Original Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF *Phyllanthusurinaria* LEAVES AGAINST *Staphylococcus aureus* AND *Pseudomonas aeruginosa* ISOLATED FROM WOUNDS

Comment [u1]: Include authority and family

ABSTRACT

The emergence and spread of antibiotic resistance has been on the increase, and as such there is the 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 focused on exploring the antimicrobial properties of *Phyllanthusurinaria* plant against selected bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* found in human wounds. The plant extracts were obtained by boiling, soaking and maceration of 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 3nm for 25mg/ml and *Pseudomonas aeruginosa* showed complete resistance of the plant extract. The mean efficacy of the extract showed 19.4% and 35.5% in comparison to control 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 statistical 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, the F-Cal was 8.4678 and P-value was 0.0584, Ftab at 0.05=9.55 and at 0.01=30.8, F-Cal <Ftab there were no statistically significant differences in the effects of the plant extracts on the bacterial isolates. The experiment confirmed the efficacy of the plant extract as a natural potential antimicrobial.

KEY WORD: Antibiotic, Resistance, Antimicrobial, Medicinal, Environment, Pathogen and Infection.

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 *Phyllanthusurinaria* (leaves) which can be used as medicines to treat infections. The colonization of wounds by microorganisms, such as *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) is an important cause of death among patients[1]. When there is a hole in the skin, microorganisms (more often the opportunistic ones) invade and multiply, causing delay in the wound healing and as such an infection which can lead to asymptomatic colonization, bacteremia or even death [2].

Comment [u2]: Are they resistance to standard drugs used in wound healing treatment? How did you identify the organisms? Is it a strain? If it is a strain, it should have a number.

Comment [u3]: What is the difference between soaking and maceration? How long did you do that? Why did you choose to boil (decoction)? Was this how it was used locally by ethnomedicinalpractitioneers?

Comment [u4]: 100 mg/mL.do same to all

Comment [u5]: mm

Comment [u6]: Values not correct=23%

Comment [u7]: Did you compare with a standard drug (positive control)? If yes state the name of the drug used and at what concentration?

Comment [u8]: Add a comma after method e.g method, respectively

Comment [u9]: This statement should be under methodology before stating the result for this study

Comment [u10]: Only state the significant difference

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Comment [u13]: Your Conclusion is not in agreement with the aim of the study. Are you trying to justify the use of your research plant as antimicrobial agent as reported in a locality or ethnomedicinally? State clearly the organism that the plant extract was active against because from your results, you stated that there was complete resistance to the extract by P. aeruginosa.

Comment [u14]:

Comment [u15]: Your keywords does not follow your research. I suggest: Antimicrobial, medicinal plant, S. aureus, P. aeruginosa,

Comment [u16]: The research plant in focus should be discussed separately stating the authorities and family.

Comment [u17]: Can be used or reported to have antimicrobial potentials?

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Infectious diseases are still one of the main causes of death in the world, in spite of the great advances in medicinal drugs [3]. Bacteria are considered as a group of the microoganisms that cause the most deadly diseases and widespread epidemics of human civilization. Infectious diseases caused by pathogenic bacteria, have prevalence rate and morbidity more than other pathogenic microorganisms[4]. *Staphylococcus aureus* (*S. aureus*) is a major human pathogen that causes a wide range of clinical infections. *Pseudomonas aeruginosa*(*P. 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 reisistance 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]. The result of the continuous use of the same synthetic drugs without any enhancement would result in more mortality rate and evolving of pathogens.The knowledge of this fact has spiked up interest to conduct the study to determine the effectiveness of the *Phyllanthus urinara* plant extract on wound enhancing pathogens, in essence, *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Comment [u21]: Harmonize your statements because is alomost saying the same thing (Reference 1-4).

Comment [u22]: delete

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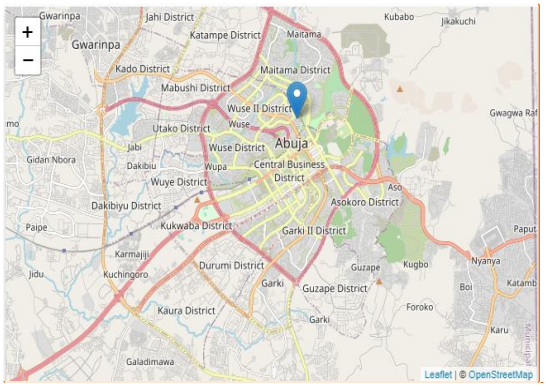
Comment [u24]: still talking about what you said already in above

Comment [u25]: General comment: your introduction does not flow. It is disjointed and not comprehensive.

MATERIALS AND METHODS

Study Area

This study was carried out among patients attending two selected medical health care centres in Abuja, Federal Capital Territory (FCT), Nigeria. The medical centres 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 sq.km, it has a GP coordinate of 9^{o} 4’20.154 and 7^{o}29’28.6872’E [8]. The inhabitant are majorly farmers in the rural settings.



Sampling site

Abuja central area

Figure 1: Map of Abuja (FCT) showing the study area.

Sample collection and Size

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

BIOCHEMICAL ANALYSIS OF THE ISOLATESS

The biochemical screening of the innoculated microorganisms was conducted to confirm the isolatess as *S. aureus* and *P. aeruginosa* respectively.

Catalase Test

Test is used to check 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 that are indicative of positive test. Mostly, catalase enzyme is produced by obligate aerobes and facultative anaerobic

Comment [u26]: I think you should also add side by side a map of Nigeria indicating Abuja your study area.

Comment [u27]: Nothing like map of Abuja...Map of Nigeria showing the study area (Abuja).

Comment [u28]: Does the ethical approval has a number?

Comment [u29]: fullstop

Comment [u30]: delete. State this under the method

Comment [u31]: check spelling

Comment [u32]: a comma before the word respectively.

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 slide or to the test tube and looking for bubble formation within 10 seconds[9]

Active bubbling indicate a postive catalyst result.This waas used to identify *S. aureus* which produces the enzyme coagulase

Comment [u33]: is it only S. aureus that generates active bubbling with this test or causes coagulation?

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 treatments mixtures were mixed thoroughly by rocking. Coagulation was an indication of 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 has the capability to produce Coagulase enzyme which causes the coagulation of human blood plasma.

Oxidase Test

Oxidase test is helpful in the identification of microorganisms having ability to produce cytochrome oxidase enzyme. The test helps to differentiate oxidase positive Pseudomonacea and negative Enterobacteriacea families. Cytochrome oxidase based on the principle of transfer of electrons from donor (Electron transport chain) to final acceptor (oxygen) and reduction will takes 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-phenylenediaminedihydrochloride acting as artificial electron donor into a filter paper and dried[9]. The bacterial colonies were smeared on paper strip and check for color change within 10 seconds and a blue coloration was formed.

Comment [u34]: How did you identify that the species is aeruginosa?

PHYTOCHEMICAL SCREENING OF EXTRACTS

Fresh plant material of *P. urinaria* were collected according to [11], and were identified accordingly. After identification, the leaves were washed using distilled water to removed dirt and dust[1]. The aqueous crude extracts were prepared according to the method of Ekpe[12]. Three

Comment [u35]: Plant Collection and Extraction. Phytochemical screening should be on its own as a heading. The plant was collected from where? Who identified it? Where was the voucher specimen deposited? Does it have authentication number? You dint describe the preparation methods of your extract. how long did you boil it? What gram of the leave did you extract? did you weigh the plant separately for the three methods adopted? In your soaking and maceration methods, how many days or hours did you do that? What solvents did you use? Is very important you state them appropriately.

methods of extract preparation were used. The boiling, soaking and maceration methods were used to The qualitative screening of the phytochemical constituents of the test plant extract was performed using chemical methods according to Harbone [13]Flavonoids, Tannins, Alkaloids, Glycosides and Terpenoids were tested.

Determination of the Minimum Inhibitory Concentartion (MIC)

In the case where *P. urinaria*extracts exhibited high activity against the isolatesd pathogenic microorganisms, (*S. aureus* and *P. aeruginosa*), it was futhered assayed for its 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 innoculum 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^{o}C. The MIC is determined as the lowest concentration of the extract which inhibits the pathogens, in essence, *S. aureus* and *P. aeruginosa*[14].

Comment [u36]: You dint state the disc diffusion and agar-well diffusion method under your methods.
Also under your methods, you dint state the material used and the grade of solvents and reagents used.

Comment [u37]: Just state the method used for MIC. During your discussion of results, you can say this.

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 towards the antibiotic[15].

RESULT

Microorganisms were identified and isolated using catalase, coagulase and the oxidase biochemical tests.

Evaluation of the antimicrobial activity of *Phyllanthusurinaria*extracts; boiled, soaked and macerated was determined initially by the disc and agar-well diffusion method against bacterial pathogens, *S. aureus*and *P. aeruginosa.* These microorganisms were found present in human wound. The research showed that all the plant extracts used in this study exhibited a varying degree of antimicrobial activity against all the microorganisms tested.

Comment [u38]: This already stated in the methods. It dosen't have business here

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 and agar well difussion method respectively.

	Plant Extracts											
Bacterial Isolates	*Phyllanthus urinaria* (Boiled) (mm)			*Phyllanthus urinaria* (Soaked) (mm)			*Phyllanthus urinaria* (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
Staphylococcus aureus	6.0	0	3.0	0	0	0	0	0	0	13.0	18.0	15.5
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0
	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean value	Plate 1	Plate 2	Mean Value
Staphylococcus aureus	5.0	6.0	5.5	0	0	0	0	0	0	13.0	18.0	15.5
Pseudomonas aeruginosa				0	0	0	0	0	0	18.0	16.0	17.0

Comment [u39]: Create separate tables for **disc diffusion method and agar well difussion method. It would have been better if you did your experiment in triplicates. From your abstract, you stated you used anova but your results does not indicate any statistics done. Mean±SD/SEM. The images of the respective plates would have complement your results.**

Comment [u40]: You cannot have 6 mm zone of inhibition in plate 1 and have complete resistance in plate 2.

Phytochemical Analysis of *Phyllanthusurinaria*

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 [15].

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 of known therapeutic properties[16]. This research has been conducted to assess the antimicrobial activity of *Phyllanthus urinaria* plant extracts against pathogenic bacteria isolatesd from human wound samples.

The antimicrobial activity of selected bacterial pathogens (*S. aureus*and *P. aeruginosa*) isolatesdfrom wound was determined with extracts of *P. urinaria* plant in accordance to[17].The leaves of the plant were used to extract. The extracts were obtained by boiling, soaking and maceration.

A statistical test was carried out using the one way ANOVA method, to show the significant differences between the extracts, bacterial isolatess 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 isolatess. The alternate hypothesis is accepted which states that there is 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 significant antimicrobial activity of the plant extract, and as such the alternate hypothesis is accepted which

Comment [u41]: Generate a table stating the test phytochemicals and the presence or absence corresponding to the degree of abundance using signs like +,++,+++,-,-/+

Comment [u42]: This will fit in when discussing the phytochemical components of your research plants. Mind you, the degree of abundance of the various tested components will definitely vary. The variability could be attributed to the method used in extraction. State authority that might justify the variability.

Comment [u43]: isolated

Comment [u44]: delete

Comment [u45]: not a complete statement...

Comment [u46]: correct term is decoction

Comment [u47]: soaking and maceration is not different

Comment [u48]: MIC not represented in your result

states that there is significant antimicrobial activity of *P. urinaria* leaves against *S. aureus* and *P. aeruginosa.*

In this study, the effect of *P. urinaria* extract showed antimicrobial properties against the bacterial pathogens\, *Staphylococcus aureus* in accordance with [17].However,the extracts of *P. urinaria*showed no antimicrobial prperty against *P. aeruginosa* using the various extracts obtained by by boiling, soaking and maceration.

Futhermore,only the plant extract obtained by boiling showed antibacterial property against *Staphylococcus aureus* in plate 1 of agar well diffusion method with an antibacterial inhibition of 6.0nm, which in comparism to the control showed an antibacterial inhibition of 13.0nm, showing that the plant extract was 46.2% efficient. In plate 2, there was no inhibition, hence a mean value for inhibition 3.0, and a total of 19.4% efficiency in comparism to the control.

In the disc diffusion method, the extract of *P. urinaria* obtained by boiling showed an antibaterial inhibition of 5.0 and 6.0 in plate 1 and plate 2 respectively. In comparism to control, plate 1 and 2 showed 38.5% and 33.3% efficiency. And a total 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 6.0nm, and at 50mg/ml and 25mg/ml, showed a 3.0nm zone of inhibition, below 25mg/ml there was no effect on the pathogen. Thus, *P. urinaria* extract effectiveness decreasess with a corresponding decrease in concentration.

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*

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 comparism to the control, it has showed the highrst 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 isolatess 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 Pvalue of 0.0584,F_{-tab} at 0.05=9.55, 0.01=30.8F_{-Cal}< F_{-tab}therefore accepting the null

Comment [u49]: Is not explicit. FtabFcal is not necessary. The results of your assay not good enough as compared to the standard. No significant activity as you claimed. Is better you delete the statement and just state your result based on the zone of inhibition. You dint do MIC. Are there no other studies done with your research plant on other microorganism that you can discuss your findings with i.e if it corroborates or not.

Comment [u50]: delete

Comment [u51]: what was the accordance with? State it

Comment [u52]: check spelling

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Comment [u54]: correct terms (decoction)

Comment [u55]: mm

Comment [u56]: mention the control as positive control and the name of the drug in bracket.

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hypothesis,which states that there is no 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 significant antimicrobial activity of the plant extract, and as such the alternate hypothesis is accepted which states that there is significant antimicrobial activity of *P. urinaria* leaves against *S. aureus* and *P. aeruginosa*.

Comment [u65]: MIC not done. I already talked about your statistics above

The plant extracts were all positive for four out of the five phytochemical tests conducted. The boiled, soaked and macertaed 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.

Comment [u66]: If contained the tested components in all. Then why the variability in results? Have you asked yourself why?

Conclusion

In this study, the antimicrobial activity of *P. urinaria*plant was assessed by disc diffusion and agar-well diffusion methods. The result showed potential antimicrobial effect of just the boiled extract of the plant on *S. aureus*, whereas, *P. aeruginosa*was resistant to all the extracts. Although shown the potency of this extract *in vitro*, it may not be translated *in vivo*.

Comment [u67]: We don't need this here. We already know the method you used. Though not stated under the methods

Comment [u68]: Mention the name of plant in full (Scientific name please).

Comment [u69]:

Comment [u70]: Write the name of organism in full in your conclusion

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Comment [u71]: Italise scientific words. Species always start with small letters. Do same to all. Your conclusion is not well packaged. Since your extract is not as effective as the standard, it should not be recommended in wound healing.

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