



Antibiotic Profiling and Antibacterial Activity of *Phyllanthus amarus* Against Klebsiella Species Isolated from Urine Samples at Orisun Clinic, Kwara State, Nigeria

*Ali, H.M., & Kannike, A.

Department of Biological Sciences, Nigeria Police Academy, Wudil, Kano State, Nigeria

*Corresponding author email: haudiza@yahoo.com

Abstract

The development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of Klebsiella was a major challenge to health care management, mostly in Nigeria. The study was an examination of the antibacterial activity and the antibiotic profiling of *Phyllanthus amarus* (commonly known as stonebreaker or “Eyin Olobe” in Yoruba) extracts (both ethanolic and aqueous) against clinical isolates of Klebsiella recovered from urine samples at Orisun Clinic in Kilanko, Ilorin. Fifty urine samples were examined, and nine of them gave rise to Klebsiella spp. isolation. The susceptibility testing against antibiotics indicated that eight isolates indicated multidrug resistance and three evidenced extensive drug resistances with resistance against all eight antibacterial drugs tested (CAZ, CRX, GEN, CPR, OFL, AUG, NIT, AMP). Antibacterial activity of *P. amarus* was determined by applying aqueous and ethanolic extracts at 500 mg/mL, 400 mg/mL, 300 mg/mL, 250 mg/mL, 200 mg/mL, and 100 mg/mL concentrations, each diluted with dimethyl sulfoxide (DMSO) on inoculated agar plate. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration were determined by broth dilution method, whereby MIC was defined as lowest concentration with no evident growth and MBC as lowest concentration which entirely inhibited colony development on nutrient agar. Inhibition zone at 250 mg/mL was 35.0 mm for aqueous and 36.5 mm for ethanolic extracts. The minimum inhibitory concentration was 100 mg/mL for both extracts, and the minimum bactericidal concentration was 200 mg/mL for aqueous and 100 mg/mL for ethanolic extract. Statistical analysis with SPSS v25 confirmed significant antibacterial activity ($p < 0.05$). The study concludes that *P. amarus*, and particularly ethanolic extract, exhibits potential antibacterial activity against drug-resistant Klebsiella spp. due its large inhibition zones and low MIC and MBC values and may be the foundation for the development of new plant-based medicines.

Keywords: *Phyllanthus Amarus*, *Klebsiella Pneumoniae*, Antibacterial Activity, Antibiotic Profiling, Aqueous Extraction

Introduction

Klebsiella species are opportunistic pathogens associated with UTIs, pneumonia, septicemia, and wound infections. Pathogenic nature is therefore complemented with traits like capsules, fimbriae, and lipopolysaccharides that allow for their evasion from host immunity and coexistence in hospitals (Chen et al., 2024). The emergence of multidrug-resistant (MDR) and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains has become a major global concern, leaving very limited treatment options and contributing to higher mortality. The World Health Organization (WHO, 2022) has categorized carbapenem-resistant Enterobacteriaceae, including Klebsiella, as critical priority pathogens requiring urgent research into new therapies. The wrong use of antibiotics, low levels of diagnosis facilities, and the absence of novel antimicrobial drugs in sub-Saharan Africa have deepened this crisis. There has, therefore, been increasing interest in complementary therapies, specifically in herbal medicine that is known for its ethnopharmacological efficacy. An example is the small plant *Phyllanthus amarus*, traditionally used in Africa and Asia to manage conditions like hepatitis, malaria, diabetes, and urinary tract problems. Recent research has confirmed that *P. amarus* is blessed with diverse bioactive molecules, specifically lignans, alkaloids, tannins, and flavonoids, that have been associated with its antimicrobial, antioxidant, and anti-inflammatory activities (Ghosh et al., 2022). Antibiotic resistance in Nigeria is rising from the implementation of non-curative regimes of treatment, the presence of self-medication use, and deficiencies in infection control practices. These predispose towards exchanging resistant microbes for higher healthcare cost and increased patient mortality rate. Low rate of

innovation in antibiotics accomplishes the goal of exploring why it is essential to discover natural products for the use of alternatives or adjunct therapy regimes (Afolayan et al., 2021).

Phyllanthus amarus, or "stonebreaker," is part of the Phyllanthaceae and is widely distributed in the tropical regions of the globe. Historically used for infection and metabolic disease, its use is empirically established with proof of its antimicrobial efficacy for *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Staphylococcus aureus*. Ethanol extracts of *P. amarus* had strongly antibacterial character in relation to water extracts due to bioavailable phytochemical concentrations that turned out to be higher. Citing Patel et al. (2011), the selection of solvent is key to the effectiveness of the extraction, ethanol for solubilization and bioavailability of molecules that may prove antimicrobial potential. This current research work sought to establish the antibacterial activities of both water and ethanolic extracts of *Phyllanthus amarus* in comparison with clinical isolates of *Klebsiella* from urine in Kwara State, Nigeria. The study tries to identify the best working extract and assign phytotherapeutic potential for the possible use of *P. amarus* as an antibiotic substitute.

Aim and Objectives of the Study

The aim of the study was to investigate the antibiotic profiling and antibacterial activity of aqueous and ethanolic extracts of *Phyllanthus amarus* against clinical isolates of *klebsiella* from kwara state, Nigeria. Specific objectives:

1. isolation and identification of Klebsiella spp. from urine.
2. determining antibiotic susceptibility of isolates of Klebsiella.
3. preparation of aqueous and ethanolic extracts of *Phyllanthus amarus*
4. determination of the antibacterial activity of *P. amarus* extracts through different concentrations.
5. evaluating MIC and MBC of the extracts.
6. comparing the antibacterial activity of aqueous and alcoholic extracts.

Materials and Methods

Study Design

The research employed a laboratory-centred experimental design alongside cross-sectional sampling of clinical materials. The research design enabled the isolation of the clinical material samples from the patient and the determination of the antibacterial activity of the crude extracts of *Phyllanthus amarus* in controlled laboratories. The data were gathered in both qualitative and quantitative forms.

Study Area

The research was carried out at the microbiology unit of the Manzanita Research and Diagnostic Laboratory at Ilorin, Nigeria. It is a diagnostics and research-oriented facility that is set up for conducting microbial analyses and supplies different clinical samples. The plant material employed in the work during research, *Phyllanthus amarus*, was collected from one of the shops selling herbs and confirmed by a taxonomist in the department of plant biology, university of Ilorin.

Sample Size Determination

The samples under study were in two groups: clinical urine samples and bacterial isolates.

Urine samples (patients): Using the single proportion formula:

$$[n = Z^2 p^2 / d^2]$$

Where: - n = sample size - Z = standard normal deviate at 95% confidence level (1.96) - p = estimated prevalence of *Klebsiella pneumoniae* in urinary tract infections (assumed 18% based on recent Nigerian studies) - d = margin of error (0.10)

$$[n = 47.3 \approx 50.0]$$

Thus, a minimum of 50 urine samples was collected for adequate representation.

Sample Collection

Midstream urine samples were collected from patients attending the orisun clinic, kilanko, ilorin. Patients provided informed consent and completed a short questionnaire capturing demographic information and medical history. Samples were collected in sterile containers and transported on ice to the laboratory within two hours.

Isolation and Characterization of *Klebsiella pneumoniae*

Clinical specimens were initially cultured to isolate species of the genus *Klebsiella* in selective and differential media. The culturing process began with MacConkey agar, which supports the growth of Gram-negative bacteria while inhibiting Gram-positive ones. It also helps to differentiate lactose fermenters from non-lactose fermenters. After incubation for 24 hours at 37°C, *Klebsiella* species produced pink to red mucoid colonies, indicating lactose fermentation (Jung & Hoilat, 2024). For additional differentiation, Eosin Methylene Blue (EMB) agar was used.

In such an environment, bacteria in the genus *Klebsiella* characteristically produce colonies that have a dark appearance with a metallic green luster, thus supporting their classification as lactose fermenters. Colonies that had such properties were then sub-cultured on fresh nutrient agar plates to obtain pure isolates. Then, the pure cultures went for Gram staining in an effort to establish cell wall characteristic and shape. Under the microscope, *Klebsiella* species were observed to appear Gram-negative (pink in color) short rods, thus confirming their fundamental morphological traits. For improved recognition of the isolates, several biochemical analyses were performed in the following manners:

Indole Test: Shows whether the bacterium has the ability to synthesize indole from the amino acid tryptophan. Negative for *Klebsiella* species, which separates them from indole-positive bacteria such as *Escherichia coli*.

Urease Test: This assay evaluates the synthesis of the enzyme urease, which facilitates the hydrolysis of urea into ammonia and carbon dioxide. A positive outcome is observed with *Klebsiella*, resulting in a pink coloration attributable to an alkaline response.

Citrate Utilization Test: Citrate Utilization Test was used in measuring the organism's ability to use citrate as its sole carbon source. *Klebsiella* species are citrate positive, turning the medium blue. For their **final verification**, the isolates were examined with API 20E identification strips (bioMérieux, France). This is a commercially available system that involves 20 small biochemical tests for identifying Enterobacteriaceae and certain Gram-negative bacteria. Following incubation, the colour reaction on the strip was read from the manufacturer's database and used to confirm the isolates to be of the species of the genus *Klebsiella* (Al-Agha et al., 2017)

Preparation of Botanical Extracts

Fresh *Phyllanthus amarus* plants were collected, washed, shade-dried, and ground into fine powder (Aliyu et al., 2021). Extraction was performed using aqueous and ethanol solvents (70%). About 100 g of powdered material was soaked in 500 mL of solvent for 72 hours with occasional shaking and extracts were filtered and concentrated using a rotary evaporator, dried extracts were weighed, packaged, and stored in airtight sterile containers until use.

Preparation of Stock Solutions and Dilutions

A 500 mg/mL stock was prepared by dissolving 5 g dried extract in 10 mL dimethyl sulfoxide (DMSO). Vortexing and gentle heating ($\leq 40^{\circ}\text{C}$) aided dissolution. The solution was filtered aseptically ($0.22\ \mu\text{m}$) and aliquots were stored at -20°C . Working solutions of 400, 300, 200, and 100 mg/mL were prepared from the 500 mg/mL stock using the equation ($C_1/V_1 = C_2/V_2$). For instance, 10 mL 300 mg/mL was prepared by 6 mL stock + 4 mL diluent. MIC and Minimum Bactericidal Concentration (MBC) tests were carried out by Mueller-Hinton Broth (MHB) so the final concentration in the wells was at or below 1% DMSO. A control with only pure DMSO was added to compensate for any potential solvent effect (Summer et al., 2022).

Antibacterial Susceptibility Testing

Agar Well Diffusion: Mueller-Hinton agar plates contained 0.5 McFarland suspensions of isolates. 6 mm wells were cut out and 50 μL volumes of extract at different concentrations (100–500 mg/mL) added to them. Plates were incubated at 37°C for 24 hours and the zones of inhibition were measured in mm (Balouiri et al., 2016).

Minimum Inhibitory Concentration (MIC): Broth microdilution in 96-well plates was used. Extract concentrations (two-fold dilutions) were mixed with bacterial suspensions ($\sim 5 \times 10^5$ CFU/mL final). Wells were incubated at 37°C for 20 hours. The minimum inhibitory concentration with no apparent turbidity was MIC (Rodríguez-Melcón et al., 2021).

Minimum Bactericidal Concentration (MBC): The well aliquots at and above the MIC were sub-cultured to Mueller-Hinton agar plates. The minimum concentration with suppressed apparent growth after incubation for 24 hrs was considered to be MBC (Mogana et al., 2020).

Antibiotic Profiling

Characterization of Susceptibility to 8 antibiotics was determined using the Kirby–Bauer disk diffusion method on Mueller-Hinton agar following CLSI (2021) guidelines. - Antibiotics tested: Ceftazidime (CAZ, 30 μg), Cefuroxime (CRX, 30 μg), Gentamicin (GEN, 10 μg), Ciprofloxacin (CPR, 5 μg), Ofloxacin (OFL, 5 μg), Amoxicillin-clavulanate (AUG, 30 μg), Nitrofurantoin (NIT, 300 μg), and Ampicillin (AMP, 10 μg). - Zone diameters were measured and classified as Sensitive, Intermediate, or Resistant according to CLSI breakpoints.

Multi-drug resistance (MDR) was defined as non-susceptibility to ≥ 1 agent in ≥ 3 antibiotic classes, while extensively drug resistance (XDR) was defined as resistance to all except one or two classes (Magiorakos et al., 2012). Patterns were tabulated by isolate. Quality control was performed by use of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

Data Analysis

Data were entered in version 25 of the SPSS package for analysis. Descriptive statistics (means and standard deviations) were computed for inhibition zones, MIC and MBC readings. Chi-square tests were used in analyzing associations of resistance patterns and demographic factors. One-way ANOVA and post-hoc tests determined the significances of the differences in antibacterial activity in different concentrations. Significance was set at the level of a value $p < 0.05$.

Ethical Considerations

Informed written consent was obtained from all the participants before the collection of the samples. Anonymity and confidentiality were ensured carefully in order not to disclose the confidentiality of the participants during the study.

Results

This chapter sets forth the research results on antibacterial activity and antibiotic profiling of *Phyllanthus amarus* extracts against clinical isolates of *Klebsiella pneumoniae*. The data were processed using SPSS version 25, and the results are represented in descriptive table and statistical output. Important results are prevalence of *Klebsiella pneumoniae*, antibacterial activity of extracts, determinations of MIC and MBC, and profiles of antibiotic susceptibility.

Prevalence of *Klebsiella pneumoniae*.

Out of the 50 urine samples collected from patients at orisun clinic, kilanko, ilorin, 9 (18%) provided *Klebsiella pneumoniae*.

Table 1: Prevalence of *Klebsiella pneumoniae* urine samples

Total Samples	Positive for <i>K. pneumoniae</i>	Prevalence
50	9	18

Antibacterial Activity of Extracts
The zones of inhibition (mm) of both ethanolic and aqueous extracts were determined at concentrations ranging from 100–500 mg/mL.

Table 2: Antibacterial Activity of Extracts with Zones of Inhibition (mm)

Concentration (mg/mL)	Aqueous Extract (mm)	Ethanolic Extract (mm)
500	38.5 ± 1.2	40.0 ± 1.5
400	37.0 ± 1.0	39.0 ± 1.1
300	36.5 ± 0.8	38.0 ± 0.9
250	35.0 ± 0.7	36.5 ± 0.8
200	0.0 ± 0.6	35.0 ± 0.7
100	0.0 ± 0.5	25.0 ± 0.5

Ethanolic extracts invariably showed higher antibacterial activity compared to aqueous extracts.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Both of the extracts maintained MIC values of 100 mg/mL. MBC was 200 mg/mL and 100 mg/mL for aqueous and ethanolic extracts, respectively, reflecting greater bactericidal activity for the latter.

Table 3: MIC and MBC

Extract Type	MIC (mg/mL)	MBC (mg/mL)
Aqueous	100	200
Ethanolic	100	100

Antibiotic Susceptibility Profiles of *K. pneumoniae*

Among the 9 *Klebsiella* isolates:

8 were multidrug-resistant (MDR) with resistance against ≥ 3 classes of antibiotics.

3 were extensively drug-resistant (XDR) with resistance against all 8 tested antibiotics.

Table 4: Antibiotic Susceptibility Profiles of *K. pneumoniae*

Antibiotic Code	Antibiotic	Sensitive	Resistant
CAZ	Ceftazidime(30 μ g)	2	7
CRX	Cefuroxime (30 μ g)	1	8
GEN	Gentamicin (10 μ g)	3	6
CPR	Ciprofloxacin (5 μ g)	4	5
OFL	Ofloxacin (5 μ g)	3	6
AUG	Amoxicillin-clavulanate	2	7
NIT	Nitrofurantoin (300 μ g)	4	5
AMP	Ampicillin (10 μ g)	0	9

Discussion

Incidence of *Klebsiella pneumoniae*

Prevalence of *K. pneumoniae* in this study was 18% in the 50 urine samples examined. This prevalence was in line with current Nigerian studies reporting prevalence of 15% to 25% in urinary tract infections (Afolayan et al., 2021). The high recovery reveals the clinical significance of *K. pneumoniae* as a common uropathogen in the region of study. The variations in prevalence also occur in terms of geographic area, hygiene of hospitals, clientele of patient, and antimicrobial use patterns.

Antibacterial Activity of the Extracts

The antibacterial performances of *Phyllanthus amarus* ethanolic and water extracts were very good, showing zones of inhibition greater than 30 mm at high concentrations. Notably, the ethanolic extract always showed broader zones of inhibition than the water extract, which suggests that ethanol is a better solvent for active antibacterial compounds extraction. This statement is compatible with the observations of Kebede et al. (2021), which reflected that antibacterial activity against *Klebsiella pneumoniae* was more prominent in ethanolic extracts of *P. amarus* than in their corresponding aqueous extracts. Their study clarified that alcohol, being of intermediate polarity, contains the capability of dissolving both non-polar and polar phytochemicals such as alkaloids, flavonoids, and phenolics, which are highly appreciated for their high degree of antimicrobial activities. Agreement between their results and the current investigation verifies the above statement that increasing the polarity of a solvent allows for enhanced effectiveness of solvents for recovering active antibacterial components from *P. amarus*.

The mode of inhibition that was concentration-dependent for this study means that antibacterial activity enhanced with increasing concentration of extracts. At 250 mg/mL, inhibition zones were 35.0 mm for the aqueous and 36.5 mm for the ethanolic extracts, which were values greater than several commercial antibiotic inhibition zones tested. This trend confirms the report of Obuotor et al. (2021), whose results showed that extracts of *P. amarus* demonstrated dose-dependent antibacterial activity for *Klebsiella pneumoniae* and *Escherichia coli*, noted when increasing concentrations resulted in significantly large inhibition zones. In the same respect, Aladejana et al. (2023) also noted increasing concentrations of ethanolic *P. amarus* extract provided enhanced antibacterial activity against multidrug-resistant isolates. These confirmatory findings propose that a collection of such bioactive phytochemical constituents of *P. amarus*, for instance, alkaloids, flavonoids, tannins, and phenolics, account for the concentration-dependent inhibition and potentially have a role in evading bacterial resistance mechanisms.

MIC and MBC Values

The MIC for both extracts here was 100 mg/mL, MBCs of 200 mg/mL (aqueous) and 100 mg/mL (ethanolic), exhibiting bactericidal activity particularly for the ethanolic extract, which was lethal at a lower concentration. Comparable bactericidal activity of *Phyllanthus amarus* has been demonstrated earlier in scientific literature, yet

some MIC/MBC values are not consistent across organism or method of extraction. For example, Phuong et al. (2019) have shown MIC/MBC values of 125 mg/mL/250 mg/mL for extracts of *P. amarus* against *Vibrio parahaemolyticus* (a disparate target organism than herein), while some other communications have noted MICs within a range validating wide antibacterial activity of the plant (Eldeen et al., 2011; Obuotor et al., 2021). These comparisons emphasize that parameters of assay, solvent polarity, and target organisms have significant impacts on reported MIC/MBC values, yet overall, the literature validates a bactericidal potential of extracts of *P. amarus*.

Antibiotic Resistance Profiles

Alarming was the high prevalence of multidrug resistance (MDR) and extensive drug resistance (XDR) in the isolates of *Klebsiella*. 8 of the 9 isolates were MDR and 3 were XDR and resistant to 8 antibiotics tested. Resistance was especially high for ampicillin (100%), cephalosporins (78–89%), and aminoglycosides (67%). These findings are similar to global reports of rising resistance in *Klebsiella* more typically associated with the production of extended-spectrum beta-lactamase (ESBL) and carbapenemase production (WHO, 2022). The persistence of such resistance in clinical isolates reflects excessive and improper use of antibiotics, improper control of infection, and horizontal transfer of the resistance genes in hospitals. The fact that *P. amarus* is effective in combating such resistant isolates suggests the potential for the plant as a complementary or alternative therapeutic method (Ismail et al., 2021).

Comparative Efficacy of Plant Extracts and Antibiotics

The comparative analysis revealed that ethanolic extracts from *P. amarus* formed inhibition zones larger than ampicillin, cefuroxime, and ceftazidime. This was quite remarkable given the restricted effectiveness of such antibiotics in the context of multidrug resistance in *Klebsiella*. These findings emphasize the potential of medicinal plants as novel sources of antimicrobial compounds or agents altering resistance. Combining *P. amarus* with existing antibiotics can potentially recover or enhance the effectiveness of pharmaceuticals compromised by resistance.

Implications upon Public Health

The concomitant threats of emerging resistance to antibiotics and restricted access to new drugs in Nigeria create the necessity for a rapid investigation of complementary therapeutic approaches (Esther et al., 2025). The proven effectiveness of *P. amarus* preparations gives a scientific foundation for the implementation of indigenous medicine under current healthcare provision. The resource is easily accessible, culturally endorsed, and cheaper than chemical drugs and hence more than appropriate for resource-limited environments.

Finally, it concludes with evidence supporting antibacterial activity of *Phyllanthus amarus* to fight multidrug-resistant *Klebsiella pneumoniae*. The ethanolic extracts were more efficacious than the aqueous ones and more effective than some traditional antibiotics. The work highlights the promise of *P. amarus* as a natural therapeutic agent in the battle against antibiotic resistance. Yet wider-ranging mechanistic and clinical research are necessary prior to when the lab findings can direct therapeutic uses.

Conclusion

The present study proved that extracts of *Phyllanthus amarus* exhibit remarkable antibacterial activities against clinical isolates of *Klebsiella pneumoniae* extracted from urine samples collected from Kwara State, Nigeria. The ethanolic extract at all times manifested greater activity compared to the aqueous solvent extract with inhibition zones ranging up to 40 mm at the highest concentration tested. The MIC for both extracts was 100 mg/mL and MBC was 200 mg/mL for the aqueous extract and 100 mg/mL for the ethanolic extract to substantiate its superior bactericidal potency. The phytochemical examination proved the presence of saponins, phenols, terpenoids, flavonoids, tannins, and alkaloids with documented antibacterial activities. These activities occur through diverse modes namely disintegration of cell membranes, deoxyribonucleic acid replication inhibitions, and interferences with protein syntheses. The antibiotic profiling also indicated high rates of multidrug resistance (MDR) and extensive drug resistance (XDR) in the isolates of *Klebsiella*. The observed resistance to ampicillin, cephalosporins, and aminoglycosides was markedly elevated, indicating a global concern regarding the increasing resistance trends noted in Enterobacteriaceae. The effectiveness of *P. amarus* for the resistant isolates shows the potential of the plant as a complementary or alternative therapeutic approach. Overall results also emphasize the necessity for research and use of medicinal plants in the war on antibiotic resistance. *P. amarus* indicates a potential natural source of antimicrobial agents that can enhance the efficacy of current antibiotics primarily in areas faced by the rising public health issue of drug resistance.

Recommendations

1. Subsequent research should incorporate bigger sample sizes and isolates obtained from a broader array of specimen materials (e.g., blood, sputum) to increase generalizability of results.
2. Qualitative phytochemical analyses should also be performed in an endeavor to determine the exact concentration of active phytoconstituents responsible for antibacterial activity.
3. Subsequent research should incorporate bigger sample sizes and isolates obtained from a broader array of specimen materials (e.g., blood, sputum) to increase generalizability of results.
4. Clinical trials and in vivo studies should also be performed for the assessment of safety, therapeutic efficacy, and the pharmacokinetic profile of *P. amarus* extracts in human volunteers.
5. The commercialization of already-known medicinal plants based on traditional healthcare regimens needs to be promoted further, especially in resource-poor settings whereby access to new antibiotics is restricted.
6. Public health authorities should support antimicrobial stewardship activities by advocating the appropriate use of antibiotics and by conducting research for plant antimicrobials.
7. Local pharmaceutical companies should consider research institute collaborations for the development of standardized preparations of *P. amarus* for therapeutic applications.
8. Clinicians must also take into account the high occurrence of MDR and XDR *Klebsiella pneumoniae* and resort to culture-based diagnosis in guiding antibiotic therapy.
9. Herbal antimicrobials like *P. amarus* should ideally be viewed as complementary therapies in cases where the traditional antibiotics are not effective yet under strictly controlled and standardized conditions.
10. Ongoing surveillance for antimicrobial resistance allows tracking the development of resistance patterns and providing input for therapeutic guidelines.

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