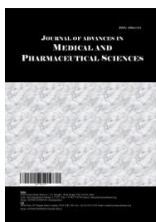

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Inhibitory Effects of *Azadirachta indica* (Neem) and *Bryophyllum pinnatum* (Oda Opue) Leaves Extracts on *Staphylococcus aureus* Isolated from Infected Wound Samples

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MII and COA designed the study, wrote the procedures and did the isolation of bacteria, author EAKO run the statistical analysis and managed the literature searches, authors ORU and IUN collect the leaves, and did extraction. All authors read and approved the final manuscript.

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ABSTRACT

Aim: to study the antimicrobial effects of different leaves extracts of *Azadirachta indica* and *Bryophyllum pinnatum* on *Staphylococcus aureus* isolated from infected wound.
Study Design: This study was done to determine inhibitory effects of extracts on *S. aureus* isolated from infected wounds.
Place and Duration of Study: General hospital Onitsha, Anambra State, Nigeria, January to March 2020.
Methodology: wound swabs were collected from 30 patients with infected wound. *S. aureus* were isolated and identified morphologically and biochemically. The leaves were collected, identified and grinded. Extraction of the leaves were done using soxhlet method with two solvents (ethanol and

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water). Phytochemical compositions were analyzed using standard methods. *In vitro* antimicrobial effect of extracts and their combination were evaluated.
Results: Phytochemical analysis of extracts showed presence of saponins, flavonoids, steroids, alkaloids, tannins, cardiac glycosides and phenols except *B. pinnatum* aqueous extract that showed presence of flavonoids, saponins and alkaloids. Minimum inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) were determined. Among the extracts analysed, *A. indica* ethanol has highest zone of inhibition against *S. aureus* with 20.00±0.00 mm at 1000 mg/ml concentration. *B. pinnatum* ethanol 10.33±0.58 mm at 1000 mg/ml, *A. indica* aqueous extract 13.33±2.08 mm but *B. pinnatum* aqueous extract has no inhibitory effect. The combination of *A. indica* aqueous and *B. pinnatum* ethanol extracts, *B. pinnatum and A. indica* ethanol extracts and *A. indica* ethanol extract and *B. pinnatum* aqueous extracts have inhibitory effects on *S. aureus* except combination of *A. indica* aqueous and *B. pinnatum* aqueous which has no inhibitory effect. The result of MIC was 500 mg/ml and the MBC showed 1000 mg/ml.
Conclusion: The extracts of *B. pinnatum* and *A. indica* possess antimicrobial properties for *S. aureus* and the plants are available and are planted worldwide. There is also need for research on other plants with antimicrobial effects.

Keywords: Bryophyllum pinnatum; Azadirachta indica; Staphylococcus aureus and Antimicrobial sensitivity.

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 sub-Saharan [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 *Azadirachta indica* and *Bryophyllum 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 8th populous state in Nigeria, located in South-East geopolitical zone of Nigeria. It is located between latitude 6^{0}20’00’’ North and longitude 7^{0}00’’00’’ East. Anambra state has a population of 2,061961.984. Anambra state has a tropical climate. The average temperature is 25.9℃ while the average rainfall in a year is 1386 mm.

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^{0}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^{0}c for 24 hours. After the incubation, the different colonies were subcultured on Nutrient agar and MacConkey agar and incubated at 37^{0}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 be by gram staining. Biochemical tests include: catalase test, oxidase test, coagulase test, methyl red test, Voges-proskaeur 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_2O_2 on colonies of Muller Hinton agar. Prompt effervescence was indicated catalase production [7].

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 [7]

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 [7].

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 24h [8].

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 [8].

2.3.4.6 Voges-proskaeur 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% α-naphtol and 6% Sodium hydroxide were added to about 1 ml of the broth culture [8].

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 [9].

2.3.4.8 Citrate test

2.4 g of citrate agar was dissolve in 100 mL of distilled water. Ten milliliter (10 ml) of citrate medium was dispensed into each tubes and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by

streaking the organisms once across the surface [8].

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±1°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^{B} and NAUH/172^{A} respectively. The leaves were washed with sterile water, shade dried, ground onto powdered form and store 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℃ 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

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 10 ml 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 10 g of extract in 10 ml of DMSO. This extract were considered as 1000 mg/ml.

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. 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. RESULTS

3.1 Frequency of the Isolates from Infected Diabetic Wound Samples

Staphylococcus aureus were isolated from infected wound samples of patients attending General hospital Onitsha, Anambra State, Nigeria.

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.

3.2 Phytochemical Analysis of the Crude Extracts

Table 2 shows the phytochemical constituent of *B. pinnatum* and *A. indica* extracts. The aqueous *Bryophyllum* extract contains saponin, flavonoids and alkaloids with the absence of steroid, phenol, tannins and cardiac glycoside. The ethanol extract of *B. pinnatum* contains saponin, flavonoids, steroids, alkaloids, tannins, phenol and cardiac glycoside while the extracts of *A. indica* both the ethanol and aqueous extracts contains saponin, flavonoids, steroids, alkaloids, tannins, phenol and cardiac glycoside.

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 *S. aureus* using agar well diffusion method. *A. indica* ethanol extract

Table1. Morphological and biochemical characteristics of bacterial isolates

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

KEY: +: positive; -: negative

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

Constituent		*Bryophyllum pinatum*		*Azadirachta indica*
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
Saponin	+	+	+	+
Flavonoid	+	+	+	+
Steroid	-	+	+	+
Alkaloids	+	+	+	+
Tannins	-	+	+	+
Cardiac glycoside	-	+	+	+
Phenol	-	+	+	+

KEY: +: positive; -: negative

showed diameter of inhibition zone of 14.00 mm, 17.33 mm, 20.00 mm , 20.00 mm and 20.00 mm at 62.5 mg/ml, 125 mg/ml, 250 mg/ml, 500 mg/ml and 1000 mg/ml concentration, *A. indica* aqueous extract showed diameter of inhibition zone of 9.67 mm, 12.67 mm and 13.33 mm at 250 mg/ml, 500 mg/ml and 1000 mg/ml , *B. pinnatum* ethanol extract showed diameter of inhibition zone of 7.33 mm, 10.00 mm and 10.33 mm at 250 mg/ml, 500 mg/ml and 1000 mg/ml but *B. pinnatum* aqueous extract has no inhibitory response on *S. aureus*.

Antimicrobial effects of the different combinations of the extracts on the isolates are shown in tables' 1-5.

4. DISCUSSION

S. aureus was isolated from the wound samples this agreed with the work of [6] which 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 similar with report of [15] 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.

In this study, the *in vitro* antimicrobial activities of the *B. pinnatum* and *A. indica* extracts (aqueous and ethanol) were done on the *S. aureus* isolate separately and combined. The result of the antimicrobial effect shows that *B. pinnatum* aqueous extract has no inhibitory effect on *S. aureus*, but there are inhibitory effect by *B. pinnatum* ethanol extract at 250 mg/ml (7.33 mm), 500 mg/ml (10.00 mm) and 1000mg/ml (10.33 mm). *A. indica* aqueous extract showed inhibitory effect on *S. aureus* at 250 mg/ml (9.67 mm), 500mg/ml (12.67 mm) and 1000 mg/ml (13.33 mm). *A. indica* ethanol extract showed inhibitory effect at 10% concentration (14.00 mm), 125 mg/ml (17.00 mm), 250 mg/ml and 100 mg/ml (20.00 mm) as shown in Table 1. This result agreed with the work of [16] which stated that zone of inhibition of the *A. indica* extracts depends on concentration. It is similar to the work of [17] which revealed that methanolic extracts of *B. pinnatum* can be used against pathogenic organisms including *S. aureus.*

For combination of *A. indica* aqueous extract and *B. pinnatum* ethanol extract, there is inhibitory effect on *S. aureus* at 62.5 mg/ml (11.33 mm), 125mg/ml (14.00 mm), 250 mg/ml (15.00%), 500mg/ml (20.00 mm) and 1000 mg/ml (21.00 mm) as shown in Table 2.

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

The combination of *A. indica* ethanol extract and *B. pinnatum* ethanol showed that there is inhibitory effect on *S. aureus* at every concentration from 62.5 mg/ml (17.67 mm) to 1000 mg/ml (21.00 mm) as shown in Table 4.

Table 3. Antimicrobial effect of extracts at different concentrations on *Staphylococcus aureus*

Concentration(mg/ml)	Mean zone of inhibition of the extracts ±SD (mm)			
	***B.pinnatum*. aqueous extract**	***B.pinnatum*. ethanol extract**	***A.indica* aqueous extract**	***A.indica*. 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 4. 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)						
	62.5 mg/ml	**125 mg/ml**	**250 mg/ml**	**500 mg/ml**	**1000 mg/ml**	**+control**	**-control**
S. aureus	11.33±0.58	14.00±1.00	15.00±0.00	20.00±0.00	21.00±1.00	23.00±0.00	0.00±0.00

Table 5. Antimicrobial effects of combination of *A. indica* and *B. pinnatum* aqueous extracts

Isolate	Mean zone of inhibition of the extracts on the isolates ±SD (mm)						
	62.5 mg/ml	**125 mg/ml**	**250 mg/ml**	**500 mg/ml**	**1000 mg/ml**	**+control**	**-control**
S.aureus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	23.33±0.58	0.00±0.00

Table 6. 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 ±SD (mm)							
	62.5 mg/ml	**125 mg/ml**	**250 mg/ml**	**500 mg/ml**	**1000 mg/ml**	**+control**	**-control**	**TOTAL**
Staphylococcus aureus	17.67±0.58	18.00±0.00	19.33±0.58	21.00±1.00	21.00±0.00	21.33±1.53	0.00±0.00	16.90±7.24

Table 7. 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 ±SD (mm)						
	62.5 mg/ml	**125 mg/ml**	**250 mg/ml**	**500 mg/ml**	**1000 mg/ml**	**+control**	**-control**
S. aureus	17.67±0.58	18.0±0.00	19.3±0.58	21.0±1.00	21.00±0.00	21.33±1.53	0.00±0.00

The combination of *A. indica* ethanol extract and *B. pinnatum* aqueous extract has inhibitory effect on *S. aureus* at all the concentration 62.5 mg/ml (17.33 mm), 125 mg/ml (18.00 mm), 250 mg/ml (19.33 mm), 500 mg/ml (21.00 mm) and 1000 mg/ml (21.00 mm) as shown in Table 5.

This result agreed with [16] which showed that there is antibacterial effect on *S. aureus.* The minimal inhibitory concentration (MIC) of the extracts on *S. aureus* is 500 mg/ml and 1000 mg/ml is the MBC. Therefore, the extracts show equal activity of 1000 mg/ml against the test isolate. This agreed with the report of [12] 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 for *S. aureus* and the plants are readily available and are planted worldwide. The plant extracts can be harnessed for production of drugs.

CONSENT

It is not applicable.

ETHICAL CONSIDERATION

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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