Evaluation of Phytochemical Constituents and Antibacterial Activity of *Chromolaena Odorata* L. Against Selected Multidrug Resistant Bacteria From Wounds

ABSTRACT

Aim: To study the antibacterial effect of *C. odorata* extracts on multidrug resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from wounds.

Place of study: University of Medical Sciences Teaching Hospital, Akure, Ondo State Nigeria, between January and June 2019.

Methodology: A total of 87 wound swabs were collected from patients of University of Medical Sciences Teaching Hospital, Akure, Air-dried and powdered *C. odorata* leaves were extracted using hot water, ethanol and methanol as extraction solvents and concentrated using a rotary evaporator. The concentrated *C. odorata* extracts were purified using chromatographic techniques. Qualitative and quantitative phytochemical screening of *C. odorata* extracts were done by standard methods. Antibiotics susceptibility pattern of bacterial isolates to a panel of ten (10) conventional antibiotics was determined by disc diffusion method.

Results: *Chromolaena odorata* methanolic extract had the highest extract yield (26.2%). From the multidrug resistance analysis, 66.7% of bacterial isolates tested had multidrug resistance index (MDRI) of 100%. *Pseudomonas aeruginosa* was susceptible to 100 mg/ml of *C. odorata* ethanolic extract but resistant (0.00±0.00) to 100 mg/ml of hot aqueous *C. odorata* extract.

Conclusion: This study reveals the inhibitory activities of *C. odorata* extracts on multidrug resistant bacteria isolated from wounds and an indication of their potential in the treatment of wound infection.

***Keywords:** Antibacterial, Chromolaena odorata leaf extracts, Multidrug resistant bacteria, Phytochemicals, Wounds.*

1. INTRODUCTION

A wound is a disruption in the structure of the skin leading to the exposure of the subcutaneous tissue and providing a condition suitable for microbial colonization and proliferation. The proliferation of microorganisms either from the body (endogenous) or from the environment (exogenous) in a wound causes wound infection [1]. The commonest pyogenic bacteria often associated with infected wounds are *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pneumococcus* species and coliform bacilli, such as *Escherichia coli*, *Proteus* species, *Pseudomonas aeruginosa* and other enteric bacilli. In chronic infection that are slow to heal and in pus showing no other microbes, there is a high possibility of infection with Mycobacterium tuberculosis and other Mycobacteria [1].

Originally, an antibiotic refers to an organic compound produced by a microorganism but has the potential to destroy another microorganism but the contemporary use of the term also includes antimicrobials produced through synthetic means [2]. Classes of antibiotics include beta-lactam, tetracycline, macrolides, quinolones, sulphonamides, oxazolidinones and glycopeptides, working either for the inhibition of cell wall, nucleic acid and protein synthesis, destruction of cell wall membrane or blockage of metabolic pathway. The condition when microorganisms (bacteria, fungi and viruses) are no longer destroyed by the use of antibiotics used against them is defined as antimicrobial resistance and has been reported to increase duration of illness and risk of death [3].

Antimicrobial resistance resulting majorly from the indiscriminate use of antibiotics is a primary threat to the success of modern health care [4] and has led to the studying of medicinal plants as a conceivable means of alternative therapy [5]. For many centuries, medicinal plants have been used as medicines in the treatment of many infections and diseases either as whole plants or plant extracts [6] because of bioactive compounds present in them which offers protection against

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microorganisms and insects. Medicinal plants are broadly used to cure various infections and also used as a precursor for the synthesis of natural drugs. Bioactive compounds responsible for therapeutic effects of plants are normally stored in plant cells as secondary metabolites and may vary in concentration depending on plant part, season, climate and growth phase [7].

Chromolaena odorata L. belongs to the family Asteraceae and is widespread over many parts of the world including Nigeria. The weed's common names include Siam weed, common floss flower and christmas bush [8]. *C. odorata* is commonly referred to as 'Obu inenawa' by the igbos (South-eastern Nigeria) , 'ewe awolowo' by the yorubas (South-western Nigeria) and 'bienqua' among the Ijaws in the Niger Delta region of Nigeria [9]. It has been been reported for its anti-inflammatory, analgesic, anthelmintic, anti-diarrhoeal, astringent, antispasmodic, antihypertensive, diuretic tonic, antipyretic, heart tonic, antigonorrheal, antioxidants and wound healing activities [10, 11]. A decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. Extract of *C. odorata* is used in the treatment of sore throat and pile and fresh juice squeezed out from its leaves is used to stop bleeding [9].

The decoction of the leaves and stems has been reported to be effective against the treatment of skin disease such as caused by *Propionibacterium acnes* [8]. Ethanol and methanol extracts of *C. odorata* has been reported for antibacterial activity against *S. aureus*, *S. pyogenes* and *P. acnes* associated with skin infection [11]. Naidoo *et al*. [12] also reports the antimicrobial potential of *C. odorata* extracts on *Bacillus cereus*, *S. aureus*, *S. epidermidis*, *Proteus vulgaris*, *Aspergillus flavus*, *A. glaucus*, *Candida albicans* and *Trichophyton rubrum*. This wound healing effect of *C. odorata* have been credited to saponins, tannins, essential oils and phenolic compounds present in the plant [13]. The problem of bacterial resistance to conventional antibiotics is growing so the outlook on its reliance in the future is still uncertain. This study investigated on antibacterial potentials of different extracts of *C. odorata* on multidrug resistant bacteria associated with wound infection.

2. METHODOLOGY

2.1 Collection of Clinical Samples and Isolation of Bacteria

Wound swabs were collected from patients in medical and surgical wards of University of Medical Sciences Teaching Hospital, Akure, Ondo State. A swab sample per patient was professionally collected by medical practitioners to avoid surface contamination. A total of 87 swabs were collected and immediately transported to the Department of Microbiology laboratory, Federal University of Technology, Akure, Nigeria. For the isolation of bacteria, wound swabs were evenly spread on prepared sterile nutrient agar plates and incubated at 37 ^{o}C for 24 hours [14]. After incubation, distinct colonies were picked from the primary isolation plates and subcultured on sterile nutrient agar plates and incubated at 37 ^{o}C for 24 hours. Pure colonies were inoculated on fresh nutrient agar slants, incubated at 37 ^{o}C for 24 hours and preserved at 4 ^{o}C for further studies. The bacteria isolates were maintained as part of the culture collection of Microbiology department, Federal University of Technology, Akure.

2.2 Presumptive of Identification of Selected Bacterial Isolates

The selected bacteria were identified using colonial morphology and biochemical characteristics. Colonial characteristics used for presumptive identification of bacterial isolates include colony opacity, colour, elevation, surface, edge and shape. Biochemical characteristics include Gram's reaction, catalase, citrate, urease, oxidase and sugar fermentation tests [15].

2.3 Collection of Typed Bacterial Isolates

Three pure typed cultures of bacterial species were collected from Federal Institute of Industrial Research, Oshodi (FIIRO), Nigeria and ascertained in Department of Microbiology, Federal University of Technology, Akure. A loopful of the bacteria was streaked on the surface of freshly prepared sterile nutrient agar plates and incubated at 37 ^{o}C for 24 hours. Colonial characteristics used for the confirmation include colony opacity, colour, elevation, surface, edge, shape and biochemical characteristics such as Gram's reaction, catalase, citrate, urease, oxidase and sugar fermentation tests [15].

2.4 Preparation of *C. odorata* Leaf Extracts

C. odorata leaves were collected from Ogbomoso, Oyo State, Nigeria and identified in the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure. The leaves were washed and air-dried at room temperature, grinded and stored in sterile paper bags. For the extraction, organic solvent extracts were prepared by soaking 100 g of the powdered *C. odorata* leaves in 1000 ml of methanol and ethanol for 72 hours and stirred frequently [12] while aqueous extracts were obtained by soaking 100 g of the pulverised plant leaves in 1000 ml of hot (100 ^{o}C) distilled water for 24 hours and stirred frequently [16]. The solutions were sieved using muslin cloth,

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filtered through Whatman No. 1 filter paper, concentrated using rotary evaporator and stored at 4 ^oC for further studies [11]. The percentage yield of crude extract was calculated as:

$$\text{Percentage Yield (\%)} = \frac{\text{Extract Yield}}{\text{Dried plant yield}} \times 100$$

2.5 Phytochemical Screening of Extracts

Phytochemical screening was carried out on the hot aqueous, methanolic and ethanolic extracts for the qualitative determination of phytochemical constituents as described by Akinmoladun *et al*. [17]. Plant extracts were screened for the presence of terpenoids, steroids, flavonoids, alkaloids, saponin, tannin, phlobatannin and phenol. Quantitative analyses of phytoconstituents in the extracts were also performed using methods described by Mir *et al*. [18].

2.6 Purification of Plant Extracts using Column Chromatography

A glass wool was fixed to the bottom of a clean column, the column was packed with silica gel and petroleum ether was passed through it for proper settling of the gel. Three grams of the extract was properly mixed with silica gel till it forms a free flowing powder, and loaded to the top of the prepared column. The packed column was eluted by gradient method using petroleum ether, chloroform, ethyl acetate and methanol as solvents. Fractions were collected in clean conical flasks [19].

2.7 Standardization of Bacterial Inoculum

A total of ten (10) *S. aureus*, twenty-seven (27) *P. aeruginosa* and thirteen (13) *K. pneumoniae* cultures were selected for this study. The bacterial inoculum was standardized to 0.5 McFarland standard using the method of Akinyemi and Ogundare [20]. A loopful of pre-cultured test bacteria was inoculated into 5 ml sterile nutrient broth and incubated for 24 hours at 37 ^oC. The broth was compared with the 0.5 McFarland standard for turbidity match.

2.8 Antibiotics Sensitivity Pattern of Bacterial Isolates

Susceptibility of isolated bacteria to conventional antibiotics was determined using the Kirby-Bauer (disc diffusion) method [20]. Exactly 0.1 ml of the standardized inoculum was introduced to prepared sterile Mueller-Hinton Agar plates using three-way swab method. Commercially available antibiotics disc inclusive pefloxacin (10 µg), gentamycin (10 µg), ampliclox (30 µg), zinnacef (20 µg), amoxicillin (30 µg), rocephin (25 µg), ciprofloxacin (10 µg), streptomycin (30 µg), septrin (30 µg) and erythromycin (10 µg) was used to determine the sensitivity of Gram positive bacteria. Disc inclusive septrin (30 µg), chloramphenicol (30 µg), sparfloxacin (10 µg), ciprofloxacin (30 µg), amoxacillin (30 µg), augmentin (10 µg), gentamycin (30 µg), pefloxacin (30 µg), ofloxacin (10 µg) and streptomycin (30 µg) was used for the determination of sensitivity of Gram negative bacteria to antibiotics. These antibiotics discs were aseptically placed on the agar plates using sterile forceps. The Mueller-Hinton plates were incubated at 37 ^oC for 18 hours. After incubation, diameters of zones of inhibition were measured to the nearest millimetre (mm) using a transparent meter rule.

2.9 Resistance Profile of Bacterial Isolates

Following the method of Oladoja and Onifade [21], the resistance of bacteria to conventional antibiotics was determined following the Clinical and Laboratory Standards Institute (CLSI) recommendation for each drug. The multidrug resistance index (MDRI) was calculated using the formula:

$$\text{MDRI (\%)} = \frac{\text{(Number of antibiotics indicating resistance)}}{\text{(Total number of antibiotics used)}} \times 100$$

2.10 Antimicrobial activity of *C. odorata* Extracts

One gram of *C. odorata* methanolic, ethanolic and hot aqueous extracts were reconstituted in 30% Dimethyl sulphoxide (DMSO) to obtain extract concentration of 100 mg/ml. The inhibitory activity of the extract on the bacterial isolates was determined using agar well diffusion method. 0.1 ml of the standardized inoculum of the test bacteria was transferred to the surface of sterile Mueller Hinton agar plates and streaked by three way method using a sterile swab stick to achieve a confluent growth. A 6mm cork-borer was used to bore wells on the solidified agar plates and 100 µg (0.1 ml) of purified extracts was introduced into each well. These plates were incubated at 37°C for 24 h and the zones of inhibition were measured in millimetres [5, 20]

2.11 Statistical Analysis

Data were analysed using one-way analysis of variance and presented as mean ± standard error. Statistical significance was determined at values $P \leq 0.05$ and the means were separated using Duncan's New Multiple Range Test by SPSS 20.0.

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3. RESULTS AND DISCUSSION

The bacterial isolates tested against conventional antibiotics and *C. odorata* extract in this study are common bacteria associated with wound infection and has been reported by many papers as the most prevalent pathogens [14, 22, 23]. In this study, methanol had the highest % yield of *C. odorata* extract as shown in Figure 1. This is in accordance with the findings of Mbajiuka *et al.* [10] who reported that the yield of *C. odorata* ethanol extract (3.8%) was higher than its aqueous extract (3.5%) but negates the findings of Mohd *et al.* [24] who reported higher % yield from *Orthosiphon stamineus* aqueous extract. The difference recorded on the percentage yield may be due to the difference in plant used or as a result of the varied polarity of the tested solvents; methanol and ethanol solvents are less polar compared to water. It could also be due to the disparity of the quality and quantity of their phytoconstituents.

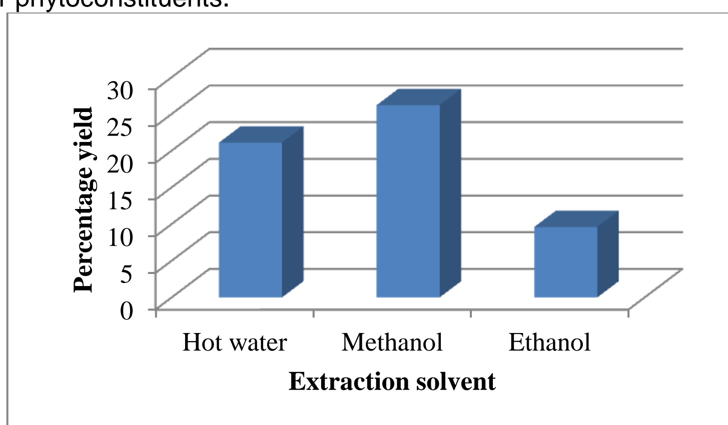

Figure 1: Percentage yield (%) of extracts of *Chromolaena odorata*

The qualitative and quantitative phytochemical components of *C. odorata* presented in Tables 1 and 2 shows the presence of phenol, saponin, tannin, glycosides, steroids, terpenoids and flavonoids in extracts of *C. odorata*. The presence of these phytochemicals in *C. odorata* has also been reported by other researchers [25-27]. These phytoconstituents have been reported for antibacterial activities such as formation of complex with extracellular soluble proteins and bacterial cell wall, disruption of cell membrane, precipitation of microbial protein and inactivation of microbial adhesion, enzymes, and cell envelope transport proteins [26, 28].

Table 1: Qualitative phytochemical components of *Chromolaena odorata* extracts

Solvent	Phenol	Saponin	Tannin	Glycoside	Steroid	Terpenoid	Flavonoid	Alkaloid	Phlobatannin
Methanol	+	-	+	+	+	+	+	-	-
Ethanol	+	+	+	+	+	+	+	-	-
Cold water	+	+	+	+	-	+	+	-	-

Key:

'+' indicates presence of phytochemical constituent

'-' indicates the absence of phytochemical constituent

Table 2: Quantitative phytochemical constituents of *C. odorata* extracts

Phytochemical	Cold water	Ethanol	Methanol
Saponin	130.73±0.18^{c}	34.00±0.18^{a}	110.73±0.18^{b}
Flavonoid	0.82±0.02^{a}	0.79±0.02^{a}	1.09±0.02^{b}
Terpenoid	29.55±0.03^{b}	36.17±0.03^{c}	0.00±0.00^{a}
Steroid	0.00±0.00^{a}	2.35±0.03^{b}	2.29±0.02^{b}
Tannin	0.55±0.01^{a}	1.90±0.01^{c}	1.50±0.01^{b}

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Glycosides	9.03±0.34^{b}	0.77±0.32^{a}	0.74±0.32^{a}
Phenol	13.41±0.06^{a}	13.88±0.06^{a}	17.51±0.06^{b}

Results are presented as mean±SE. Values carrying the same alphabet in the same row are not significantly different (p≤0.05)

Table 3 shows the zones of inhibition of bacterial isolates and multidrug resistance index of 100% was observed in all tested clinical bacterial isolates as shown in Table 4. As presented in figure 2, the ten (10) *S. aureus* isolates tested were resistant to erythromycin (10 µg) while only 50% were resistance to ciprofloxacin (10 µg) and amoxicillin (30 µg). As presented in figure 3, all twenty seven (27) *P. aeruginosa* isolates were resistant to augmentin (10 µg). As shown in figure 4, the highest antibiotics resistance of *K. pneumoniae* was to augmentin (10 µg) (76.9%) while the least was to ciprofloxacin (30 µg) (30.8%). Resistance in bacteria can be either vertical by mutating existing genes or horizontal by acquiring new genes from other strains or species [29, 30] and multidrug resistance is a present threat in antimicrobial therapy (Table 4).

Table 3: Antibiotics susceptibility profile of selected bacteria isolated from wounds

Antibiotics	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus* NTCC 6571	*P. aeruginosa* ATCC 10145	*K. pneumoniae* ATCC 13883
CPX	7.33±0.67^{c}	7.33±0.33^{cde}	9.33±0.33^{e}	16.67±0.33^{c}	15.67±0.33^{f}	9.00±0.58^{abc}
S	0.00±0.00^{a}	4.00±0.00^{b}	0.00±0.00^{a}	15.67±0.33^{d}	0.00±0.00^{a}	9.67±0.33^{bc}
SXT	2.67±1.33^{b}	7.67±0.33^{de}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	10.00±0.00^{abc}
E	0.00±0.00^{a}	NA	NA	12.33±0.33^{b}	NA	NA
PEF	6.67±0.33^{c}	9.33±0.33^{f}	9.33±0.33^{e}	14.00±0.58^{c}	13.33±0.33^{c}	9.00±0.00^{abc}
CN	0.00±0.00^{a}	4.33±0.33^{b}	3.67±0.33^{b}	13.33±0.33^{c}	4.67±0.33^{b}	9.00±0.00^{abc}
APX	4.33±0.33^{b}	NA	NA	0.00±0.00^{a}	NA	NA
Z	3.67±0.33^{b}	NA	NA	0.00±0.00^{a}	NA	NA
AM	3.00±0.00^{a}	4.00±0.00^{b}	5.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	8.67±0.33^{ab}
R	4.33±0.33^{b}	NA	NA	14.00±0.00^{c}	NA	NA
CH	NA	6.33±0.33^{c}	0.00±0.00^{a}	NA	0.00±0.00^{a}	9.33±0.33^{abc}
SP	NA	7.00±0.00^{cd}	0.00±0.00^{a}	NA	8.33±0.33^{c}	9.67±0.33^{bc}
AU	NA	1.00±1.00^{a}	0.00±0.00^{a}	NA	0.00±0.00^{a}	8.33±0.33^{a}
OFX	NA	8.33±0.33^{ef}	7.33±0.33^{d}	NA	11.33±0.67^{d}	9.33±0.33^{abc}

Results are presented as mean±SE. Values carrying the same alphabet in the same row are not significantly different (*p≤0.05*)

Key: PEF= Pefloxacin (10 µg); CN= Gentamycin(10 µg); APX= Ampliclox (30 µg); Z= Zinnacef (20 µg); AM= Amoxicillin (30 µg); R= Rocephin (25 µg);CPX= Ciprofloxacin (10 µg); S= Streptomycin (30 µg); SXT= Septrin (30 µg); E= Erythromycin (10 µg); SP= Sparfloxacin (10 µg); AU= Augmentin (10 µg); OFX= Ofloxacin (10 µg); CH= Chloramphenicol (30 µg); NA= Not applicable

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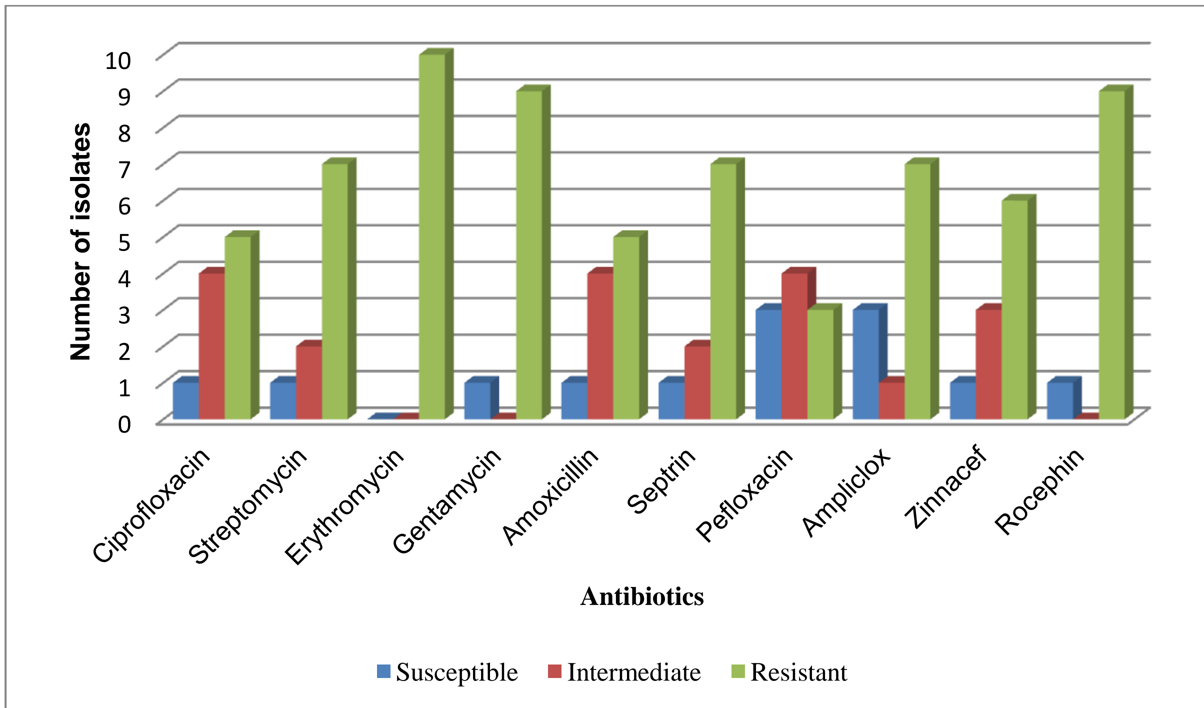

Figure 2: Antibiotics resistance pattern of *Staphylococcus aureus* isolated from wounds
Total number of isolates =10

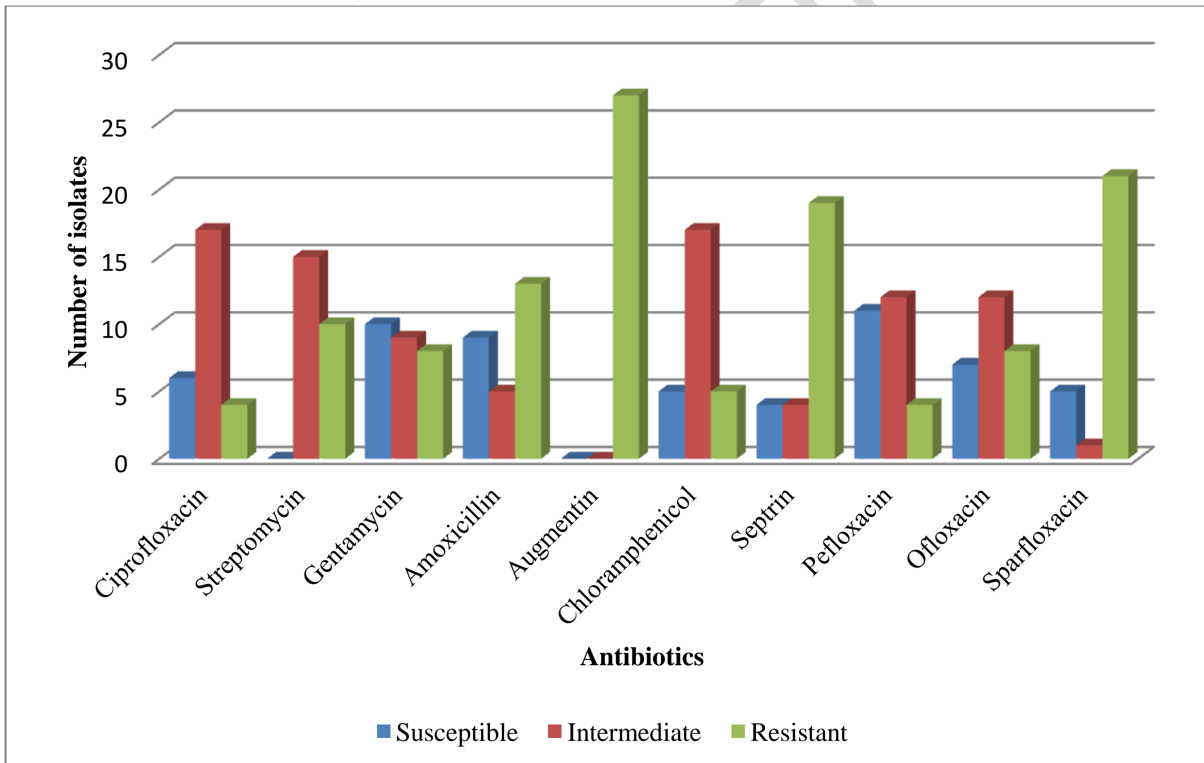

Figure 3: Antibiotics resistance pattern of *Pseudomonas aeruginosa* isolated from wounds
Total number of isolates =27

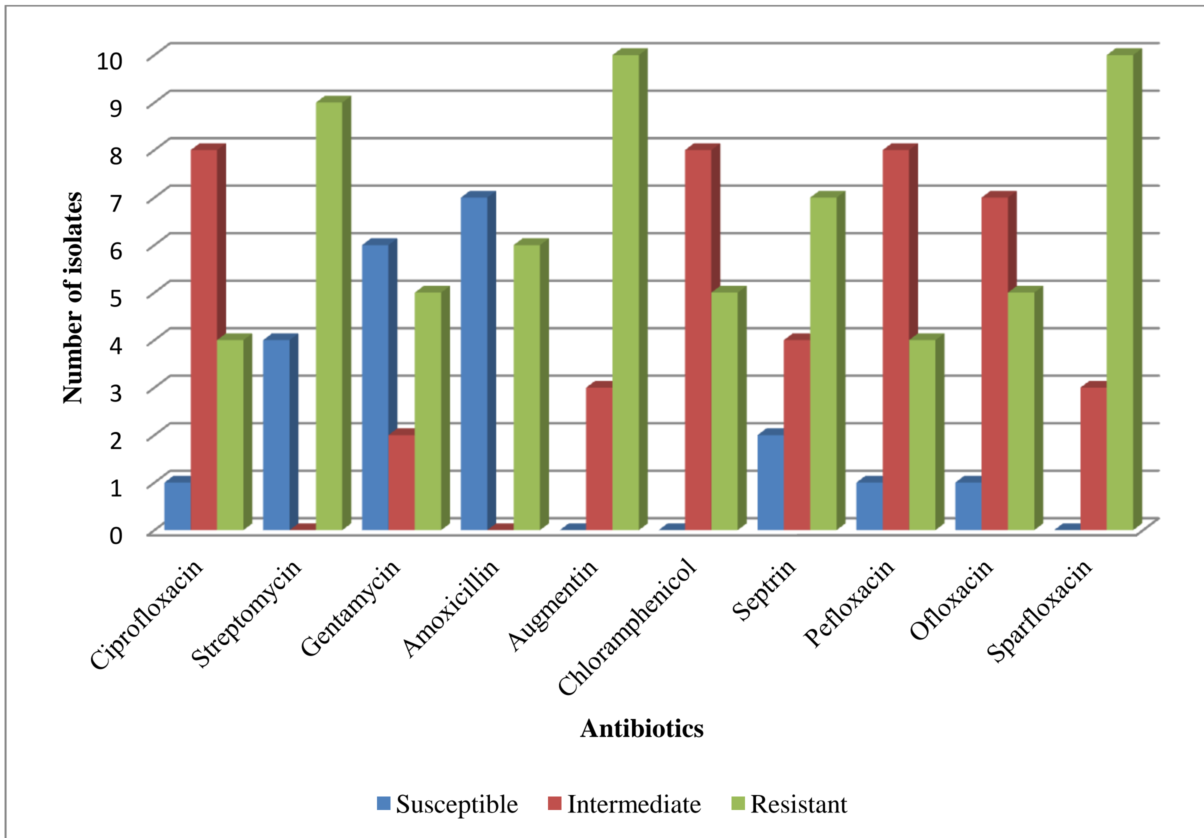

Figure 4: Antibiotics resistance pattern of *Klebsiella pneumoniae* isolated from wounds Total number of isolates =13

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Table 4: Antibiotics susceptibility profile of bacteria isolated from wounds

Antibiotics	SA	PA	KP	TSA	TPA	TKP
Ciprofloxacin	R	R	R	I	I	R
Streptomycin	R	R	R	I	R	R
Septrin	R	R	R	R	R	R
Erythromycin	R	NA	NA	R	NA	NA
Pefloxacin	R	R	R	R	R	R
Gentamycin	R	R	R	I	R	R
Ampliclox	R	NA	NA	R	NA	NA
Zinnacef	R	NA	NA	R	NA	NA
Amoxicillin	R	R	R	R	R	R
Rocephin	R	NA	NA	I	NA	NA
Chloramphenicol	NA	R	R	NA	R	R
Sparfloxacin	NA	R	R	NA	R	R
Augmentin	NA	R	R	NA	R	R
Ofloxacin	NA	R	R	NA	R	R
MDRI (%)	100	100	100	60	90	100

Key:
SA= *S. aureus*; PA= *P. aeruginosa*; KP= *K. pneumoniae*; TSA= *S. aureus* NTCC 6571; TPA= *P. aeruginosa* ATCC 10145; TKP= *K. pneumoniae* ATCC; MDRI= Multidrug resistant index; S= Susceptible; I= Intermediate; R= Resistant

The present high rate of antimicrobial resistance has been attributed to indiscriminate use of antibiotics and infections with drug-resistant bacteria remain an important problem in clinical practice. Ciprofloxacin has the least resistance across bacterial isolates tested compared to other antibiotics used in this study. Its effectiveness on bacteria isolated from wounds was also reported by Akinkunmi *et al*. [22] and Goswami *et al*. [31].

Zones of inhibition of purified *C. odorata* extracts on typed and clinical bacterial isolates are presented in Table 5. No zone of inhibition was observed with hot aqueous extract. This can be attributed to the inability of water to dissolve and extract the bioactive component of the plant. This findings corresponds with the result of Naidoo *et al*. [12] and Mbajiuka *et al*. [10] who reported no zones of inhibition against bacteria by *C. odorata* aqueous extract. The 100 mg/ml *C. odorata* methanolic and ethanolic extracts exhibited antimicrobial potential with better activity observed with typed bacterial isolates than with the clinical. This observed antibacterial activity of organic solvent extracts can be due to the accessibility of solvent to the bioactive component of the plant leaf which could have acted singly or in combination. This result corresponds with the finding of Srisuda *et al*. [11] stating the best extraction solvent of *C. odorata* leaves for antibacterial activity to be methanol and ethanol. The efficiency of extraction procedure depends on the accessibility of the constituent to the solvent [1]. Methanolic and ethanolic extracts of *C. odorata* showed antibacterial effect on both Gram positive and Gram negative bacteria tested which may suggest that its bioactive component possess broad spectrum antibacterial activity [32].

Table 5: Antibacterial activity of Purified *C. odorata* extracts on bacteria isolated from wounds

	Zones of inhibition (mm)		
Isolates	**Hot water**	**Methanol**	**Ethanol**
Staphylococcus aureus	0.00±0.00^{a}	9.33±0.33^{b}	10.00±0.58^{b}
Pseudomonas aeruginosa	0.00±0.00^{a}	16.00±0.58^{c}	9.67±1.21^{b}
Klebsiella pneumoniae	0.00±0.00^{a}	9.67±0.33^{b}	12.33±0.33^{c}
S. aureus NTCC 6571	0.00±0.00^{a}	12.67±0.58^{b}	12.00±0.58^{b}
P. aeruginosa ATCC 10145	0.00±0.00^{a}	15.00±0.58^{c}	12.33±0.33^{b}
K. pneumoniae ATCC 13883	0.00±0.00^{a}	14.67±0.33^{c}	13.00±0.58^{b}

Results are presented as mean±SE. Values carrying the same alphabet in the same row are not significantly different ($p \leq 0.05$)

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4. CONCLUSION

This study revealed the multidrug resistant bacteria associated with wounds. The study also reveals the presence of phenol, saponin, tannin, glycoside, steroid, terpenoid, and flavonoid in methanolic and ethanolic extracts of *C. odorata*. The antibacterial activity exhibited by methanolic and ethanolic extracts of *C. odorata* against multidrug resistant bacteria from wounds suggests their usage as a wound healing agent in alternative to conventional antibiotics. The study confirmed the susceptibility of multidrug resistant bacteria to *C. odorata* methanolic and ethanolic extracts. Against the daily increasing threat of antimicrobial resistance, *C. odorata* can serve as an alternative medicine and can also be used to discover bioactive components which will serve as leads for the manufacture of new drugs rather than removal of the weed by destruction. Further studies are recommended on biosafety assay of the extracts.

Ethical Approval

Ethical approval for the collection of wound swab samples from patients of University of Medical Sciences Teaching Hospital, Akure, Nigeria was obtained from Ondo State Health Research Ethics Committee, Ministry of Health, Ondo State, Nigeria.

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