

Original Research Article

In Vitro Antimicrobial Activity Of *Phyllanthus urinaria* (Linnaeus, *Phyllanthaceae*) Leaves Against *Staphylococcus aureus* And *Pseudomonas aeruginosa* Isolated From Wounds

ABSTRACT

The emergence and spread of antibiotic resistance have been on the increase, and as such there is a need for new and safer antimicrobials. Commonly used medicinal plants found in surrounding environments and communities can be used as medicines to treat infections. This research is aimed on exploring the antimicrobial properties of the *Phyllanthus urinaria* plant against selected bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* found in human wounds. The plant extracts were obtained by boiling, soaking and decoctioning the plant leaves. These extracts were subjected to a series of tests for their antimicrobial and active components. The antimicrobial assay was carried out by disc and agar-well diffusion methods. The results indicated that the extract exhibited antimicrobial properties. The highest and only potential was observed in the boiled extract against *S. aureus* with zones of inhibition at 6mm for disc diffusion method and 5mm for agar-well diffusion method at 100mg/mL and 3mm for 25mg/mL and *Pseudomonas aeruginosa* showed complete resistance of the plant extract. The mean efficacy of the extract showed 23.0% and 35.5% in comparison to Chloramphenicol in the agar-well diffusion method and disc diffusion method, respectively. A statistical test was carried out using the one-way ANOVA method, to show the statistically significant differences between the extracts, bacterial isolates, and also zones of inhibition. The results showed that in both the disc and agar-well diffusion methods, p-value was 0.0584; hence there were no statistically significant differences in the effects of the plant extracts on the bacterial isolates. This experiment confirmed the efficacy of the plant extract as a natural potential antimicrobial against *Staphylococcus aureus*.

Keywords: Antimicrobial, Medicinal plant, *Staphylococcus aureus*, *Pseudomonas aeruginosa* .

INTRODUCTION

The incidence of antibiotic resistance among bacteria to synthetic drugs is on the increase, as such there is the need for new and safer antimicrobials especially from natural sources like plants, such as *Phyllanthus urinaria* (leaves) which has been used as medicines to treat infections. *Phyllanthus urinara* commonly called chamber bitter, gripeweed, shatterstone, stonebreaker or leafflower, is a herb species in the family *Phyllanthaceae*. *Phyllanthus urinaria*, one of the species belonging to the genus *Phyllanthus*, is used as a traditional folk medicine for the treatment of several diseases including hepatitis B, nephrolithiasis, and some painful disorders. Extensive studies have shown that this plant exhibits many biological and pharmacological functions *in vitro* and *in vivo*, such as anticancer, cardioprotective, hepatoprotective, antiangiogenic, antioxidant, antisemicarbazide-sensitive amine oxidase, and antihypertensive effects. The colonization of wounds by microorganisms, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* is an important cause of death among patients. When there is a hole in the skin, microorganisms (more often the opportunistic ones) invade and multiply, causing a delay in the wound healing and as such an infection that can lead to asymptomatic colonization, bacteremia, or even death^[1, 2].

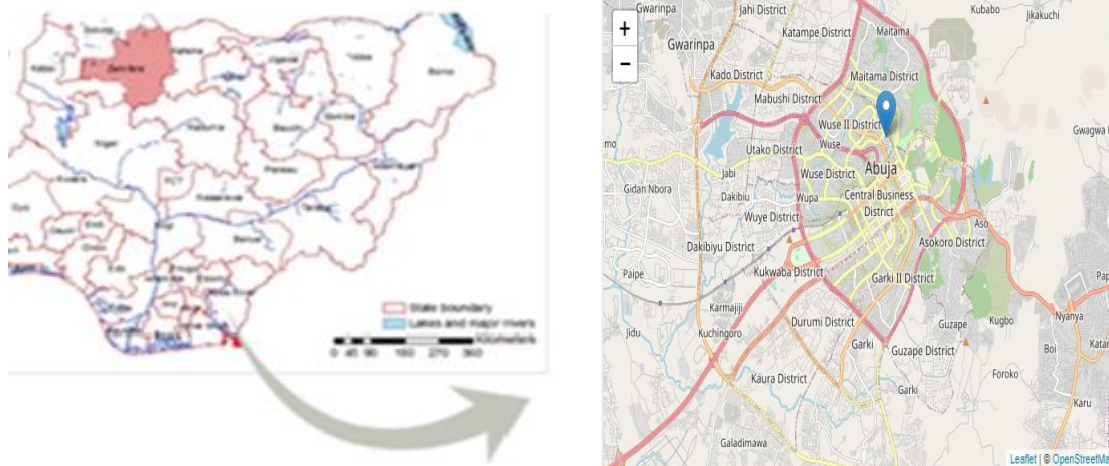
Infectious diseases caused by pathogenic bacteria, have a prevalence rate and morbidity higher than any other pathogenic microorganisms^[3, 4]. *Staphylococcus aureus* is a major human pathogen that causes a wide range of clinical infections. *Pseudomonas aeruginosa* is a common cause of nosocomial infections such as pneumonia, urinary tract infections, and bacteremia^[5]. In general, bacteria have the genetic ability to transmit and acquire resistance to antibiotics, and as such, medicinal plants may offer a new source of antibacterial agents for use^[6].

The continual rise in antibiotic resistance among patients with wound infection has resulted in the search for safer, cheaper and new medicines outside synthetic drugs^[1] and, spiked interest to conduct the study to determine the effectiveness of the *Phyllanthus urinaria* plant extract on wound-enhancing pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Study Area

This study was carried out among patients attending two selected medical health care centers in Abuja, Federal Capital Territory (FCT), Nigeria. The medical centers include: Maitama District Hospital and Garki Hospital, Abuja, located at Abuja Municipal Area Council (AMAC). The FCT is the capital of Nigeria and was formed in 1976^[7]. Abuja covers a total land area of approximately 7315 km², it has a GP coordinate of 9° 4'20.154 and 7° 29'28.6872'E^[8]. The inhabitants are majorly farmers in rural settings.



Sampling site

Abuja central area

Figure 1: Map of Nigeria showing the study area Abuja .

Sample collection and Size

The sample population includes all age groups, a total number of 60 human wound samples were used (deep cuts, open sores and burns). Ethical approval was sought from the Research Ethics Committee of the two selected medical centers. The participants were randomized and their consent was sought before their participation in the study and a consent

form was issued to them accordingly. Wound samples were collected using a sterile swab, with the aid of the medical laboratory assistant, and transferred to the Department of Biological Sciences, Bingham University. The samples were screened using the disc diffusion and agar-well diffusion method after which it was stored for further processing.

Determination of Antimicrobial Activity using Disc Diffusion Method

For the disc diffusion assay 1 ml of each bacterial suspension was evenly spread on a solidified 20 ml Mueller-Hinton agar (MHA). Discs (4mm in diameter) were punched from sheets of Whatmann filter paper, sterilized and impregnated with 20µl each of 100mg/mL plant extract and dried at room temperature for 12 hours. Thereafter, the discs were placed on the surface of inoculated Mueller-Hinton agar plates, and were incubated at 37°C for 24-48 hours for the observation of the formation of inhibition zones around the discs.⁹

Determination of Antimicrobial Activity using Agar-well Diffusion Method

In this method, MHA plates were prepared and inoculated as in the disc diffusion method. Four (4) holes of 6mm in diameter were made on the inoculated MHA plates using a sterile cork borer and the agar discs removed. Holes were aseptically filled with 20µl of 100mg/mL of plant extract by means of microliter pipette, and allowed to diffuse at room temperature for 2 hours and thereafter incubated at 37°C for 24-48 hours. Antibacterial activity was assessed by the appearance of inhibition zone around the hole, without bacterial growth⁹.

BIOCHEMICAL ANALYSIS OF THE ISOLATES

The biochemical screening of the inoculated microorganisms was conducted to confirm the isolates as *S. aureus* and *P. aeruginosa*, respectively.

Catalase Test

The test is used to distinguish microorganisms that produce the catalase enzyme, such as Staphylococci from non catalase producing bacteria such as Streptococci. Catalase enzyme produced by these bacteria will neutralize the hydrogen peroxide and bubbles will be produced indicating a positive test. Mostly, the catalase enzyme is produced by obligate aerobes and facultative anaerobic bacteria. The test is performed by tube or slide method by mixing the

colony of bacteria using a sterile glass rod with few drops of 3% hydrogen peroxide on a slide or to the test tube and looking for bubble formation within 10 seconds^[9]

Active-bubbling indicate a positive catalyst result. This was used to identify *S. aureus* which produces the enzyme catalase.

Coagulase test

In this study, the slide method test was used. A drop of saline on two separate spots was placed on a grease-free slide. Then, a speck of growth of the test organism was picked and emulsified in both spots, to one spot a drop of plasma was added, and to the other a drop of saline was added. Both treatment mixtures were mixed thoroughly by rocking. Coagulation was an indication of a positive test to which plasma was added. The presence of clotting indicates positive test for *Staphylococcus aureus*^[10]. This test was based on our understanding that the microorganism can produce Coagulase enzyme which causes the coagulation of human blood plasma.

Oxidase Test

The oxidase test is helpful in the identification of microorganisms having the ability to produce cytochrome oxidase enzyme. The test helps to differentiate oxidase-positive Pseudomonaceae and negative *Enterobacteriaceae* families. Cytochrome oxidase is based on the principle of transfer of electrons from a donor (Electron transport chain) to a final acceptor (oxygen) and a reduction will take place in the form of water. Cytochrome oxidase will oxidize the electron donor and the color will change to dark purple. This test is performed by impregnation of 1 percent tetra-methyl-p-phenylenediamine dihydrochloride acting as an artificial electron donor into a filter paper and dried^[9]. The bacterial colonies were smeared on a paper strip and checked for color change within 10 seconds, when a blue coloration is formed.

Test is used to check microorganisms that produces the catalase enzyme. Catalase enzyme produced by these

bacteria will neutralize the hydrogen peroxide and bubbles will be produced that are indicative of positive

test. Mostly, catalase enzyme is produced by obligate aerobes and facultative anaerobic bacteria.

PLANT COLLECTION, IDENTIFICATION, EXTRACTION AND PHYTOCHEMICAL SCREENING OF EXTRACTS

Fresh plant material of *P. urinaria* were collected from Bingham University, Karu in June 2019 from the lecturers parking lot. The procedure for plant collection was carried out as described by ^[11] The plant was identified by Dr. Ihuma, J. O., of the Department of Biological Sciences, Bingham University. After identification, the leaves were washed using distilled water to remove dirt and dust^[11]. The aqueous crude extracts were prepared according to the method of Ekpe^[12]. Three methods of extract preparation were used. The boiling, soaking and maceration methods were used. The qualitative screening of the phytochemical constituents of the test plant extract was performed using chemical methods ^[13,]Flavonoids, Tannins, Alkaloids, Glycosides, and Terpenoids were tested.

Plant extract (Boiling extraction method)

The fresh leaves were blended using a sterile electric blender to increase extract concentration. The plant was not be exposed to sunlight so that the active compounds are not lost. The boiling extract was prepared by putting 10g of the blended leaves into a conical flask to which 100ml of distilled water was added. This was heated to boil for one hour using a hot plate and stirred regularly for three to five minutes. After which, the mixture was filtered using Whatmann No 1 filter paper and the supernatant recovered. The extract was stored at 5°C in the refrigerator for further use¹⁴

Plant extract (Soaking extraction method)

The soaking extract was prepared by adding 10g of the blended leaves of the plant to 100ml of the distilled water. The mixture was allowed to stand at room temperature for 48 hours with occasional stirring. The mixture was then filtered using the Whatmann No 1 filter paper to remove the residue. The filtrate was allowed to settle for 30 minutes at room temperature. The extract was therefore stored at 5°C in the refrigerator for further use.¹⁵

Plant extract (Maceration extraction method)

The maceration extract was prepared by adding pieces of the plant leaves to 100ml of distilled water. The mixture was allowed to stand at room temperature for 72 hours with

occasional stirring. The mixture was then filtered to separate the plant material from the liquid. The filtrate was then allowed to sediment and decanted thereafter. The extract was stored in the refrigerator at 5°C for further use¹⁶

Determination of the Minimum Inhibitory Concentration (MIC)

This was carried out by the four-fold serial dilution of the tested extracts in distilled water (2ml volume), then inoculated with 20µl inoculum size with the test organisms. The extracts were prepared at concentrations of 100; 50; 25; 12.5; 6.25% (w/v). The MIC is determined by the broth dilution method. The tubes were incubated for 24 hours at 37°C. The MIC is determined as the lowest concentration of the extract which inhibits the pathogens, in essence, *S. aureus* and *P. aeruginosa*^[17].

Zone Of Inhibition

The Zone of inhibition is a circular area around the spot of the antibiotic in which the bacteria colonies do not grow. The zone of inhibition can be used to measure the susceptibility of the bacteria to the antibiotic^[18].

RESULT

The research showed that all the plant extracts used in this study exhibited a varying degree of antimicrobial activity against all the microorganisms tested.

Table 1 shows the highest zone of inhibition at 6.0mm of the microorganisms against the plant extracts in comparison to the control, in this case, chloramphenicol which was at 18.0mm, using the disc diffusion method. Table 2 shows the highest zone of inhibition at 6.0mm in comparison to the control, chloramphenicol which was at 18.0mm against the microorganisms, using the agar-well diffusion method.

Although *P. aeruginosa* was resistant to all the extracts, *S. aureus* showed zones of inhibition in the boiled extract of the plant, but was resistant to the soaked and macerated extracts of the plant as shown below in Table 1 and 2.

Table 1: Diameter of zones of inhibition (mm) of *Phyllanthus urinaria* extracts against *Staphylococcus aureus* and *P. aeruginosa* at 100mg/mL by disc diffusion method.

Bacterial Isolates	Plant Extracts											
	<i>Phyllanthus urinaria</i> (Boiled) (mm)			<i>Phyllanthus urinaria</i> (Soaked) (mm)			<i>Phyllanthus urinaria</i> (Macerated) (mm)			Control (Chloramphenicol) (mm)		
	Plate 1	Plate 2	Mean Value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value
<i>Staphylococcus aureus</i>	6.0	0	3.0	0	0	0	0	0	0	13.0	18.0	15.5
<i>Pseudomonas aeruginosa</i>	0	0	0	0	0	0	0	0	0	0	0	0

Table: 2 Diameter of zones of inhibition (mm) of *Phyllanthus urinaria* extracts against *Staphylococcus aureus* and *P. aeruginosa* at 100mg/mL by disc argar well difussion methods.

Bacterial Isolates	Plant Extracts											
	<i>Phyllanthus urinaria</i> (Boiled) (mm)			<i>Phyllanthus urinaria</i> (Soaked) (mm)			<i>Phyllanthus urinaria</i> (Macerated) (mm)			Control (Chloramphenicol) (mm)		
	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value
<i>Staphylococcus aureus</i>	5.0	6.0	5.5	0	0	0	0	0	0	13.0	18.0	15.5
<i>Pseudomonas aeruginosa</i>				0	0	0	0	0	0	18.0	16.0	17.0

Minimum Inhibitory Concentration of *Phyllanthus urinaria* Boiled extract on *Staphylococcus aureus*

It was observed that the boiled extract of *P. urinaria* plant was the most effective among the extracts tested. It showed zones of inhibition against the bacterial pathogen *S. aureus*, while there was no activity against *P. aeruginosa*. The effectiveness of the extracts in the tested bacterial pathogens was determined by measuring the Minimum Inhibitory Concentration (MIC). MIC was performed on only *S. aureus*, as it showed a zone of inhibition and was sensitive to the plant extract in the previous antimicrobial assay by disc and agar-well diffusion method. The MIC of *S. aureus* was 50 mg/mL and 25 mg/mL against the boiled extract *P. urinaria* as shown in table

Table 3; MIC values of boiled extract of *P. urinaria* plant against *S. aureus*

MIC Values (mg/mL)	Zone of Inhibition (mm)
100 (stock)	6.0
50	3.0
25	3.0
12.5	-
6.25	-

Key: - = 0.0mm

Phytochemical Analysis of *Phyllanthus urinaria*

The phytochemical analysis of *P. urinaria* plant extracts revealed that flavonoids, tannins, alkaloids and terpenoids are present in the tested extracts, in this case, boiled, soaked, and macerated.

These five phytochemicals are naturally occurring in most plants, and are known to be biologically active and have bactericidal and fungicidal activities, conferring the antibacterial property to the tested plants ^[18].

Table 4; phytochemical screening of *Phyllanthus urinaria* extracts (boiled, soaked and macerated)

Phytochemical Test	<i>Phyllanthus urinaria</i> Extracts		
	Boiled Extract	Soaked Extract	Macerated Extract
Flavonoids	+	+	+
Tannins	+	+	+
Alkaloids	+	+	+
Glycosides	-	-	-
Terpenoids	+	+	+

Key: (+) Positive

(-) Negative

DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

Discussion

The emergence and continuous spread of multi-drug resistant pathogens have substantially threatened the current antibacterial therapy. This has necessitated a search for new and safer antimicrobial substances such as plants as they produce a variety of bioactive compounds with known therapeutic properties^[19]. This research has been conducted to assess the antimicrobial activity of *Phyllanthus urinaria* plant extracts against pathogenic bacteria isolated from human wound samples.

The antimicrobial activity of selected bacterial pathogens (*S. aureus* and *P. aeruginosa*) isolated from wounds was determined with extracts of leaves of the *P. urinaria* plant^[20]. The extracts were obtained by boiling, soaking, and decoction.

A statistical test was carried out using the one-way ANOVA method, to show the significant differences between the extracts, bacterial isolates and also zones of inhibition and the MIC concentrations and zones of inhibition.

The results showed that in both the disc and agar-well diffusion methods, the F_{Cal} was 1.714 and F_{tab} was 0.050, therefore, there were significant differences in the effects of the plant extracts on the bacterial isolates. The alternate hypothesis is accepted which states that there is a significant antimicrobial activity of *Phyllanthus urinaria* leaves against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The results for MIC showed that upon further assay of the antimicrobial effect of the plant extract, the F_{Cal} was 16.506 and F_{tab} was 0.050, which deduces that there is a significant antimicrobial activity of the plant extract, and as such the alternate hypothesis is accepted which states that there is a significant antimicrobial activity of *P. urinaria* leaves against *S. aureus* and *P. aeruginosa* with the antibiotic Chloramphenicol.

In this study, the effect of *P. urinaria* extract showed antimicrobial properties against the bacterial pathogen, *Staphylococcus aureus* ^[21]. However, the extracts of *P. urinaria* showed no antimicrobial properties against *P. aeruginosa* using the various extracts obtained by boiling, soaking, and maceration.

Furthermore, only the plant extract obtained by boiling showed antibacterial properties against *Staphylococcus aureus* in plate 1 of the agar well diffusion method with an antibacterial inhibition of 6.0mm, which in comparison to the Chloramphenicol control that showed an antibacterial inhibition of 13.0mm, showing that the plant extract was 46.2% efficient. In plate 2, there was no inhibition, hence a mean value for inhibition of 3.0, and a total of 23.0% efficiency in comparison to the Chloramphenicol control.

In the disc diffusion method, the extract of *P. urinaria* obtained by boiling showed an antibacterial inhibition of 5.0 and 6.0 in plate 1 and plate 2 respectively. In comparison to Chloramphenicol control, plates 1 and 2 showed 38.5% and 33.3% efficiency., respectively. And a mean value of 35.5%

Using serial dilution, the extract of *P. urinaria* obtained by boiling at 100mg/mL (stock) showed the highest zone of inhibition at 6.0mm, and at 50mg/mL and 25mg/mL, showed a 3.0mm zone of inhibition, below 25 mg/mL there was no effect on the pathogen. Thus, *P. urinaria* extract effectiveness decreases with a corresponding decrease in concentration.

It was observed that the boiled extract of the *P. urinaria* plant was the most effective among the extracts tested. It showed zones of inhibition against the bacterial pathogen *S. aureus*, while there was no activity against *P. aeruginosa*.

It is worthy to note that the only extract method that was effective in this study is the boiling process, and this extract only showed antibacterial activity against *Staphylococcus aureus*. However, in comparison to the control, it showed the highest mean antibacterial inhibition of 35.5% meaning that, the extract is only 46.2% at 100mg/mL effective against the pathogen *Staphylococcus aureus* and 0% effective against *P. aeruginosa*

A statistical test was carried out using the one-way ANOVA method, to check for significant differences between the extracts, bacterial isolates and also zones of inhibition and the MIC concentrations and zones of inhibition.

The results showed that in both the disc and agar-well diffusion methods, the F_{-Cal} was 8.4678 with a p-value of 0.0584, F_{-tab} at 0.05=9.55, 0.01=30.8 $F_{-Cal} < F_{-tab}$ therefore accepting the null hypothesis, which states that there is no significant antimicrobial activity of *Phyllanthus urinaria* leaves against *Pseudomonas aeruginosa*.

The results for MIC showed that upon further assay of the antimicrobial effect of the plant extract, the F_{-Cal} was 16.506 and F_{-tab} was 0.050, which deduces that there is a significant antimicrobial activity of the plant extract, and as such the alternate hypothesis is accepted which states that there is a significant antimicrobial activity of *P. urinaria* leaves against *S. aureus*.

The plant extracts were all positive for four out of the five phytochemical tests conducted. The boiled, soaked, and macerated extracts were positive for flavonoids with a yellow coloration, tannins with a blue-black coloration, alkaloids with an orange precipitate, and terpenoids with a reddish-violet coloration. The extracts were however all tested negative for the glycoside test.

Conclusion

The result showed a potential antimicrobial effect of just the boiled extract of *Phyllanthus urinaria* on *Staphylococcus aureus* whereas, *Pseudomonas aeruginosa*, was resistant to all the extracts. *Phyllanthus urinaria* is not efficacious to be used on open wounds and thus not an effective replacement for chloramphenicol, a recommendation of a further study

of a medicinal plant that has antimicrobial property against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Although shown the potency of this extract *in vitro*, it may not be translated *in vivo*.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

Consent

As per international standard or university standard, Participants' written consent has been collected and preserved by the author(s).

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