of *Bryophyllum pinnatum* aqueous and ethanolic Leaf Extract

Bioactive Compounds	Ethanolic extract	Aqueous extract
Alkaloid	Present	Present
Flavonoid	Present	Present
Steroid	Present	Absent
Tannins	Present	Absent
Saponins	Present	Present
Glycosides	Present	Present

Table 2: Antibacterial activity of *Bryophyllum pinnatum* aqueous and ethanolic leaf extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Concentration (mg/ml)	Inhibition zone (mm)			
	Staphylococcus aureus		*Pseudomonas aeruginosa*	
	Aqueous	Ethanolic	Aqueous	Ethanolic
500	18	19	15.5	17
250	14	15	NZ	12
125	11	13	NZ	11
62.5	NZ	12	NZ	NZ

Key: mg/ml = milligram per millilitre, mm= millimetre, NZ = no zone of inhibition

Table 3: Minimum Inhibitory Concentration (MIC) of *Bryophyllum pinnatum* aqueous and ethanolic leaf extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Concentration (mg/ml)	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract
500	No Growth	No Growth	No Growth	*MIC
250	No Growth	*MIC	*MIC	Growth
125	*MIC	Growth	Growth	Growth
62.5	Growth	Growth	Growth	Growth
31.25	Growth	Growth	Growth	Growth

Key: mg/ml = milligram per millilitre, *MIC = Minimum Inhibitory Concentration

Table 4: Minimum bactericidal Concentration (MBC) of *Bryophyllum pinnatum* aqueous and ethanolic Leaf Extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Concentration (mg/ml)	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract
500	No Growth	No Growth	No Growth	Growth
250	No growth	*MBC	*MBC	Growth
125	*MBC	Growth	Growth	Growth
62.5	Growth	Growth	Growth	Growth
31.25	Growth	Growth	Growth	Growth

Key: mg/ml = milligram per millilitre, *MBC = Minimum Bactericidal Concentration

DISCUSSION

Both ethanolic and aqueous extract from the plant leaf in this study contained alkaloids, flavonoids, saponins and glycosides, with the additional presence of steroids and tannins in the ethanolic extract. The outcome of this study conforms to the detectable phytochemicals present in the leaves of *Bryophylum pinnatum* such as Alkaloids, Saponins, Flavonoids, Tannins, Steroid and Glycosides as reviewed by several authors (Fernandes *et al*., 2019; Latif *et al*., 2019; Punit *et al*., 2019; Raman *et al.,* 2014). The variations in some phytochemicals could be due to the variety of plant, geographical conditions, methods of extraction and solvent used (Jane and Patil, 2012). For example, Akinnibosun and Edionwe (2015) reported positive test for Saponins, Flavonoids, Alkaloids, Steroids and Tannins in ethanolic extract but absence of Steroids and Tannins in aqueous extracts of *Bryophyllum pinnatum.* Higher polarity possessed by the ethanol gives it higher penetration property and therefore the extract removes more bioactive compounds from the powdered leaf than the aqueous solvent.

The extracts have potential antibacterial activity against the otitis media pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as indicated by the result of antibacterial susceptibility test.

The result showed that aqueous extract has activity against *Pseudomonas aeruginosa* only at the highest concentration (500mg/ml) with inhibition zone of 15.5mm but showed more activity against *Staphylococcus aureus* at 500, 250 and 125mg/ml with inhibition zones of 18, 14 and 11mm respectively. Similarly, ethanolic extract showed activity against *Pseudomonas aeruginosa* at 500, 250 and 125mg/ml with inhibition zones of 17, 12 and 11mm respectively and significant activity on *Staphylococcus aureus* at all the four different concentrations 500, 250, 125 and 62.5mg/ml with inhibition zones of 19, 15, 13 and 12mm respectively. The aqueous extract has lesser activity against Gram-negative bacteria, *Pseudomonas aeruginosa* compared with the Gram-positive bacteria, *Staphylococcus aureus.* The ethanolic extract follows similar pattern with significant activity at different concentrations on each of the test organisms (*Staphylococcus aureus* and *Pseudomonas aeruginosa*). Both extracts are active at high concentrations and less or inactive at lower concentrations with activity increasing with increase in concentration. Thus, this study suggests that the inhibition of the test organisms is concentration dependent and the activity is more against the representative Gram-positive bacteria than Gram negative bacteria. The result of this study is therefore in line with the report of Joshi *et al*. (2016) who investigated the *in vitro* antibacterial activity of *Bryophyllum pinnatum* leaf extract and found that it is a potent agent that is effective against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli.* It was also reported that, *Staphylococcus aureus* and *Pseudomonas aeruginosa* alongside other organisms including *Klebsiella pneumoniae, Aspergillus niger* and *Candida albicans* were found to be sensitive to the bioactive compounds of the plant (El Abdellaoui *et al*., 2010). However, a research conducted by Ogochukwu (2011) revealed that the aqueous extract did not demonstrate any antimicrobial activity against *Pseudomonas aeruginosa.* Therefore, this result can serve as an improvement and explain the reason why the aqueous extract was not effective against *Pseudomonas aeruginosa* except at the highest concentration. The phytochemicals present in the plants are responsible for the antibacterial activity observed against the otitis media causing pathogens which validate the use of the traditional use of the leaves juice in the treatment of ear infections (Akinpelu, 2000).

The results obtained from this research showed significantly higher zones of bacterial inhibition with ethanolic extract when compared with aqueous extract. This result is in-line with the work of Ladan *et al*. (2016) who found that ethanolic extract significantly inhibited the test organisms (*S. aureus* and *P. aeruginosa*) more than aqueous extract and concluded that solvent of extraction influences the antimicrobial activity of *Bryophyllum pinnatum.* This indicates that ethanolic extract contains more significant bioactive ingredients than aqueous extract, therefore giving the ethanolic extract higher inhibitory property.

The activity of the extracts against both Gram-positive and Gram-negative bacteria tested may suggest broad spectrum of antibacterial activity. This is a very important observation that can be applied possibly in the development of promising therapeutic substances that will be active against ear infections including OM.All these findings are indications or evidences supporting the promising medicinal properties of *Bryophyllum pinnatum* (Okwu and Nnamdi, 2011). The findings supported the ethnomedicinal use of the plant by "*Hausa*" (one of the three major Nigerian ethnic groups) people in rural areas in the management of otitis media and its application on the navel of new-borns to prevent infection and facilitates healing.

The MIC against *Staphylococcus aureus* was higher in aqueous extract (250mg/ml) than in ethanolic extract (125mg/ml), suggesting that *Staphylococcus aureus* is more susceptible to the ethanolic extract than

aqueous. The MIC against *Pseudomonas aeruginosa* was also higher on aqueous extract (500mg/ml) than on ethanolic extract (250mg/ml), suggesting also that *Pseudomonas aeruginosa* is more susceptible to the ethanolic extract than aqueous extract. The MIC indicated that the ethanolic extract is more efficacious than the aqueous extract against both organisms. This better inhibition potential shown by ethanolic extract at lower concentration against the test organisms could be due to its higher phytochemical content.

MBC was seen against *Staphylococcus aureus* at 250mg/ml and 125mg/ml concentrations of ethanolic and aqueous extracts respectively. Similarly, MBC seen against *Pseudomonas aeruginosa* was at 250mg/ml in ethanolic extract with no MBC in aqueous extract. This showed that MBCs of the extracts were higher than the MICs, suggesting that the extracts are bactericidal at higher concentration and bacteriostatic at lower concentrations.

The result obtained showed that Gram-negative bacteria represented by *Pseudomonas aeruginosa* are relatively more resistant to the extracts than the Gram-positive bacteria, and this may be due to structural variation between their cell wall. This finding is supported by Tortora *et al.* (2001) who reported that the principle factor in the relative resistance to antimicrobials by Gram-negative bacteria is the external lipopolysaccharide and characteristic changes of the porins, structural openings in the cell wall of Gram-negative bacteria that are highly selective of molecules that they permit to enter the cell. Therefore the active compounds in the extracts may not be able to penetrate the bacterial cell wall because the size of the porin channel determines the size of the bioactive molecule that can pass through it and thus the outer membrane serving as barrier to the passage of many molecules and selectively exclude many toxic compounds, rendering the extract inactive and hence making the Gram-negative organism less sensitive. However, Gram positive bacterium has a relative thick membrane layer, peptidoglycan that is permeable to many compounds like ions, amino acid among others regardless of its thickness and thus making it more sensitive to the extract.

CONCLUSION AND RECOMMENDATION

The findings from this study shows the antibacterial activities of ethanolic and aqueous extracts of *Bryophyllum pinnatum* against otitis media pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Phytochemicals detected in the extracts from the plant leaves were found to be more in the ethanolic extract than the aqueous extract making the ethanolic extracts more efficacious. Also, the activity of the extracts increases with increase in concentrations as shown by the zones of inhibition produced. This suggest a promising potential on the use of the plant leaves in the treatment of OM lending credence to the traditional use of the plant in the management of OM and other ear infections. And the plant, if further exploited can help in the development of phytomedicine for the control and management of otitis media. This will require a series of clinical trials and identification of the specific metabolites that exert the pharmacologic effects.

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