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Original Research Article

Detection and Screening of Some Medicinal Plants Against Multiple Drug-Resistant *Pseudomonas aeruginosa* from Selected Sources

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ABSTRACT

Multi-drug resistant (MDR) *Pseudomonas aeruginosa* strain continues to be a global health challenge due to the rise in their ability to produce extended-spectrum β-lactamase and AmpC β-Lactamas. In Nigeria, *Heterotis rotundifolia* (Sunflower), *Chromolaena odorata* (Awolowo leaf), and *Helianthus annuus* (Pink lady) have been used traditionally to treat *P. aeruginosa*-associated diseases. Still, their effectiveness has not been scientifically validated. This study investigated the prevalence and effect of crude extracts of these medicinal plants on MDR *P. aeruginosa*. A total of 500 samples (350 clinical, 100 animal and 50 environmental samples) were collected in Lagos State between January and June 2023. Samples were analysed microbiologically, and isolates were subjected to antibiotic susceptibility by standard methods. *P. aeruginosa* isolates were screened for AmpC and ESBL production using standard procedures. The plants were screened for their anti-*P. aeruginosa* activity and their phytochemical constituents. This study revealed a 2.4% overall prevalence of *P. aeruginosa*, with varying prevalence including 1.7% in clinical, 6% environmental, and 3% in animal samples. *P. aeruginosa* isolates were 100% resistant to ceftriaxone, augmentin, and colistin sulphate, 100% susceptible to imipenem and 83.3% AmpC producers. Both methanol and aqueous extracts of the three plants were effective against MDR *P. aeruginosa* strains at 100 mg/ml and 50 mg/ml concentrations. The existence of flavonoids, alkaloids, saponins, terpenoids, and other compounds at different concentrations was shown from the phytochemical screening. Higher effectivity of *H. rotundifolia* aqueous extract was detected against AmpC-producing strains. Molecular, phyto-kinetic and toxicological analyses of AmpC-producing *P. aeruginosa* should be carried out to ascertain the basis of the effectiveness of *H. rotundifolia*.

***Keywords*:** Antibiotics, Resistance, Phytochemicals and Susceptibility

Introduction

The World Health Organization's surveillance report on antimicrobial resistance indicates a significant rise in the global threat of antimicrobial resistance, jeopardising the effectiveness of treating common hospital-acquired or community-acquired infections.[1] *Pseudomonas aeruginosa*, one of the ESKAPE pathogens (*Enterobacter* species, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus faecium*), an opportunistic gram-negative bacterium [1-2] that thrives in moist environments, particularly aquatic ones, making healthcare facilities an ideal environment for its growth and spread. [2-3]

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While *P. aeruginosa* is widely found in the environment, it rarely infects healthy individuals, with a skin colonisation rate of 0% to 2%. However, it is more likely to colonise hospitalised patients, with colonisation rates exceeding 50%. [4] This bacterium significantly contributes to healthcare-associated infections (HCAIs), leading to severe and potentially fatal acute or chronic infections. These include recurrent exacerbations in patients with cystic fibrosis, hospital-acquired and ventilator-associated pneumonia, urinary tract infections, wound and soft tissue infections, and bacteremia. [5] *Pseudomonas aeruginosa* is naturally resistant to several structurally distinct antibiotics due to its extensive array of chromosomal and plasmid-mediated antibiotic resistance mechanisms [6] and its dynamic tendency to acquire new resistance genes from other genera like *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Salmonella* species. [7] The formation of biofilms enhances resistance to certain classes of antibiotics or antibacterial drugs, complicating infection control [8]. Treating patients with *P. aeruginosa* infections that are resistant to conventional medications can be challenging. [9] The prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XRD) *P. aeruginosa* strains is on the rise, with rates varying from 15% to 30% in certain parts of the world. [7] Due to the widespread use of broad-spectrum antibiotics in Nigeria and increasing bacterial resistance to affordable and accessible antimicrobials, multidrug-resistant *P. aeruginosa* and other resistant nosocomial pathogens that are linked to severe infections have emerged. [10] Antibiotic resistance is now understood to be a danger to world health. Therefore, efforts to combat the threat of antibiotic resistance while searching into different sources of antimicrobial compounds, such as medicinal plants. [11] In

addition to antibiotics, various additional approaches are being used or suggested as potential substitutes for the treatment of infectious diseases, such as Honey, [12] lantibiotics and Bacteriocin. [13] Nanoparticles [14] Coinfection and Probiotics, [15] CRISPR-Cas System, [16] RNA silencing, photodynamic antimicrobial chemotherapy, Sonodynamic antimicrobial chemotherapy, Phage therapy and Monoclonal antibody (mAb) therapy. [17]

Natural phytochemicals (secondary metabolites), which are present in seeds, roots, leaves, stems, flowers, and fruits, are the primary means by which plants defend themselves against microbes. [11] The pharmaceutical and scientific communities have studied a wide variety of plant extracts and oils as prospective antibacterial and antibiotic resistance-modifying agents to understand better the potential usefulness of plant-derived chemicals as therapeutic options. [18] It is crucial to investigate additional inexpensive, widely available indigenous plants as potential sources of novel antibiotics targeted specifically against *P. aeruginosa* to aid in the efficient treatment of diseases brought on by this organism. As part of this research objectives, the antimicrobial properties of methanolic and aqueous extracts from three medicinal plants: *Heterotis rotundifolia* (commonly known as pink lady), *Chromolaena odorata* (referred to as Awolowo leaf), and *Helianthus annuus* (sunflower) was investigated. This study focused on the effectiveness of these medicinal plants against multidrug-resistant (MD-R) strains of *P. aeruginosa* isolated from various sources.

Materials and Methods

Study Design

The research was carried out at the microbiology department of Lagos State University from January to June 2023. A total of 500 samples were processed from Randle General Hospital in Surulere, Lagos State Teaching Hospital (LASUTH), and Cele abattoir to detect multi-drug resistant *Pseudomonas aeruginosa*. The two hospitals investigated serve as the primary referral centres for the Ikeja and Surulere districts in Lagos State.

Ethical Approval

The Lagos State Teaching Hospital Ethics Committee granted the ethical approval for sample collection. No.: LREC/06/10/2164 for three months.

Sample collection

Clinical samples, which included wound swabs, sputum and urine, were randomly collected from patients considered in this research work. This comprised patients with chronic orthopaedic and post-surgical wound infections, urine catheter, ventilated-associated pneumonia and burn wound infection, patients in the Intensive Care Unit (ICU) who were eighteen years and above of age, and both sexes with prolonged hospital admission (at least 14 days or more) from the two selected hospitals in Lagos. Altogether, the study population comprised 350 patient samples collected by the laboratory scientists aseptically from these hospitals. Moreover, a total of 50 hospital effluents were collected from the previously mentioned two hospitals from various points and on different days. From Cele Abattoir, 100 cow rectal swabs were collected by veterinary doctors using sterile swab sticks.

Sample processing, isolation, and identification of P. aeruginosa

A total of 100 wound swab samples collected were carefully removed from the container using sterile forceps and placed into a tube with sterile saline, which helps in eluting the collected material from the swab. A total of 200 urine samples were visually examined to determine their quality, including colour, clarity, and the presence of any particle matter. A centrifugation procedure was used to concentrate any potential pathogens present in urine samples that appeared turbid or had visible sediment. A total of 50 sputum samples were thoroughly mixed through homogenisation to ensure an equitable distribution of pathogens present. These clinical samples were first cultured on selective agars, specifically MacConkey agar (Oxoid, U.K) and Cetrimide agar (Oxoid, U.K). Subsequently, colonies suspected to be of interest were further cultured on Blood agar (Oxoid, U.K) and Muller Hinton agar (MHA) (Oxoid, U.K) to examine hemolysis and pigmentation. Incubation of all the plates took place at 37°C for 18-24 hours, and the resulting growths were assessed. Identification of the isolates was conducted using standard bacteriological techniques, which included evaluating characteristics such as morphology, colony appearance, hemolysis, and pigment production [19]. A total of 50 samples of hospital effluents were collected, each consisting of 100ml of water collected from various points on different days. We combined a 10 mL aliquot of each effluent sample with 90 mL of Tryptone Soya Broth. The mixture was then incubated aerobically at 37 ℃ for 24 hours. Following incubation, a 100 µL volume of the resulting bacterial suspension, which underwent ten-fold dilutions, was streaked onto Cetrimide agar plates. These plates were then incubated aerobically at 37°C for 24-48 hours. After incubation, colonies displaying presumptive characteristics such as being significant, flat, blue-green pigmented, and having irregular margins were selected. These colonies were subsequently sub-cultured onto non-selective blood agar plates, following the methodology described by Roulová *et al.* [20] A total of 100 cattle rectal swab samples were collected using sterile swab sticks then transferred to the Microbiology Research Laboratory at Lagos State University, Ojo. *P. aeruginosa* isolates were identified from the cattle rectal samples as described by Chika *et al.* [21] Additional identification techniques were employed, including Gram staining and biochemical tests such as the Triple sugar iron (TSI) test, catalase test, oxidase test using oxidase strips, citrate utilisation, indole production, and urea hydrolysis for further identification. [22]

Antimicrobial Susceptibility Testing

The susceptibility testing using the disc diffusion method was performed by adhering to the Clinical and Laboratory Standards Institute guidelines. [23] Antibiotic disks from Mast (U.K.) were employed, including cefepime (30 µg), Augmentin (30 µg), ceftriaxone (30 µg), Cefotaxine (30 µg), imipenem (10 µg), colistin sulfate (10 µg), ciprofloxacin (5 µg), gentamycin (10 µg) and tegecycline (15 µg). The interpretation of the results was based on evaluating inhibition zone diameters. [23] The control strain for the testing procedure was *Escherichia coli* ATCC 25922. For the identification of multidrug-resistant (MDR) *P. aeruginosa*, organisms displaying resistance to at least three additional antibiotic classes were selected.

Phenotypic identification of AmpC Beta-lactamase (AmpC) and extended-spectrum Beta-lactamase (ESBL)

The ESBL-producing isolates were identified phenotypically using the double disc synergy test as described by El Aila *et al*. [24] The procedure involved spreading the test organism on a Mueller-Hinton agar (Oxoid, U.K) plate. Four antibiotic discs were placed—Ceftriaxone (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), and Amoxicillin/Clavulanic acid (20/10 µg)—at a 20 mm distance from the central Amoxicillin/Clavulanic acid disc, with edges touching. Following 24 hours of incubation, the test was considered positive if there was an increased zone of inhibition observed between any of the Cephalosporin antibiotics and the Amoxicillin/Clavulanic acid disc. This enhanced zone of inhibition indicated the presence of synergistic activity with Clavulanic acid and confirmed the presence of an ESBL-producing organism. The evaluation of Amp-C production was conducted using the AmpC Disk Test as described by Black *et al.* [25] In-house-produced filter paper disks were treated with a mixture of 20µl saline and 100x Tris-EDTA. These disks were then allowed to dry and stored at a low temperature (2 to 8°C). A lawn of Cefoxitin-susceptible *E. coli* strain ATCC 25922 was spread on the surface of a Mueller-Hinton agar plate. Just before the test, the prepared AmpC disks were rehydrated with 20µl of saline. After that, a few colonies of the test organism were applied to one of these AmpC disks. A disk containing 30µg of Cefoxitin was placed on the inoculated surface of the agar. The AmpC disk with the inoculated face in contact with the

agar was positioned near the Cefoxitin disk. The inverted agar plates were then incubated overnight at 35°C in ambient air. After incubation, the plates were examined for results. A positive result was indicated by an indentation or flattening of the zone of inhibition around the Cefoxitin disk, whereas a negative result showed the absence of such a distortion. [25]

Plant Collection and Identification

The leaves of *Heterotis rotundifolia* (pink lady), *Chromolaena odorata* (Awolowo leaf), and *Helianthus annuus* (sunflower) were collected in June 2023 from the farm opposite the ETF Research Building, Faculty of Science, LASU Ojo, Lagos (latitude 6.459836°N, longitude 3.197896°E) and authenticated at the Herbarium by Dr. Omotosho K. Oluwa in Department of Botany Lagos State University, Ojo. Voucher specimens of the *Heterotis rotundifolia*, LSH001030; *Chromolaena odorata*, LSH001029; and *Helianthus annuus*, LSH001028, with their voucher specimen numbers were deposited at the Herbarium of the Botany Department, Lagos State University, Ojo. The collected portions of medicinal plants were carefully washed using running water to remove any dirt particles. These plant parts were air-dried at room temperature (28-30ºC) for two weeks, then milled into a fine powder, and the samples were stored in an airtight container until ready for use.

Soxhlet Extraction of plant material

Air-dried ground leaves of each of the three plants [*Heterotis rotundifolia, Chromolaena odorata, and Helianthus annuus*] (20.00 g) and distilled water (100 mL)/ methanol (100 mL) were used for each extraction. Following Soxhlet protocol as described by Manousi *et al.*[26], the extraction process took approximately six hours, with the exception of sonication, which was completed within one hour. Following the freeze-drying of the aqueous extracts using a rotary evaporator, the resulting crude material was stored at -30ºC for future use. [26]

Screening for Phytochemical Constituents of plant samples

The dried plant extracts were screened for the presence of phytoconstituents like alkaloids, saponins, terpenoids, glycosides, flavonoids, sterols and steroids, tannins, phenolic resin compounds, and carbohydrates according to the method described. [27]

Quantitative determination of phytochemicals in plant samples

Different methods were used to quantify the phytochemicals in plant samples. For saponin, the samples were extracted with 20% ethanol and purified with diethyl ether and n-butanol, and the dry weights of the residues were measured [28]. For alkaloids, the samples were extracted with 10% acetic acid in ethanol and precipitated with ammonium hydroxide, and the dry weights of the sediments were measured. [29] For flavonoids, the samples were extracted with 80% methanol and filtered, and the dry weights of the filtrates were measured. [30]

Assessing the Antimicrobial Activities of the Extracts

The efficacy of the extracts against *P. aeruginosa* was evaluated using an Agar disc diffusion assay [31]. A standard inoculum of 0.5 McFarland standards ($1.0 \times 10^{8)}$) was used to inoculate the surface of a Mueller-Hinton agar plate (Oxoid; UK) uniformly. Sterile paper discs soaked in extracts at specified concentrations (50 and 100 mg/ml) were carefully placed at various locations on an agar plate containing *P. aeruginosa* to ensure proper contact. The culture plates were incubated for 18 to 24 hours at 37°C. Subsequently, the diameter of the zones of inhibition was assessed using a ruler. [31]

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extract

The MIC and MBC of the efficient extract were evaluated using the broth dilution method [31]. Test tubes were filled with an equal mixture of plant extract and the base medium (brain heart infusion broth). A standardised inoculum of 10^8 CFU/ml was added to a series of test tubes using a micropipette. Incubation was done aerobically at 37°C for 18 to 24 hours. Two control tubes were maintained for each test batch. These included test tubes containing physiological saline, the Inoculum (organism control), growth media without Inoculum (antibiotic control), and tubes containing plant extract and growth medium. The lowest concentration (maximum dilution) of the extract that showed no visible growth (any turbidity) when compared to the control tube was considered the minimum inhibitory concentration (MIC). The test dilution, however, was sub-cultured on nutrient agar and then incubated for an additional 18 to 24 hours at 37°C to assess the MBC. MBC is referred to as the lowest concentration at the maximum dilution of the extract that produces no single bacterial colony on a solid medium.

Statistical analysis

In this study, the collected data underwent computer-based analysis using IBM SPSS software (version 20). The results were presented in multiple formats, including frequency tables, charts, and written descriptions. Key variables were cross-tabulated, and the Chi-square test was employed to assess the relationship between these variables.

Results and Discussion

This study investigated three medicinal plants for their antibacterial properties against MDR *P. aeruginosa*. Out of the 500 samples analysed, a total prevalence of 2.4% (12/500) of *P. aeruginosa* was recorded from various sources (Table 1). Of these, 1.7% (6/350) was estimated from 350 clinical samples, 6% (3/50) from 50 environmental samples, and 3% (3/100) from 100 animal samples (Table 2). This result confirms the ubiquitous nature of *P. aeruginosa* and its potential to transmit from animals to humans, as well as the environment. This is because *P. aeruginosa* has been known to be one of the critical priority "1" pathogens and as the most significant toxigenic pathogen due to the quantity and variety of invasive infections it causes, high morbidity and mortality rates have been linked to this organism, as well as its capacity to establish different mechanisms of antibiotic resistance. [32] However, the result from this study is similar to the findings of Odumosu *et al.* [33,] who recorded *P. aeruginosa* with a total prevalence of 39.7% (100/252) from different sources. In their study, 100% (17/17) prevalence was obtained from poultry, 66% (54/82) from vegetables, 19.6% (22/112) from clinical, and 17% (7/41) from cow dungs in Southwestern Nigeria states. Other studies such as Singh *et al.* [34], Adesoji *et al.* [35], and Pondei *et al.* [36] also isolated *P. aeruginosa* at different prevalence rates from animal (9.30%), environmental (66.7%) and clinical (38.6%) sources respectively. The disparity in the prevalence might be associated with differences in the number of samples collected and study location.

In this study, 100% of the *P. aeruginosa* were multiple drug resistant (MDR), resisting at least three antibiotics from all the nine antibiotics tested. Although imipenem was highly potent against all strains, ciprofloxacin and gentamycin were ineffective against two *P. aeruginosa* strains. These two resistant strains, W14 and A9, originated from clinical and animal sources, respectively (Table 3 and Figure 1). Similarly, the strains U04 and W14, both from clinical sources, were resistant to gentamycin. Furthermore, Cefotaxine, Ceftriaxone, and Augmentin were not effective on twelve (100%), eleven (92%), and ten (83%) *P. aeruginosa* strains, respectively. Also, tigecycline, cefepime, and colistin sulfate were resisted by seven (58.3%), six (50%), and five (42%) *P. aeruginosa* strains, respectively. This result is in agreement with Isichei-Ukah *et al.* [37,] who isolated MDR *P. aeruginosa* from environmental and clinical sources. In their report, the highest (100%) resistance was recorded on penicillin, cephalosporins: ceftriaxone and cefuroxime (48% - 100%) and the lowest resistance to imipenem and meropenem. Adesoji *et al.* [35] also recovered MDR *P. aeruginosa* from selected sewages in Kastina state, Nigeria, with the highest (100%) resistance cephalosporin (cefuroxime) and nitrofurantoin reported.

In contrast, the slightest resistance was demonstrated on gentamycin. The high resistance of *P. aeruginosa* to beta-lactam antibiotics observed in these studies may be due to the ability of the organism to

produce a beta-lactamase enzyme that breaks the beta-lactam ring, thereby inactivating the antibiotic. This study recorded zero resistance to imipenem (a carbapenem), agreeing with the study of Odumosu *et al.* [33] on the high level of activities of Imipenem to *P. aeruginosa* affirming this antibiotic as the potential antipseudomonal agent for the treatment of diseases caused by this organism, hence the need for national alert to conserve the efficacy of this antibiotic.

Interestingly, there were no instances of extensively drug resistance (XDR) or pan-drug-resistant (PDR) *P. aeruginosa* strains found in this study. This is because none of the tested *P. aeruginosa* strains exhibited resistance to all antibiotics tested or were susceptible to only two or fewer antibiotics from various categories of antimicrobial agents screened. However, 5% XDR- *P. aeruginosa* has been reported by Awanye *et al.*[38] from clinical samples in Nigeria.

Furthermore, 8 distinct resistance profiles (A to H) were exhibited by the 12 *P. aeruginosa* isolates from different sources (Table 3). Of these, resistance profile A is the most common, consisting of four *P. aeruginosa* isolates from clinical and environmental sources. For instance, W16, U13, and SP7 from the clinical source, as well as E01 from the environmental source, manifested the same resistance profile A (Table 3). This is followed by resistance profile D found in two *P. aeruginosa* isolates from animal (A12) and environmental (E03) sources (Table 3). The most diminutive resistance profile was found in B, C, E, and H, with one isolate each from different sources (Table 3). This shows the possible transmission dynamic of *P. aeruginosa* from the environment to animals and to humans, and vice-versa.

It is noteworthy that, of the 12 *P. aeruginosa* phenotypically screened for ESBL and AmpC beta-lactamase production, none (0%) and 10(83.3%) were positive for ESBL and AmpC, respectively (Table 4, Plate 1). This is similar to the findings of Oliveira *et al.* [39,] who reported twenty-two AmpC-positive isolates of *P. aeruginosa* from twenty-seven isolates from a domestic wastewater treatment plant tested phenotypically for inducible AmpC beta-lactamase production in Southeastern Brazil. Also, Chika *et al.* [21,] in the study conducted in Abakaliki Southeastern Nigeria, reported 25% AmpC beta-lactamase-producing *P. aeruginosa* from Abattoir, which were resistant to multiple antimicrobial agents. The disparity might be attributed to the sample source and the study location. However, this study and other studies agreed that both AmpC producers and non-AmpC producers showed high levels of resistance to different antibiotics tested, hence the risk of MDR with AmpC producers' transmission from human to animal and the environment.

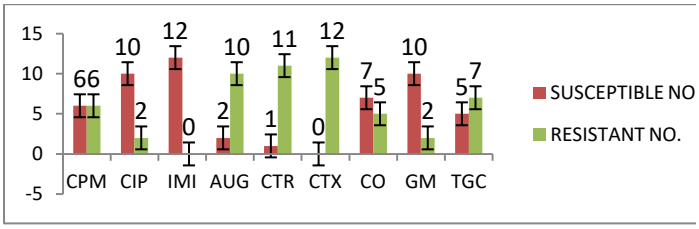

Figure 1: Susceptibility and resistance of 12 isolates of *P. aeruginosa* from different sources to nine different antibiotics

Plate 1. Showing AmpC-positive and negative plates of *Pseudomonas aeruginosa* from different sources. A, B & C=AmpC positive plates of *P. aeruginosa* isolated from RGH urine, cow rectal swab & LASUTH effluent, respectively. D=AmpC-negative plate of *Pseudomonas aeruginosa* isolated from LASUTH urine.

The antimicrobial activity of three medicinal plants against multidrug-resistant *P. aeruginosa* strains, isolated from clinical, environmental, and animal sources, was investigated (Tables 5 and 6). At 100 mg/mL, the aqueous extract of *H. rotundifolia* exhibited the highest inhibition zone diameter (20 mm) against the animal-sourced strain (A13) (Tables 7 and 8). In contrast, at 50 mg/mL, the same extract recorded an 8 mm inhibition zone. Both methanol and aqueous extracts from the three plants were active against *P. aeruginosa* strains from wound and animal sources (W16 and A13) at 50 mg/mL (Tables 7 and 9). However, only the aqueous extracts were effective against urine and effluent strains (U13 and E01) (Table 7). At this concentration, the methanol extracts were ineffective. Furthermore, at 100 mg/mL, the aqueous and methanol extracts of the three plants were generally effective against most strains, except the aqueous extract of *H. annuus* (A), which was ineffective against the W16 strain (Tables 7 and 10). Moreover, the combination of aqueous extracts from all three plants at 100 mg/mL demonstrated efficacy against the tested *P. aeruginosa* strains, while methanol extract combinations were ineffective (Plate 2). The minimum inhibitory concentration (MIC) of these plant extracts ranged from 20 to 30 mg/mL, and the minimum bactericidal concentration (MBC) ranged from 60 to 100 mg/ml (Table 8). The higher effectivity of *H. rotundifolia* aqueous extract to isolate (A13) might be attributed to the source of isolation, higher flavonoids (20.19%) content, and presence of other biologically active chemicals such as alkaloid, glycosides, steroids, tannins, saponin and phenol (Table 6). The presence of high alkaloids, tannins, and saponin was reported in different extracts of *Bryophyllum pinnatum* and *Rauvolfia vomitoria* on bacterial pathogens.[40] This, however, confirmed the presence of these secondary metabolites in different crude extracts of medicinal plants. Nevertheless, at 50 mg/ml concentration, the same plant extract was resisted by AmpC-producing *P. aeruginosa* strains E01 and U13. This might be a result of low concentration of the extract and sample sources. In agreement with this, Dougnon *et al.* [41] reported that *H. rotundifolia* was active on enteropathogenic bacteria with inhibition zone diameters varying between 9.5 mm and 15 mm (aqueous extract) and 9.5 mm and 12 mm (ethanol extract) at 100 mg/ml. The difference in the inhibition zone diameters may be due to location and the plant part used. Also, at 100 mg/ml, both methanol and aqueous extract of *H. annuus* showed inhibition zones diameters of 12 mm and 10 mm, respectively, on AmpC-producing *P. aeruginosa* strains from different sources (U13, E01 and A13). The sensitivity of these AmpC-producing strains to crude extract of *H. annuus* might be a result of the higher percentage of saponin (2.94%) compared to (1.18%) present in *H. rotundifolia* and with an appreciable amount of alkaloids (6.80%). In addition, a lesser inhibition zone diameter of 8mm was recorded in this plant's aqueous and methanol extracts at 50 mg/ml concentration against *P. aeruginosa* strains W16 and A13. This result is in concordance with the findings of Mahamba and Palamuleni. [42] who reported direct proportionality on the effect of *Helianthus annuus* dosage on the test organism in their study; thus, the higher the dosage, the higher the zone of inhibition. Furthermore, at 50 mg/ml, methanol and aqueous extracts of *C. odorata* were effective on both AmpC-positive and negative stains of *P. aeruginosa* with inhibition zones diameters of 8

mm and 10 mm, respectively. Moreover, at 100 mg/ml, both methanol and aqueous extracts were effective on the isolates with 10mm and 12 mm diameters, respectively. The higher effect of *C. odorata* extracts at both concentrations might be connected to the presence of high concentrations of alkaloids (15.8%), flavonoids (11.3%) and saponin (8.24%). This finding is similar to the antibacterial effect of C. *odorata* methanolic extract on *P. aeruginosa*, as reported by Omeke *et al*. [43] and Adewumi *et al.* [44] with varying zones of inhibitions. This suggests that the bioactive components of *C. odorata* are methanolic, ethanolic, and water-soluble and possess broad-spectrum antibacterial activity. Conversely, this study was not in agreement with the findings of Adewumi *et al.* [45], who reported that methanol and ethanol are the best solvents for *C. odorata* extraction. This is because an appreciable number of glycosides, steroids, carbohydrates, and a trace amount of terpenoids were detected in the aqueous extract of *C. odorata.* In contrast, these bioactive compounds were not detected in the methanolic extract of the same plant as observed in this study. Water remains the best extraction solvent for *C. odorata* and for its antibacterial activity due to its accessibility to more bioactive components of the plant leaves. The combination of these medicinal plants showed no synergistic activities, and the discs from the combined extracts of the two plants showed no inhibition zone diameter. Notwithstanding, when the three plant extracts were combined, only the aqueous extract showed an inhibition zone diameter of 10 mm on the isolates tested. This suggests that the two of these plants might be antagonistic to each other. Interestingly, the MIC of the three medicinal plants evaluated in this study ranged from 20 to 40 mg/ml and MBC 60 to 100 mg/ml, indicating the bacteriostatic activity of these plants at lower concentrations and bactericidal activity at higher concentrations, an assertion that has been documented by Abubakar *et al.* [46] Consequently, the leaf extracts from these three plants exhibited potential therapeutic effects against *P. aeruginosa*-induced diseases, including urinary tract infections. It is a known fact that extracts of the different plant parts have been recognised for their medicinal properties and as useful tools in the control of pathogenic organisms, particularly in the treatment of diseases brought on by MDR organisms. [11] The phytochemical analysis of methanol and aqueous extract of *C. odorata, Heterotis rotundifolia*, and *Helianthus annuus* (Figure 2) showed the presence of Flavonoids, Alkaloids, Saponins, Terpenoids, Glycosides, Steroids, Tannins, Phenol, and Carbohydrates. Other researchers have documented these phytochemical Components for their antibacterial activities, including the inactivation of microbial cell envelope transport proteins and disruption of the cell membrane [44, 46- 48]. Therefore, this study revealed the potential of these medicinal plants for novel drug discovery against *Pseudomonas*-associated diseases upon further studies.

Limitations of this study: Due to the scarcity of funds, this study could not carry out molecular/genomic assay on the MDR-*P. aeruginosa* to determine the genetic basis of their resistance, phytochemical kinetic analysis to detect the time of kill/inhibition of the isolates, and the toxicological assay on the plants' crude extract to determine their toxicity level.

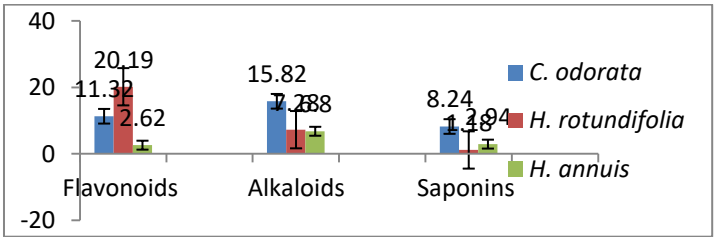

Figure 2: The percentage composition of phytochemicals found in *Heterotis rotundifolia, Chromolaena odorata,* and *Helianthus annuus* leaves

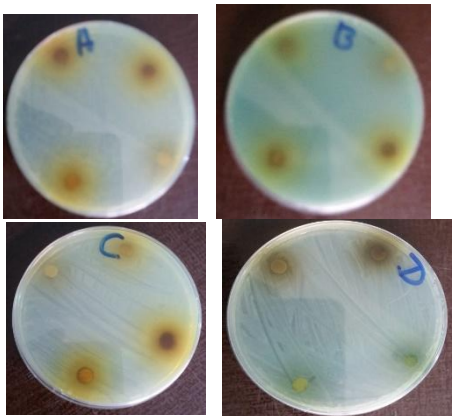

Plate 2: Plates showing antibacterial activities of plant extract on *Pseudomonas aeruginosa* from different sources. A=zones of inhibition of aqueous extract of *H. annuus* on *Pseudomonas aeruginosa* from LASUTH wound at 100 mg/ml and 50 mg/ml, B=Zones of inhibition of methanol extract of *H. annuus* on *Pseudomonas aeruginosa* from RGH effluent at 100 mg/ml and 50 mg/ml, C= zones of inhibition of aqueous extract of *C. odorata* on *Pseudomonas aeruginosa* from RGH effluent at 100 mg/ml and 50 mg/ml and D= zones of inhibition of aqueous extract of *H. rotundifolia* on *Pseudomonas aeruginosa* from animal sources at 100 mg/ml and 50 mg/ml.

Table 1: Biochemical tests for isolates from different sources.

Slant	Gram stain	Butt	Gas	H2S	Lactose	citrate	Urease	Indole	Catalase	Oxidase	Isolate
Alkali	-	Alkali	-	-	-	+	-	-	+	+	W16
Alkali	-	Alkali	-	-	-	+	-	-	+	+	E01
Alkali	-	Alkali	-	-	-	+	-	-	+	+	U13
Alkali	-	Alkali	-	-	-	+	-	-	+	+	A13
Alkali	-	Alkali	-	-	-	+	-	-	+	+	A9
Alkali	-	Alkali	-	-	-	+	-	-	+	+	A12
Alkali	-	Alkali	-	-	-	+	-	-	+	+	E03
Alkali	-	Alkali	-	-	-	+	-	-	+	+	U04

Alkali	-	Alkali	-	-	-	+	-	-	+	+	SP7
Alkali	-	Alkali	-	-	-	+	-	-	+	+	W14
Alkali	-	Alkali	-	-	-	+	-	-	+	+	U71
Alkali	-	Alkali	-	-	-	+	-	-	+	+	E05

Key: W16; LASUTH wound *P. aeruginosa* isolate, U04 and U71; LASUTH urine *P. aeruginosa* isolate, SP7; LASUTH sputum *P. aeruginosa* isolate, E01 and E03; LASUTH effluent *P. aeruginosa* isolate, U13 , W14 and E05; urine , wound and effluent respectively Randle General Hospital *P. aeruginosa* isolate, A13, A12andA9; Cow rectal swab *P. aeruginosa* isolate

Table 2: Numbers and types of collected samples and positive *Pseudomonas aeruginosa* isolates distributed across the subjects' age and sex used in the current study sampling from different sources

Parameters			**Types and number of collected samples (positive sample)**					
			Urine	**Sputum**	**Wound swab**	**Effluent**	**Cow rectal swab**	**Total**
Clinical	Hospital	LASUTH	120(2)	30(1)	70(1)	30(2)	0(0)	250(6)
		RGH	80(1)	20(0)	30(1)	20(1)	0(o)	150(3)
		Total	200(3)	50(1)	100(2)	50(3)	0(0)	400(9)
	Age in years	18-30	50(1)	10(0)	20(1)	NA.	NA.	80(2)
		31-49	60(0)	20(0)	30(0)	NA	NA	110(0)
		≥50	90(2)	20(1)	50(1)	NA	NA	160(4)
		Total	200(3)	50(1)	100(2)			350(6)
	Sex	Male	90(1)	20(0)	40(1)	NA	NA	150(2)
		Female	110(2)	30(1)	60(1)	NA	NA	200(4)
		Total	200(3)	50(1)	100(2)			350(6)
Animal	Abattoir	Cele Abattoir	0(0)	0(0)	0(0)	0(0)	100(3)	100(3)

KEY; RGH – Randle General Hospital, LASUTH–Lagos State University Teaching Hospital, NA-Not applicable

Table 3: Resistance profile of 12 isolates of *Pseudomonas aeruginosa* from different sources

Sample types	**No. of isolates**	**Strains ID**	**Antibiotics**	**No. of antibiotic resisted**	**Resistance index (%)**	**Res. Profile**
Wound, effluents, urine & sputum	4	W16, E01, U13 & SP7	AUG-CTR-CTX-TGC	4	0.4 (44.4)	A
cow rectal swab	1	A13	CPM-AUG-CTR- CTX	4	0.4 (44.4)	B
cow rectal swab	1	A9	CPM-CIP-AUG-CTR-CTX-TGC	6	0.7(66.7)	C
cow rectal swab & effluent	2	A12 & E03	CPM-AUG-CTR-CTX-CO	5	0.6(55.6)	D
Urine	1	U04	CPM-CIP-CTR-CTX-CO-GM	6	0.7(66.7)	E
Wound	1	W14	CPM-AUG-CTR-CTX-GM-TGC	6	0.7(66.7)	F
Urine	1	U71	AUG-CTR-CTX-CO	4	0.4 (44.4)	G
Environmental (effluent)	1	E05	CTX-CO-TGC	3	0.3 (33.3)	H
Total	12					

KEY; RES: Resistance, No: Number, CPM: cefepime, CIP: ciprofloxacin, AUG: Augmentin CTR: ceftriaxone, CTX: Cefotaxime, CO: colistin sulphate, GM: gentamycin, TGC: tigecycline, W16; LASUTH wound P. *aeruginosa* isolate, U04 and U71; LASUTH urine P. *aeruginosa* isolate, SP7; LASUTH sputum P. *aeruginosa* isolate, E01 and E03; LASUTH effluent P. *aeruginosa* isolate, U13, W14 and E05; urine, wound and effluent respectively Randle General Hospital P. *aeruginosa* isolate, A13, A12andA9; Cow rectal swab *P. aeruginosa* isolate.

Table 4: Effects of plant extracts on MDR & AmpC producing *Pseudomonas aeruginosa* with sample sources

Source of Sample	**Samples types**	**No. Tested (%)**	**No. Positive (%)**	**MDR+ No. (%)**	**Strains ID**	**AmpC+ No. (%)**	**Strains ID**	**MP + No. (%)**			**Strains ID**
								H. r	***H. a***	***C. o***	
Clinical	Urine	200(40)	3(25)	3(100)	U04, U71 & U13	3(100)	U04, U71 & U13	1(33)	1(33)	1(33)	U13

	Sputum	50(10)	1(8.3)	1(100)	SP7	1(100)	SP7	0(0)	0(0)	0(0)	
	W. S.	100(20)	2(16.7)	2(100)	W14 & W16	1(50)	W14	1(50)	1(50)	1(50)	W16
Envi.	Hos. E.	50(10)	3(25)	3(100)	E01, E03 & E05	2(66.7)	E01 & E03	1(33)	1(33)	1(33)	E01
Animal	C. R. S.	100(20)	3(25)	3(100)	A13, A12&A9	3(100)	A13, A12&A9	1(33)	1(33)	1(33)	A13
Total		500(100)	12(2.4)	12(100)		10(83.3)		4(100)	4(100)	4(100)	

Keys: Hos..E.; Hospital effluent, Envi.; Environmental, C. R. S; cow rectal swab, W. S.; wound swab, M. P.; medicinal plant, *C.o*.; *Chromolaena odorata, H.a.; Helianthus annuus, H. r.; Heterotis rotundifolia*, zone diameter ≥8mm was considered sensitive, ESBL; Extended Spectrum ß-lactamase; MDR: Multidrug resistant; AmpC; AmpC ß-lactamase, No.; Number, W16; LASUTH wound P. *aeruginosa* isolate, U04 and U71; LASUTH urine P. *aeruginosa* isolate, SP7; LASUTH sputum P. *aeruginosa* isolate, E01 and E03; LASUTH effluent P. *aeruginosa* isolate, U13 , W14 and E05; urine , wound and effluent respectively Randle General Hospital P. *aeruginosa* isolate, A13, A12andA9; Cow rectal swab P. *aeruginosa* isolate.

Table 5: Profile of three medicinal plants used/ percentage yields

Botanical Name	Family name	Local name	Plant part used	Voucher code	Extract yield (%)	
					Methanol	Aqueous
Helianthus annuus	*Asteraceae*	Sunflower	Leaves	LSH001028	20.8	17.6
Heterotis rotundifolia	*Melastomataceae*	Pink lady	Leaves	LSH001030	8.2	19.7
Chromolaena odorata	*Asteraceae*	Awolowo leaf	Leaves	LSH001029	17.3	17.1

Table 6: Qualitative phytochemical composition of the three medicinal plant extracts used

Plant screened		Phytochemicals								
Plant Species	Plant part	Flavonoids	Alkaloids	Saponins	Terpenoids	Glycosides	Steroids	Tannins	Phenol	Carbohydrates
					Aqueous extract					
C. odorata (C)	Leaves	+	+	+	+	+	+	+	+	+
H. rotundifolia (A)	Leaves	+	+	+	+	+	+	+	+	+
H. annuus (B)	Leaves	-	+	+	+	+	+	+	+	+
					Methanol extract					
C. odorata (C)	Leaves	+	+	+	-	-	-	+	+	-
H. rotundifolia (A)	Leaves	+	+	-	+	-	-	+	+	+
H. annuus (B)	Leaves	+	+	+	+	-	-	+	+	+

Key: + Appreciable amount; + Moderate amount; + Trace amount; - Complete absence; ME: Methanol extract; AE: Aqueous extract.

Table 7: Antibacterial activities of plant extracts on *Pseudomonas aeruginosa* from different sources.

Plants used	*Pseudomonas aeruginosa*							
	W16		E01		U13		A13	
	ME	AE	ME	AE	ME	AE	ME	AE
Plant extract 50 mg/ml.								
H. rotundifolia (A)	8	8	-	8	-	8	8	8
H. annuus (B)	8	8	-	8	-	8	8	8
C. odorata (C)	8	10	8	10	8	8	8	10
Plant extract 100 mg/ml.								
H. rotundifolia (A)	10	10	10	10	10	10	10	20
H. annuus (B)	-	-	12	10	12	10	12	10
C. odorata (C)	10	12	10	12	10	12	10	12
(A+B)=E	-	-	-	-	-	-	-	-

(A+C)=F	-	-	-	-	-	-	-	-
(B+C)=G	-	-	-	-	-	-	-	-
(A+B+C)=H	-	10	-	10	-	10	-	10
ciprofloxacin(5µg)		18		30		22		20

Key: Figures indicating the average diameter of zone of inhibition in mm; - = No inhibition; AE = aqueous extract; ME = methanol extract; ciprofloxacin = commercial antibiotics used as control, zone diameter ≥8mm was considered sensitive, W16; LASUTH wound *P. aeruginosa* isolate, U13; RGH urine *P. aeruginosa* isolate, E01; LASUTH effluent *P. aeruginosa* isolate, A13; Cow rectal swab *P. aeruginosa* isolates.

Table 8: The inhibitory and bactericidal concentration of plant extract on *Pseudomonas aeruginosa* from different sources

Plant	**W16**				**E01**				**U13**				**A13**			
	MIC		**MBC**		**MIC**		**MBC**		**MIC**		**MBC**		**MIC**		**MBC**	
	In mg/ml															
	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE
H. rotundifolia (A)	ND	ND	ND	ND	30	20	100	80	30	ND	80	ND	30	20	100	80
H. annuus (B)	30	20	100	80	30	20	100	80	ND	ND	ND	ND	30	20	80	60
C. odarata (C)	30	20	80	60	ND	20	ND	80	20	30	100	80	30	20	100	80

Key: MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; ND. = not determined; A.E. = aqueous extract; M.E. = methanol extract.

Table 9: Chi-square result showing the level of significance of Antibacterial activities of plant extracts on *Pseudomonas aeruginosa* from different sources.

Plants used	**Aqueous extract (50 mg/ml)**		**Chi-Square Tests**		
	8 (mm)	**10(mm)**	***P*-Value**	**Df**	**Pearson Chi-Square (2-sided)**
C. odorata (C)	1	3	0.05	2	.018
H. rotundifolia (A)	4	0			
H. annuus (B)	4	0			
Total	9	3			
	Methanol extract (50 mg/ml)				
	0(mm)	8(mm)			
C. odorata (C)	2	2			
H. rotundifolia A	2	2			
H. annuus (B)	2	2	0.05	2	1.000
Total	6	6			

Table 10: Chi-square result showing the level of significance of Antibacterial activities of plant extracts on *Pseudomonas aeruginosa* from different sources

Plant used	**Aqueous extract (100 mg/ml)**				**Chi-Square Tests**		
	0(mm)	**10(mm)**	**12(mm)**	**20(mm)**	***P*-Value**	**Df**	**Pearson Chi-Square (2-sided)**
C. odorata (C)	1	0	3	0			
H. rotundifolia A	0	3	0	1	0.05	6	.062
H. annuus (B)	1	3	0	0			
Total	2	6	3	1			
	Methanol extract (100 mg/ml)						
	0(mm)	10(mm)	12(mm)				

C. odorata (C)	1	3	0			
H. rotundifolia A	0	4	0	0.05	4	.030
H. annuus (B)	1	0	3			
Total	2	7	3			

Conclusion

All *P. aeruginosa* identified in this study were MDR, and 83.3% were AmpC producers with 100% sensitivity to imipenem. The three medicinal plants, *H. rotundifolia*, *C. odorata*, and *H. annuus* extracts, were effective on all the isolates tested. Also, there is no statistical difference between the methanolic and aqueous extracts of the three plants examined. However, higher effectivity of *H. rotundifolia* aqueous extract was detected against AmpC-producing MDR- *P. aeruginosa* (A13) from animal sources. Therefore, molecular screening and phyto-kinetic analysis of the AmpC and MDR-*P. aeruginosa* is recommended to be carried out to ascertain the basis of resistance and time of kill/inhibition of the plant extracts, as well as a toxicological assay of the plant extracts for drug production facilitation.

Conflict of interest:

The authors have disclosed that they do not have any conflicts of interest.

Authors' Declaration

The authors assert that the content presented in this article is original, and they accept responsibility for any claims related to its accuracy.

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