

**Inhibitory effects of *Azadirachta indica* (Neem) and *Bryophyllum pinnatum* (Oda opue) leaves extracts on *Staphylococcus aureus* isolated from infected wound samples.**

**ABSTRACT**

The antimicrobial effect of *Bryophyllum pinnatum* and *Azadirachta indica* extracts were evaluated on infected wound samples of patients attending General hospital Onitsha. Out of 40 wound swabs, 30 bacteria isolates were obtained which include 33.33% of *Staphylococcus aureus*. The phytochemical analysis of the leaves extracts showed the presence of saponin, flavonoid, steroid, alkaloid, tannin, cardiac glycoside and phenols. The *in vitro* antimicrobial effect of the extracts: *Bryophyllum pinnatum* extracts (ethanol and aqueous), *Azadirachta indica* extracts (ethanol and aqueous) and their combinations to *Staphylococcus aureus* were evaluated using agar well diffusion method. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined. Among the leaves extracts analysed, *A. indica* ethanol has the highest zone of inhibition against *Staphylococcus aureus* with  $20.00 \pm 0.00$  mm at 1000 mg/ml concentration. *B. pinnatum* ethanol  $10.33 \pm 0.58$  at 1000 mg/ml, *A. indica* aqueous extract  $13.33 \pm 2.08$  mm but *B. pinnatum* aqueous extract has no inhibitory effect on *S. aureus*. The combination of *A. indica* aqueous and *B. pinnatum* ethanol extracts, *B. pinnatum* ethanol and *A. indica* ethanol extract combinations and *A. indica* ethanol extract and *B. pinnatum* aqueous extracts has inhibitory effects on *Staphylococcus aureus* except for combination of *A. indica* aqueous and *B. pinnatum* aqueous has no inhibitory effect. The result of MIC was 500 mg/ml and the MBC showed 1000 mg/ml.

The leaves extracts of *B. pinnatum* and *A. indica* possess antimicrobial properties especially for *Staphylococcus aureus* and the plants are readily available and are planted worldwide. The plant extracts can be harnessed for production of drugs. There is also need for research on other plants with antimicrobial effects.

Key words: *Bryophyllum pinnatum*, *Azadirachta indica*, *Staphylococcus aureus* and Antimicrobial sensitivity.

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## 1. INTRODUCTION

There have been increase in antibiotic resistant strains of clinical important pathogens in recent times and this have led to emergence of new bacterial strains that are multidrug resistant.

Plants have formed the essential part of African and global society since civilization in traditional healthcare delivery systems (1). Recently, African traditional medicine has been brought into recognition and acceptance as alternative healthcare delivery practice. Herbal medicines have been the basis of treatment and cure for various diseases and physiological disorders in traditional methods among the people of subSaharan (2).

Herbal remedies are known to treat many infectious diseases throughout the history of mankind. Plant material continues to play a major role in the primary health care as therapeutic remedies in many developing countries. Thus, the discovery of medicinal plants as antimicrobial agents is useful in expanding the wide varieties of antibiotics available (3). Various parts of the neem tree have been used as traditional Ayurvedic medicine in India. For instance, the leaves, seed and bark possesses a wide spectrum of antibacterial action against Gram negative and Gram positive microorganisms. However, the leaves are in concern for its medicinal properties for wound healing. Neem leaf is effective in the healing of chronic wounds, diabetic foot and gangrene developing conditions (4).

The availability of antibiotics over the counter has led to increase in the antimicrobial resistance of pathogens to conventional drugs. Due to this increase in resistance, there is need to for research on natural products which have therapeutic effectiveness which plants and there products are one of them. This work aimed at antimicrobial effects of different leaves extracts of *A. indica* and *B. pinnatum* on *Staphylococcus aureus* isolated from infected wound.

## 2. MATERIALS AND METHODS

### 2.1 Study Area

The study was carried out in Anambra State, which is the 8<sup>th</sup> populous state in Nigeria, located in South-East geopolitical zone of Nigeria. It is located between latitude 6°20'00" North and longitude 7°00'00" East. Anambra state has a population of 2,061961.984. Anambra state has

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a tropical climate. The average temperature is 25.9°C while the average rainfall in a year is 1386mm.

## **2.2. Study Design**

This study was done to determine inhibitory effects of *Azadirachta indica* and *Bryophyllum pinnatum* extracts on *Staphylococcus aureus* isolated from infected wounds.

## **2.3 Isolation of Microorganisms**

### **2.3.1 Sample Collection**

Forty (40) wound swabs of patients with infected wounds and are attending different clinics at General hospital were collected. Wound exudates were collected using sterile cotton tipped swab before antiseptic dressing. Sterile normal saline were used to moisten the swab stick before collecting the specimen (5)

### **2.3.2 Preparation of Culture Media**

All culture media employed in this study were prepared according to the manufacturer's instructions. They were sterilized by autoclaving at 121°C, 15psi for 15 minutes.

### **2.3.3 Isolation and Identification of Bacteria**

Each sample was inoculated onto Nutrient agar and MacConkey agar and incubated at 37°C for 24 hours. After the incubation, the different colonies were subcultured on Nutrient agar and MacConkey agar and incubated at 37°C for 24 hours to get pure cultures. Then the colonies were identified macroscopically, microscopically, using biochemical analysis and by culturing on selective media, and Mannitol salt agar. Macroscopic identification includes the color and the size of the colonies. Microscopic identification was by gram staining. Biochemical tests include: catalase test, oxidase test, coagulase test, methyl red test, Voges-proskauer test, indole test, citrate test and sugar fermentation and assimilation tests (6)

### **2.3.4 Biochemical Analysis**

#### **2.3.4.1 Catalase Test**

This was done by placing a drop of 3% H<sub>2</sub>O<sub>2</sub> on colonies of Muller Hinton agar. Prompt effervescence was indicated catalase production (8).

#### **2.3.4.2 Oxidase Test**

It was done by placing a drop of freshly prepared 1% solution of oxidase reagent on a piece of filter paper using a sterile loop, a test colony was picked and rub on the paper in the area impregnated with the oxidase reagent. The paper became deep purple blue showing that the organism is oxidase positive (8).

#### **2.3.4.3 Coagulase Test**

It was done by placing three separate drops of saline on a clean slide, a loopful of test colony was suspended in two of the saline drops and a loop of control organism in the third, a drop of citrated rabbit plasma was added on one test and the control suspension. Occurrence of clumping within 10seconds will indicate a positive result (8).

#### **2.3.4.4 Motility Test**

A sterile needle was used to pick a loop of a 24 h old culture and was stabbed onto nutrient agar in glass vials. The vials were incubated at 37°C for 24-48 h (9).

#### **2.3.4.5 Methyl Red Test**

Five millimeters of glucose phosphate broth (1 g glucose, 0.5% KH PO, 0.5% peptone and 100 mL distilled water) was dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 h. At the end of incubation, few drops of methyl red solution was added to each test and colour change indicates a positive result (9).

#### **2.3.4.6 Voges-Proskauer Test**

Five millimeter of glucose phosphate broth (1 g glucose, 0.5% KH PO, 0.5% peptone and 100 mL distilled water) was dispensed in clean test tubes and sterilized. The tubes were inoculated with the test organisms and incubated at 37°C for 48 h. After incubation, 6%  $\alpha$ -naphthol and 6% Sodium hydroxide were added to about 1 ml of the broth culture (9).

#### **2.3.4.7 Indole Test**

Tryptone broth (5 ml) was placed into different test tubes after which a loopful of the bacterial isolates were inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as control. The test tubes were then incubated at 37°C for 48 h. After incubation, 0.5 ml of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 min to permit the reagent to rise (7).

#### **2.3.4.8 Citrate Test**

2.4 g of citrate agar was dissolved in 100 mL of distilled water. Ten milliliters (10 ml) of citrate medium was dispensed into each tube and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface (9).

#### **2.3.4.9 Sugar Fermentation**

Sugar indicator broth was prepared using peptone water medium containing 1% fermentable sugar and 0.01% phenol red. About ten milliliters of sugar broth were dispensed into each of the test tubes, Durham tube which would trap the gas if produced was inverted carefully. The test tubes were autoclaved and inoculated with a loopful of 24 h old culture of the test organisms after then incubated for 2-7 days at  $36\pm 1^{\circ}\text{C}$  and observed daily for acid and gas production (10).

#### **2.4 Plant Collection and Identification**

The leaves of *Azadirachta indica* and *Bryophyllum pinnatum* were collected from the bush in Aguluezechukwu, Anambra State. The identification and authentication of the plant was carried out by Mr. Chisom of Department of Botany, Nnamdi Azikiwe University, Awka with voucher specimen number NAUH/14<sup>B</sup> and NAUH/172<sup>A</sup> respectively. The leaves were washed with sterile water, shade dried, ground into powdered form and stored in an air tight container for future use (11).

#### **2.4.1 Preparation of Crude Extract**

Two sets of 100g of *B. pinnatum* leaves were washed, shredded and blended with electric blender. Each set of the crushed leaves was soaked in 300ml of ethanol (95%) and 300ml deionized distilled water in two 1000ml capacity flasks respectively. The flasks were then left standing on a bench with occasional shaking for 72 hours. The aqueous extraction and ethanol extraction were done using a Soxhlet extractor. At the end of the extraction process, the pure extract collected were filtered with No. 1 Whatman filter paper and evaporated to dryness by gentle heating at  $40^{\circ}\text{C}$  on a hot plate. The dried concentrates (ethanol and aqueous extracts) were weighed and for antimicrobial assay on the bacterial isolate (12).

#### **2.4.2 Phytochemical Screening**

##### **2.4.2.1 Qualitative Analysis of Phytochemical Constituents**

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This was carried out to determine the presence of saponins, tanins, flavonoids, glycosides, phytosterols, cardiac glycoside, triterpenoids and alkaloids. The solvent to be used are ethanol and distilled water (13).

### 2.5 Preparation of Inoculum

The isolated microorganisms (bacteria and fungi) was aseptically scraped and transferred into 10ml of sterile water in a sterile bottle and agitated vigorously. The suspension was diluted serially tenfold and used for antifungal and antibacterial screening (14).

### 2.6 Reconstitution of Plant Extract

The extracts were reconstituted using the described by (14). The dried extracts were reconstituted by dissolving 10g of extract in 10ml of DMSO. This extract were considered as 1000mg/ml.

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### 2.7 *In Vitro* Antibacterial Activity Assessment of the Extracts on the Isolate

The sensitivity of the test organism to the various extracts was determined using the agar well diffusion method as described by Prescott et al., 2000. Nutrient agar plates were prepared in triplicates for each of the test organisms and labelled appropriately. A volume of 0.1 ml of each standardized (McFarland standard) test organism was inoculated into separate plates using the spread plate method. A sterile cork borer of 3 mm in diameter was used to bore 5 equidistant holes on the surface of the plate with one at the centre. One-tenth of a millilitre (0.1 ml) of each extract concentration was introduced into the four peripheral holes while the hole in the middle had 0.1 ml of gentamycin (40 mg/ml) to serve as control. After leaving the plates for an hour to allow for diffusion of the extracts through the medium, they were incubated at 37°C for 24hours. For each triplicate culture plate, the inhibition zone around each well was measured and the mean value obtained and recorded in millimetres (mm).

### 2.8 Statistical Analysis

The data obtained were subjected to one-way ANOVA using Statistical Package for Social Sciences (SPSS) 21 for window evaluation. P-values < 0.05 was considered significant.

## 3. RESULT

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### 3.1 Frequency of the Isolates from Infected Diabetic Wound Samples

Out of 40 swab samples of infected diabetic wounds, 30 isolates which include 10(33.33%) *Staphylococcus aureus*, 4(13.33%) *Streptococcus pyrogenes*, 5(16.67%) *Pseudomonas aeruginosa*, 7(23.33%) *Escherichia coli* and 4(13.33%) *Klebsiella pneumonia*

Table 1 show the results of the morphological and biochemical characteristics of the bacterial isolate. It also shows the ability of the bacteria to ferment and assimilate various carbohydrates added in a basal medium. Ability to produce acid was indicated by yellowish colour change of the bromothymol blue which was used as the indicator while gas accumulation in the Durham tubes submerged in the medium indicates production of gas.

**Table1: Morphological and Biochemical Characteristics of Bacterial Isolates**

Test	Characteristics
Gram Staining	+ve
Catalase	+ve +
Oxidase	-ve
Urease	+ve
Methyl red	+ve
Voges-Proskauer	+ve
Indole	-ve
Motility	-ve
Citrate	+ve
Coagulase	+ve
Morphology	Cocci
Sucrose	+ve
Glucose	+ve
Lactose	+ve
Maltose	+ve

KEY: +ve: positive

-ve: negative

### 3.2 Phytochemical analysis of the crude extracts

Table 2 shows the phytochemical constituent of *Bryophyllum pinnatum* and *Azadirachta indica* extracts. The aqueous *Bryophyllum* extract contain saponin, flavonoids and alkaloids with the absence of steroid, phenol, tannins and cardiac glycoside. The ethanol extract of *Bryophyllum*

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*pinnatum* contains saponin, flavonoids, steroids, alkaloids, tannins, phenol and cardiac glycoside while the extracts of *Azadirachta indica* both the ethanol and aqueous extracts contain saponin, flavonoids, steroids, alkaloids, tannins, phenol and cardiac glycoside.

**Table 2: Phytochemical constituents of *Bryophyllum pinnatum* and *Azadirachta indica* extracts**

Constituent	<i>Bryophyllum pinnatum</i>		<i>Azadirachta indica</i>	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
Saponin	+ve	+ve	+ve	+ve
Flavonoid	+ve	+ve	+ve	+ve
Steroid	-ve	+ve	+ve	+ve
Alkaloids	+ve	+ve	+ve	+ve
Tannins	-ve		+ve	+ve
Cardiac glycoside	-ve	+ve	+ve	+ve
Phenol	-ve	+ve	+ve	+ve

KEY: +ve: positive

-ve: negative

### 3.3 Antimicrobial Effects of the Crude Extracts on the Isolates

Table 3 shows the antimicrobial effects of *Bryophyllum* and *Azadirachta* extracts (ethanol and aqueous) on *Staphylococcus aureus* using agar well diffusion method. *Azadirachta indica* ethanol extract showed inhibitory response of 14.00mm, 17.33mm, 20.00mm, 20.00mm and 20.00mm at 62.5mg/ml, 125mg/ml, 250mg/ml, 500mg/ml and 1000mg/ml concentration, *Azadirachta indica* aqueous extract showed inhibitory response of 9.67mm, 12.67mm and 13.33mm at 250mg/ml, 500mg/ml and 1000mg/ml, *Bryophyllum pinnatum* ethanol extract showed inhibitory response of 7.33mg/ml, 10.00mg/ml and 10.33mg/ml at 250mg/ml, 500mg/ml and 1000mg/ml but *Bryophyllum pinnatum* aqueous extract has no inhibitory response on *Staphylococcus aureus*.

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Antimicrobial effects of the different combinations of the extracts on the isolates are shown in tables' 3a-3e.

**Table 3a: Antimicrobial Effect Of Extracts At Different Concentrations On *Staphylococcus aureus***

Concentration(mg/ml)	Mean zone of inhibition of the extracts ±SD (mm)			
	<i>B.pinnatum</i> . aqueous extract	<i>B.pinnatum</i> . ethanol extract	<i>A.indica</i> aqueous extract	<i>A.indica</i> . ethanol extract
62.5	0.00±0.00	0.00±0.00	0.00±0.00	14.00±1.00
125	0.00±0.00	0.00±0.00	0.00±0.00	17.33±1.15
250	0.00±0.00	7.33±0.58	9.67±1.53	20.00±0.00
500	0.00±0.00	10.00±0.00	12.67±2.52	20.00±0.00
1000	0.00±0.00	10.33±0.58	13.33±2.08	20.00±0.00
+control	30.00±0.00	30.00±0.00	30.00±0.00	30.00±0.00
-control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Positive control: Gentamicin: 30.00±0.00mm

Negative control: sterile distilled water: 0.00mm, Result represented as Mean±SD

**Table: 3b Antimicrobial Effect of Combination of *A. indica* aqueous Extract and *B. pinnatum* ethanol Extracts**

Isolates	Mean zone of inhibition of different concentration of the extracts ±SD (mm)						
	62.5mg/ml	125mg/ml	250mg/ml	500mg/ml	1000mg/m	+control	-control
<i>S. aureus</i>	11.33±0.5	14.00±1.0	15.00±0.0	20.00±0.0	21.00±1.0	23.00±0.0	0.00±0.0
	8	0	0	0	0	0	0

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**Table: 3c Antimicrobial effects of combination of *A. indica* and *B. pinnatum* Aqueous extracts**

Isolate	Mean zone of inhibition of the extracts on the isolates $\pm$ SD (mm)						
	62.5mg/ml	125mg/ml	250mg/ml	500mg/ml	1000mg/ml	+control	-control
<i>S.aureus</i>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	23.33 $\pm$ 0.58	0.00 $\pm$ 0.00

**Table: 3d Antimicrobial Effect of the combination of *B. pinnatum* Ethanol Extract and *A. indica* Ethanol Extract**

Isolates	Mean zone of inhibition of extracts on the isolates $\pm$ SD (mm)							TOTAL
	62.5mg/ml	125mg/ml	250mg/ml	500mg/ml	1000mg/ml	+CONTROL	-CONTROL	
<i>Staphylococcus aureus</i>	17.67 $\pm$ 0.58	18.00 $\pm$ 0.00	19.33 $\pm$ 0.58	21.00 $\pm$ 1.00	21.00 $\pm$ 0.00	21.33 $\pm$ 1.53	0.00 $\pm$ 0.00	16.90 $\pm$ 7.24

**Table: 3e Antimicrobial Effect of Combination of *A. indica* ethanol Extract and *B. pinnatum* aqueous extracts**

Isolates	Mean zone of inhibition of different concentrations of the combined extract $\pm$ SD (mm)						
	62.5mg/ml	125mg/ml	250mg/ml	500mg/ml	1000mg/m	+control	-control
<i>S. aureus</i>	17.67 $\pm$ 0.5	18.0 $\pm$ 0.0	19.3 $\pm$ 0.5	21.0 $\pm$ 1.0	21.00 $\pm$ 0.0	21.33 $\pm$ 1.5	0.00 $\pm$ 0.0
	8	0	8	0	0	3	0

#### 4. DISCUSSION

Forty (40) infected wound swab samples were collected and analysed. Out of these samples, 30 isolates which include 10(33.33%) *Staphylococcus aureus*, 4(13.33%) *Streptococcus pyrogenes*, 5(16.67%) *Pseudomonas aeruginosa*, 7(23.33%) *Escherichia coli* and 4(13.33%) *Klebsiella pneumoniae*. Aynalem *et al.*, 2017 also revealed that *S. aureus* is the most predominant bacteria in infected wounds.

The result of the phytochemical analysis of *B. pinnatum* and *A. indica* aqueous and ethanol extracts shows the presence of saponin, flavonoids, steroid, alkaloids, tannins, cardiac glycoside and phenol except for *B. pinnatum* aqueous extract that has no tannins, steroids, cardiac glycoside and phenol.

This is in concordance with report of Okwu and Josiah (16) which stated that stronger antibacterial activity of methanol extracts have been attributed to the ability of the solvent to extract some of the active properties of these plants like phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial.

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In this study, the *in vitro* antimicrobial activities of the *B. pinnatum* and *A. indica* extracts (aqueous and ethanol) were done on the *Staphylococcus aureus* isolate separately and combined. The result of the antimicrobial effect shows that *B. pinnatum* aqueous extract has no inhibitory effect on *Staphylococcus aureus*, but there are inhibitory effect by *B. pinnatum* ethanol extract at 250mg/ml (7.33mm), 500mg/ml (10.00mm) and 1000mg/ml (10.33mm). *A. indica* aqueous extract showed inhibitory effect on *S. aureus* at 250mg/ml (9.67mm), 500mg/ml (12.67mm) and 1000mg/ml (13.33mm). *A. indica* ethanol extract showed inhibitory effect at 10% concentration (14.00mm), 125mg/ml (17.00mm), 250mg/ml and 100mg/ml (20.00mm) as shown in table 3a. This result agreed with the work of Uwimbabazi *et al.*, 2015 which stated that zone of inhibition of the *A. indica* extracts depends on concentration. It is also in concordance with the work of Azuonwu *et al.*, 2017 which revealed that methanolic extracts of *Bryophyllum pinnatum* can be used against pathogenic organisms including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* which can cause slow healing of wounds.

For combination of *A. indica* aqueous extract and *B. pinnatum* ethanol extract, there is inhibitory effect on *Staphylococcus aureus* at 62.5mg/ml (11.33mm), 125mg/ml (14.00mm), 250mg/ml (15.00%), 500mg/ml (20.00mm) and 1000mg/ml (21.00mm) as shown in table 3b.

The combination of *A. indica* aqueous leave extract and *B. pinnatum* aqueous leave extract has no inhibitory effect on the isolate as shown in table 3c.

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The combination of *A. indica* ethanol extract and *B. pinnatum* ethanol showed that there is inhibitory effect on *S. aureus* at every concentration from 62.5mg/ml (17.67mm) to 1000mg/ml (21.00mm) as shown in table 3d.

The combination of *A. indica* ethanol extract and *B. pinnatum* aqueous extract has inhibitory effect on *S. aureus* at all the concentration 62.5mg/ml (17.33mm), 125mg/ml (18.00mm), 250mg/ml (19.33mm), 500mg/ml (21.00mm) and 1000mg/ml (21.00mm) as shown in table 3e.

This result agreed with Uwimbabazi *et al.*, 2015 which showed that there is antibacterial effect on *S. aureus* only while *E.coli* and other isolates can alter their genetic makeup with astonishing rapidity.

The minimal inhibitory concentration (MIC) of the extracts on *S. aureus* is 500mg/ml and 1000mg/ml is the MBC. Therefore, the extracts show equal activity of 1000mg/ml against the test isolate. This agreed with the report of Lawrence *et al.*, 2016 which revealed that there is correlating increase in antimicrobial activity with increase in extract concentration.

## 5. CONCLUSION

The leaves extracts of *B. pinnatum* and *A. indica* possess antimicrobial properties especially for *Staphylococcus aureus* and the plants are readily available and are planted worldwide. The plant extracts can be harnessed for production of drugs. ~~There is also need for research on other plants with antimicrobial effects.~~

### Ethical Consideration

Ethical approval was gotten from ethical committee of Anambra State Ministry of Health with ethical approval no. SHMB/AD.196/IV/58.

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