Original Research Article

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

ABSTRACT

The emergence and spread of antibiotic resistance ~~has~~ havebeen on the increase, and as such, there is ~~the~~ aneed 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 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 maceratingon~~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~~ 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, the F-Cal was 8.4678 and the p~~P~~-value was 0.0584,Ftab at 0.05=9.55,and at 0.01=30.8,F-Cal <Ftabthere 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.

Keywords~~EY 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 *Phyllanthus urinaria*(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 a delay in the wound healing and as such an infection ~~which~~ thatcan lead to asymptomatic colonization, bacteremia, or even death [2].

Infectious diseases are still one of the main causes of death in the world, ~~in spite of~~despite the great advances in medicinal drugs [3]. Bacteria are considered ~~as~~ a group of ~~the~~ microorganisms that cause the most deadly diseases and widespread epidemics of human civilization. Infectious diseases caused by pathogenic bacteria~~,~~ have a prevalence rate and morbidity higher~~more~~ than any 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 re~~i~~sistance 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 the ~~evolving~~ evolutionof pathogens. The knowledge of this fact has spiked ~~up~~ interest to conduct the study to determine the effectiveness of the *Phyllanthus urinaria* plant extract on ~~wound~~ wound-enhancing pathogens, in essence, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Study Area

This study was carried out among patients attending two selected medical health care ~~centres~~ centers in Abuja, Federal Capital Territory (FCT), Nigeria. The medical ~~centres~~ 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 ~~sq.~~km^2, it has a GP coordinate of 9^o 4’20.154 and 7^{o}29’28.6872’E [8]. The inhabitants are majorly farmers in ~~the~~ rural settings.

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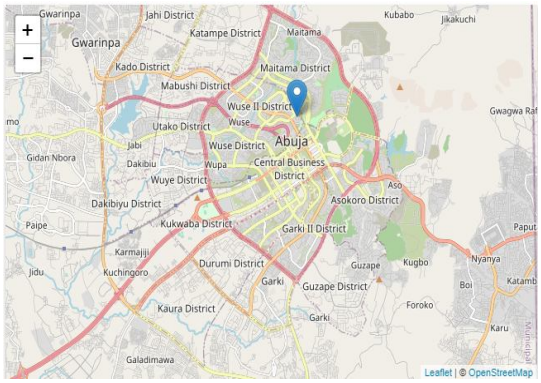

Sampling site

Abuja central area

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

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 ~~Commettiee~~ Committee of the two selected medical ~~centres~~centers. The participants were randomized and their consent was sought ~~for~~ 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.

BIOCHEMICAL ANALYSIS OF THE ISOLATE~~S~~S

The biochemical screening of the i~~n~~noculated microorganisms was conducted to confirm the isolate~~s~~s as *S. aureus* and *P. aeruginosa* respectively.

Catalase Test

The test is used to ~~check~~ distinguishmicroorganisms that produce the catalase enzyme, such as ~~s~~*S~~s~~taphylococci* from non-~~catalase~~ catalase-producing bacteria such as *S~~s~~treptococci*. Catalase enzyme produced by these bacteria will neutralize the hydrogen peroxide and bubbles will be produced ~~that are indicative of~~indicatinga positive test. Mostly, the catalase enzyme is produced

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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 a few drops of 3% hydrogen peroxide on a slide or to the test tube and looking for bubble formation within 10 seconds[9]

.ActiveActive bbubbling indicates a positive catalyst result.This waas used to identify *S. aureus* which produces the enzyme coagulasecatalase.

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 a positive test to which plasma was added. The presence of clotting indicates a positive test for *Staphylococcus aureus*[10] thisThis test was based on our understanding that the microorganism has the capability tocan produce cCoagulase 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 oxidase-positive*Pseudomonacea* 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 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-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 and whena blue coloration iswas formed.

PHYTOCHEMICAL SCREENING OF EXTRACTS

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Fresh plant material of *P. urinaria* ~~were~~ was collected and identified accordingly ~~according to [11], and were identified accordingly~~. After identification, the leaves were washed using distilled water to remove~~d~~ dirt and dust[1]. 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 ~~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~~ Concentration (MIC)

In the case where *P. urinaria*extracts exhibited high activity against the isolate~~s~~d pathogenic microorganisms, (*S. aureus* and *P. aeruginosa*), it was further~~ed~~ assayed for its Minimum Inhibitory Concentration (MIC). This was carried out by the ~~four~~ four-fold serial dilution of the tested extracts in distilled water (2ml volume), then inoculated with 20µl i~~n~~noculum 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].

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~~wards~~ the antibiotic[15].

RESULT

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

Evaluation of the antimicrobial activity of *Phyllanthus urinaria*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 the 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.

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~~diffusion 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

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 [15].

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

The antimicrobial activity of selected bacterial pathogens (*S. aureus* and *P. aeruginosa*) isolate~~s~~d from wounds was determined with extracts of leaves of the *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~~ one-way ANOVA method, to show the significant differences between the extracts, bacterial isolate~~s~~s, 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 isolate~~s~~s. 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*.

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 properties against *P. aeruginosa* using the various extracts obtained by by boiling, soaking, and maceration.

Furthermore,only the plant extract obtained by boiling showed antibacterial property properties against *Staphylococcus aureus* in plate 1 of the agar well diffusion method with an antibacterial inhibition of 6.0nm, which in comparism comparisonto the Chloramphenicol control that 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 of 3.0, and a total of 19.4% efficiency in comparism comparisonto the Chloramphenicolcontrol.

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 comparism comparisonto Chloramphenicolcontrol, plates 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* extracts effectiveness decreasess 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 comparism comparisonto the control, it has showed the highrst 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 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 p-~~P~~value of 0.0584,F_{-tab} at 0.05=9.55, 0.01=30.8F_{-Cal}< F_{-tab}therefore accepting the null 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 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*~~.

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

In this study, the antimicrobial activity of the *P. urinaria*plant was assessed by disc diffusion and agar-well diffusion methods. The result showed a 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*.

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