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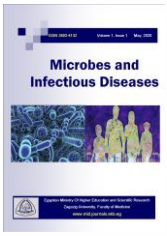

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Original article

Unlocking the antibacterial efficacy of *Chromolaena odorata* extract in wound healing

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ABSTRACT

Background: Wound complications are a worldwide problem which has morbidity, mortality and financial consequences and there is a need for alternative means of wound care. Plants are natural resources for applications against resistant bacteria due to their multiple mechanisms of action. Therefore, this study evaluated the antibacterial activity of *C. odorata* acetone extract in its crude and purified state against Gram-negative bacteria isolated from wounds. **Methods**: Bacteria were molecularly identified using the 16S rRNA sequencing. The antibiotic susceptibility of bacteria was assayed using disc diffusion methods. Plant leaves were collected and extracted, and the extract was screened for phytochemical constituents. The extract was purified using column chromatography. The antibacterial activities of crude and purified extracts were assayed using agar well diffusion methods. **Results**: The bacterial isolates were molecularly identified as *Pseudomonas aeruginosa*, *Providencia vermicola* and *Proteus mirabilis.* All three bacteria were resistant to all antibiotics tested. Phytochemicals present in the *C. odorata* acetone extract include phenol, saponin, tannin, glycoside, steroid, terpenoid and flavonoid. The percentage yield of *C. odorata* leaf acetone extract was 12.3%. The antibacterial activity of purified *C. odorata* acetone extract was better than the crude. The lowest MIC was 12.5 mg/ml and the lowest MBC was 100 mg/ml. **Conclusion**: *C. odorata* extract contains phytochemicals that possess antibacterial activity and should be exploited for antibacterial activities and used to discover bioactive natural products that serve as lead for the development of new antibacterial drugs.

Introduction

Wound infection is one of the primary reasons for hospital stays and makes treatment expensive and time-consuming for both patients and their families [1], which has been linked to the increase in the rate of antibiotic resistance. This increase in the absolute number of bacterial pathogens presenting multidrug resistance to antibacterial agents is a global health concern [2]. Resistance, which can result from mutation or acquired by transfer from other species [3], may take place irrespective of the presence of antibacterial agents. Nonetheless, exposure to these drugs provides the necessary selective pressure for the rise and spread of resistant pathogens [4]. Antibacterial agents, whether used by patients, in livestock or released into the environment is the driving force for antimicrobial resistance which has become a global health threat and requires concerted effort to tackle it at its very root [5]. Antimicrobial compounds are

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mainly from cultivable microbial strains. However, the rapid establishment of antimicrobial resistance has led to the search to exploit the untapped natural resources of different origin which produces biologically active metabolites [6].

Many pathogenic bacteria have developed different mechanisms to render ineffective the antibiotics used against them [7]. Interestingly, the use of natural products as medicine has been considered an effective and safer alternative to conventional antibiotics [8]. Natural products have also been utilized as preservatives which ensures the safety and quality of food and serves as alternative to other systems of preservation such as chemical or thermal ones [9]. Plants are well utilized natural resources for applications in pharmaceutical science due to their accessibility, abundance and possession of multiple mechanisms of action [10]. *Chromolaena odorata* has been demonstrated to exhibit antimicrobial activities. It has been reported for several ethnomedicinal uses and its extracts have also been reported for broad spectrum antimicrobial activity against multidrug-resistant bacteria [11–14]. The leaf extracts contain coumarins, tannins, steroids, saponins, terpenoids, terpenes, flavonoids and cardiac glycosides which are responsible for their antibacterial, anti-inflammatory and wound healing activities [15,16].

Metabolites in plants and other natural products function either by compromising the bacterial membrane integrity or disrupting essential components inside the cells. This differs from the specific receptors targeted by conventional antibiotics which allow the pathogenic bacteria to develop resistance more rapidly [17]. Towards the need for alternative medicine, there is a need for study for the discovery of novel compounds possessing antimicrobial properties. Therefore, this study aimed to evaluate the antibacterial activity of *C. odorata* acetone extract in its crude and purified state against Gram-negative bacteria isolated from wounds.

Methods

Culturing and biochemical characterization of bacterial isolates

Three Gram-negative bacteria isolated from wound samples denoted WSA1, WSA2 and WSA3 were used in this study. The bacteria were cultured on sterile nutrient agar and incubated at 37 ^{o}C for 24 hours. Bacterial isolates were biochemically characterized and pure fresh cultures were stored on nutrient agar slants at 4 ^{o}C [12].

16S rRNA identification of bacterial isolates

Bacterial isolates were cultured on sterile nutrient agar plates and incubated at 37 ^{o}C for 24 hours. Purified bacterial strains were used for DNA extraction. Genomic DNA extraction was carried out with JENA Bioscience Bacteria DNA Preparation Kit following the manufacturer's guide. 16S rRNA polymerase chain reaction (PCR) amplification was conducted following due protocol. Two universal primers; 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) were used for amplification of gene [18]. The amplicons were electrophoresed in 1.5% agarose gel containing ethidium bromide at 80 volts for 90 minutes and DNA bands were visualised using a transilluminator. A 100 bp DNA ladder was used as DNA molecular weight marker. The PCR products were purified with Exo sap purified PCR products and further amplified in one direction with the 16S primers using Big Dye terminator ready reaction mix. Sequencing of amplified product was done. Partial 16S rRNA gene sequence of studied bacteria was analysed with nucleotide BLAST search in GenBank [19].

Antibiotics sensitivity test of bacterial isolates

Susceptibility of isolated bacteria to conventional antibiotics was determined using the Kirby-Bauer method. Isolates were cultured at 37 ^{o}C for 24 hours and standardized to 0.5 McFarland standard. One-tenth of a millilitre of the standardized inoculum was aseptically introduced to the surface of Mueller-Hinton agar plates and swabbed for even distribution. Commercially available antibiotics discs were used to determine the susceptibility of bacteria. The antibiotics discs were aseptically placed on the inoculated agar plates using sterile forceps. The seeded plates were then incubated at 37 ^{o}C for 18 hours. After incubation, diameters of zones of inhibition were measured to the nearest millimetre (mm) using a transparent meter rule and interpreted according to Clinical and Laboratory Standard Institute (CLSI) standard [20]. The multidrug resistance index (MDRI) was calculated using the formula:

$$\text{MDRI (\%)} = \frac{\text{(Number of antibiotics indicating resistance)}}{\text{(Total number of antibiotics used)}} \text{X100}$$

Collection of plant leaves and preparation of plant extract

Leaves of *C. odorata* were collected from a farmland and identified at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure. The leaves were washed and air-dried at 25±2 ºC for 28 days after which they were pulverised and stored in a clean polythene bag. A hundred grams of the powdered *C. odorata* leaves was soaked in 1000 ml of acetone for 72 hours and stirred frequently. The sample was sieved using muslin cloth, filtered through Whatman No. 1 filter paper, concentrated using rotary evaporator and stored at 4 ºC [21]. The percentage yield of crude extract powder was calculated using the equation:

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

Phytochemical screening of *Chromolaena odorata* acetone extract

Qualitative and quantitative screening of *C. odorata* acetone extract for terpenoid, steroid, flavonoid, alkaloid, saponin, tannin, phlobatannin, phenol and cardiac glycoside was carried out following standard procedure [16].

Purification of *Chromolaena odorata* acetone extract

A glass wool was fixed to the bottom of the column after which the column was packed with silica gel and sufficient amount of petroleum ether was run through the packed column in order to make the silica gel completely settle. The sample was prepared by mixing 3 g of *C. odorata* acetone extract with silica gel and properly stirred till it forms a free-flowing powder. The sample was loaded to the top of the prepared column and the column was eluted by gradient method using petroleum ether, chloroform, ethyl acetate and methanol (in that order). Fractions were collected in clean conical flasks [22].

Antibacterial activity of crude and purified *C. odorata* acetone extract

Antibacterial activity of *C. odorata* leaf acetone extract was determined using agar well diffusion method. One-tenth of a millilitre of each standardized test organism was streaked on the surface of sterile Mueller-Hinton agar plates and allowed to stand for 15 minutes. A sterile 6 mm cork-borer was used to bore wells on the solidified Mueller-Hinton agar plates and 0.1 ml of both crude and purified *C. odorata* extract was introduced into each well. Ciprofloxacin (50 µg) was used as positive control and sterile distilled water as negative control. The plates were incubated at 37 ºC for 18 hours. The experiment was conducted in triplicate. Clear zones around the wells were indicative of inhibition and were measured in millimetre (mm) using a transparent meter rule [23].

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *C. odorata* leaf purified acetone extract samples were determined using the broth dilution method. Different concentrations of the extract were prepared by reconstituting in dimethyl sulphoxide (DMSO). One tenth of a millilitre of the standardized inoculum was added to each tube. These test tubes were incubated at 37 ºC for 18 hours alongside the control test tubes. The test tube containing growth medium and *C. odorata* leaf acetone extract without inoculum was used as negative control while the test tube containing the growth medium and the inoculum without *C. odorata* leaf acetone extract was used as the positive control. MIC was determined as the lowest concentration (mg/ml) of the extracts with no visible growth when compared with the positive and negative control tubes. The MBC was determined by sub-culturing the test tubes with no growth on sterile Mueller-Hinton agar plates and further incubated at 37 ºC for 24 hours. The lowest concentration (mg/ml) with no growth after culturing on Mueller-Hinton agar was regarded as the MBC [12].

Statistical analysis

Analysis was performed using Statistical Package for Social Sciences (SPSS) version 20.0. Data obtained were subjected to one-way analysis of variance (ANOVA) and result presented as mean ± standard error.

Results

The three Gram-negative bacterial isolates used in this study were molecularly identified as *Pseudomonas aeruginosa*, *Providencia vermicola* and *Proteus mirabilis* with 99.9, 99.4 and 92.0 percentage of identity, respectively **(Table 1 and 2)**.

All three bacteria were resistant to all antibiotics tested (MDRI=100%) **(Table 3)**. Although there were zones of inhibition, they were still resistant based on the CLSI interpretation. As presented in **Table 4**, phytochemicals present in the *C. odorata* acetone extract include phenol, saponin, tannin, glycoside, steroid, terpenoid and flavonoid; with phenol (35.87±0.06) and terpenoid (22.40±0.03) being highly abundant. After

extraction and concentration, the percentage yield of *C. odorata* leaf acetone extract was calculated to be 12.3%. **Table 5** presents antibacterial activity of *C. odorata* extract and purified extract presented better activity compared to crude extract. The lowest MIC was 12.5 mg/ml and the lowest MBC was 100 mg/ml **(Table 6)**.

Table 1. Biochemical characterization of bacterial isolates from wound swab samples.

Isolate number	Gram's reaction	Catalase	Citrate	Oxidase	Urease	Glucose	Fructose	Lactose	Sucrose	Mannitol	Maltose
WSA1	-	+	+	-	+	-	-	-	-	-	-
WSA2	-	+	+	-	+	AG	AG	AG	AG	AG	AG
WSA3	-	+	-	-	-	AG	AG	AG	AG	AG	AG

Key:+ = positive; - = negative; A = Acid production; AG = Acid and Gas production

Table 2. Molecular identification of bacterial isolates from wound swab samples.

Isolate code	Number of bases	Molecular identity	% identity	Accession number
WSA1	886	*Pseudomonas aeruginosa*	99.9%	MN786329
WSA2	778	*Providencia vermicola*	99.4%	MN786331
WSA3	222	*Proteus mirabilis*	92.0%	MN786330

Table 3. Antibiotics susceptibility of bacterial isolates.

Bacterial isolates	Antibiotics tested						MDRI (%)
	Ciprofloxacin (10µg)	Streptomycin (30µg)	Septrin (30µg)	Pefloxacin (10µg)	Gentamicin (10µg)	Amoxicillin (30µg)	
	Zones of inhibition (mm)						
Pseudomonas aeruginosa	7.33±0.33 (Resistant)	4.00±0.00 (Resistant)	7.67±0.33 (Resistant)	9.33±0.33 (Resistant)	4.33±0.33 (Resistant)	4.00±0.00 (Resistant)	100
Providencia vermicola	7.33±0.67 (Resistant)	0.00±0.00 (Resistant)	2.67±1.33 (Resistant)	6.67±0.33 (Resistant)	0.00±0.00 (Resistant)	3.00±0.00 (Resistant)	100
Proteus mirabilis	4.33±0.33 (Resistant)	0.00±0.33 (Resistant)	0.00±0.00 (Resistant)	3.33±0.33 (Resistant)	0.00±0.00 (Resistant)	0.00±0.00 (Resistant)	100

Table 4. Qualitative and quantitative phytochemical analysis of *Chromolaena odorata* leaf acetone extract.

Phytochemical	Qualitative	Quantitative
Phenol	Present	35.87±0.06
Saponin	Present	1.00±0.27
Tannin	Present	0.81±0.01
Glycoside	Present	2.44±0.32
Steroid	Present	3.03±0.03
Terpenoid	Present	22.40±0.03
Flavonoid	Present	3.34±0.02
Alkaloid	Absent	0.00±0.00
Phlobatannin	Absent	0.00±0.00

Table 5. Antibacterial activity of crude and purified extracts of *C. odorata* acetone extract.

Bacterial isolates	**Crude**	**Purified**	**Positive control**	**Negative control**
Providencia vermicola	8.67±0.33	15.67±0.33	25.67±0.33	0.00±0.00
Proteus mirabilis	8.67±0.33	14.33±1.20	30.33±2.73	0.00±0.00
Pseudomonas aeruginosa	10.33±0.88	17.00±0.00	30.33±0.33	0.00±0.00

Table 6. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Bacterial isolates	**MIC (mg/ml)**	**MBC (mg/ml)**
Providencia vermicola	12.5	100
Proteus mirabilis	12.5	100
Pseudomonas aeruginosa	25	200

Discussion

Pseudomonas aeruginosa has been isolated from wounds and may colonize the wound space with the capacity to severely damage tissues and negatively influence the effectiveness of therapy, thereby making it a virulence factor in wound healing [24]. Otu et al. [25] also reported the isolation of *P. vermicola* from wounds which agrees with the result of this finding. The third bacteria used in this study was identified as *P. mirabilis* and this has also been reported to be isolated from wounds [26]. All three bacteria have been reported as wound contaminants and associated with wound infection thereby delaying would healing. In exposed skin wounds following burn injuries or surgical incisions, bacteria which are most times the patient's normal flora often quickly colonize the wound. There is also the possibility of contamination from water, surfaces or healthcare personnel's hands [27]. Prolonged wound infections can impede the healing process, leading to clinical consequences such as worsening pain and diminished quality of life, as well as substantial strain on healthcare systems [28].

The bacteria tested were resistant to all six antibiotics used in this study. This indicates the high resistance of bacteria to conventional antibiotics. Although there are many contributing factors to antimicrobial resistance, there is no denying that antibiotic misuse has played a major role [29]. Shariati et al. [30] also reported increasing reports of ciprofloxacin resistance in *P. aeruginosa* which corresponds with the findings of this study. The result of this study corresponds with the result of Gahamanyi et al. [31] who reported high resistance rate to mostly used antibiotics. The increase in resistance to most antibiotics and emergence of multidrug resistant isolates could be associated with extensive use of antimicrobials not only as therapeutic agent for human infections but also for prophylaxis and growth promotion in animal husbandry. The resistance of all bacteria to ciprofloxacin at a lower concentration (10 µg) in this study, compared to the concentration used as a positive control in antibacterial assay is a concern because it is an antibiotic of choice in wide spectrum treatment in cases where other antibiotics are not effective. However, this study gives insight on the possibility to need to increase the dosage of ciprofloxacin for effective treatment which is a health concern.

Other bacteria that have been reported for resistance against ciprofloxacin *are Enterococci, Bacillus anthracis, Neisseria gonorrhoeae, Klebsiella pneumoniae* and *Escherichia coli* [32]. This resistance could develop by efflux pumps or mutations in DNA gyrase genes (*gyrA*) [30]. Conversely, Ahmed et al. [33] reported the sensitivity of *P. aeruginosa* to ciprofloxacin which negates the finding of this study. This can be as a result of the difference in the exposure of bacteria to antibiotics as exposure always precedes resistance. However, their study reported 52.4% multiple drug resistance indicating the resistance of other bacteria to other antibiotics asides ciprofloxacin. The use of antibiotics has brought the consequence of development of resistant bacterial strain which has prompted continuous effort to exert control over antibiotics usage. Multidrug-resistant bacteria have emerged from the use of antibiotics in treating infections and is presently a global health concern [5].

The complication of wound healing due to infection coupled with the health care challenge of antibacterial resistance necessitates the discovery of novel compounds with antibacterial potential and plants have been scientifically proven as rich source of these secondary metabolites. Saponin, phenol, tannin, glycoside, steroids, terpenoids and flavonoid are phytochemicals present in the *C. odorata* acetone extract. Hridhya and Kulandhaival [34] also reported the presence of saponin, terpenoids, glycosides, flavonoids, tannin and steroids in *C. odorata* collected from Palakkad. Phytochemical screening provides an overview of the phytochemicals present in plants and in relation to their quantity. The presence of these bioactive compounds depicts that *C. odorata* leaf extracts may exhibit antibacterial effects.

Acetone has been presented as a good choice of solvent mainly credited to its ability to extract compounds of a wide range of polarities, its low toxicity to bioassay systems and because it is easy to remove from extracts [35]. Furthermore, the efficiency of an extraction procedure depends on its accessibility of the constituent; hence, the antibacterial activity of *C. odorata* acetone extract suggests that the bioactive components of *C. odorata* are soluble in acetone. *C. odorata* acetone extract had zones of inhibition against bacteria tested indicating antibacterial activity. This agrees with the findings of Hanphanphoom and Krajangsang [21] who reported the activity of *C. odorata* leaves on *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Proteus vulgaris.* The range of bacteria (Gram positive and negative) in their study suggested that its bioactive component possess broad spectrum antibacterial activity. Udaya Prakash et al. [36] also reported the highest activity of *C. odorata* acetone extract against *S. aureus* and *P. aeruginosa* which corresponds to the finding of this study.

The purification of extracts can improve their antibacterial activity. In this study, there were higher values of zones of inhibition in purified extracts compared to the crude. This suggests the possibility of impurities in crude extracts hindering the activity of the contained metabolites which are able to exert their effects better when the extracts have been purified. This observation is supported by the study of Zhang et al. [37] who also purified extracts for better activity of extracts of waste peanut shells. The antibacterial potency of plant extract is determined in terms of MIC. The lower the value, the more active the extract is and vice versa. MIC values in this study ranged from 12.5 to 50 mg/ml and MBC values ranged from 100 to 200 mg/ml. Mbajiuka et al. [38] reported lower MIC values compared to this study, ranging from 0.125 to 0.25 mg/ml of *C. odorata* ethanol and aqueous extract against *E. coli, S. aureus* and *C. albicans*. Omeke et al. [39] reported higher MIC values which agrees with the result of this study. They reported MIC values ranging from 12.5 to 200 mg/ml and MBC values ranging from 12.5 to 400 mg/ml. Although MIC values in this study for *C. odorata* acetone extract are high they can still be exploited for antibacterial activities and serve as lead for the development of the new pharmaceuticals.

Conclusions

P. aeruginosa, *P. vermicola* and *P. mirablis* are common contaminants of wounds which can be from surfaces, the skin of patient or hands of health care providers. They can colonize wound surfaces resulting in infection and delaying wound healing. The increase in the use of antibiotics in health care and agriculture is resulting in antibiotic resistance, and this requires concerted effort to reduce the burden of this global menace, including regulation of sales of antibiotics over the counter, in developing and underdeveloped countries especially. High effect antibiotics such as ciprofloxacin are gradually becoming ineffective at lower dosages and this will lead to increased dosage which may increase the problem of resistance. *C. odorata* extract contains phytochemicals that have been reported for medicinal properties such as antibacterial, antioxidant, anticancer etc. Acetone extract of *C. odorata* leaves presented antibacterial activity; therefore, the plant extract can be harnessed in the discovery of new antibacterial remedies. It is recommended that extracts are purified for better effectiveness and specific bioactives are tested for their antibacterial activity.

Declaration of interest

The authors report no conflicts of interest.

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Authors' contribution

Muftau Kolawole Oladunmoye conceptualised and supervised this study. Mercy Adewumi Alabi conducted the study, wrote the drafts of this manuscript and analysed data. Olubukola Olusola-Makinde supervised the study,

confirmed data analysis and corrected the draft. All authors approved the final draft of this manuscript.

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