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Evaluation of the antimicrobial activities of *Napoleona imperialis* leaf extracts against multi-drug resistant bacterial isolates from diabetic foot ulcer

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Abstract

Background: The increased resistance of Gram-negative bacteria, particularly those that produce extended beta-lactamase, are limiting the efficacy of antimicrobial drugs in treating infected diabetic foot ulcers. This study evaluates antimicrobial activities of *Napoleona imperialis* leaf extracts on bacterial isolates of diabetic foot ulcers as a way of developing novel antimicrobials that will be effective in treating infections caused by multi-drug-resistant (MDR) bacterial isolates.
Methodology: Fresh leaves of *N. imperialis* were collected and identified by a plant taxonomist at the Department of Plant Biology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria. The leaves were washed and air-dried under shade and pulverized into fine powder using local milling machine. The pulverized plant was extracted using methanol, hexane, ethyl-acetate, and water. The test organisms used were MDR bacteria isolated from fresh clinical samples collected from patients with diabetic ulcer. The samples were processed using conventional cultures, biochemical identification and molecular detection by PCR methods, and antimicrobial susceptibility testing was done by the disc diffusion technique. The phytochemical compositions of the extract were assessed using standard methods. Antibacterial activity of the extracts was performed at a concentration of 400mg/ml of the extracts using agar well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MIC) of the extracts were determined using serial (doubling) dilution technique. The time-kill assay of the plant extract was also evaluated.
Results: The MDR isolates recovered from the diabetic ulcer were *Escherichia coli, Klebsiella pneumoniae*, *Streptcoccus pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The plant extract yield showed that aqueous fraction of the leaf extract gave higher yield of 37.3%, followed by ethyl acetate fraction of the leaf extract at 26.8%, hexane fraction of the leaf extract at 22.3%, and the least was crude methanol leaf extract at 13.4%. The phytochemical analysis showed that the leaf extracts contained phenols, flavonoid, tannins, glycosides, alkaloids, saponin, terpenoids and triterpenes. The methanol extract produced highest mean inhibition zone diameter of 19.7±00mm against *E. coli,* 19.3±1.2mm against *P. aeruginosa*, 19.3±1.2mm against *S. pneumoniae*, 18.7±1.2mm against *S. aureus* and 17.7±1.5mm against *K. pneumoniae*. The bacteriostatic (MIC) activities of this methanol extract at different concentrations ranged from 3.125 to 25.00mg/ml, and bactericidal (MBC) activities ranged from 6.25 to 50.00mg/ml. The time- kill assay of the crude methanol leaf extract at 1xMIC, 2xMIC and 3x MIC showed a decrease in the number of viable cells count of the initial inoculum within 2-8 hours of incubation, indicating high activity.
Conclusion: The methanol leaf extracts of *N. imperialis* could be used as a complementary source of antimicrobials to the conventional antibiotic in the treatment of wound infections caused by MDR bacteria due to the contents of essential secondary metabolites in the plant extract and the antibacterial activities observed.

Keywords: *Napoleona imperialis,* extract, bacteriostatic, bactericidal, time kill assay

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Évaluation des activités antimicrobiennes des extraits de feuilles de *Napoleona imperialis* contre des isolats bactériens multi-résistants aux antibiotiques provenant d'ulcères du pied diabétique

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Résumé:

Contexte: La résistance accrue des bactéries Gram-négatives, en particulier celles qui produisent une bêta-lactamase prolongée, limite l'efficacité des médicaments antimicrobiens dans le traitement des ulcères du pied diabétique infectés. Cette étude évalue les activités antimicrobiennes des extraits de feuilles de *Napoleona imperialis* sur des isolats bactériens d'ulcères du pied diabétique comme moyen de développer de nouveaux antimicrobiens qui seront efficaces dans le traitement des infections causées par des isolats bactériens multirésistants (MDR).
Méthodologie: Des feuilles fraîches de *N. imperialis* ont été collectées et identifiées par un taxonomiste végétal du Département de biologie végétale et de biotechnologie de l'Université Nnamdi Azikiwe, à Awka, au Nigéria. Les feuilles ont été lavées et séchées à l'air libre à l'ombre et pulvérisées en poudre fine à l'aide d'une machine à moudre locale. La plante pulvérisée a été extraite à l'aide de méthanol, d'hexane, d'acétate d'éthyle et d'eau. Les organismes d'essai utilisés étaient des bactéries MDR isolées à partir d'échantillons cliniques frais prélevés chez des patients atteints d'ulcère diabétique. Français Les échantillons ont été traités en utilisant des cultures conventionnelles, une identification biochimique et une détection moléculaire par des méthodes de PCR, et des tests de sensibilité aux antimicrobiens ont été effectués par la technique de diffusion sur disque. Les compositions phytochimiques de l'extrait ont été évaluées à l'aide de méthodes standard. L'activité antibactérienne des extraits a été réalisée à une concentration de 400 mg/ml des extraits en utilisant la méthode de diffusion en puits d'agar. La concentration minimale inhibitrice (CMI) et la concentration minimale bactéricide (CMI) des extraits ont été déterminées en utilisant la technique de dilution en série (doublement). Le test de time-kill de l'extrait de plante a également été évalué.
Résultats: Les isolats MDR récupérés de l'ulcère diabétique étaient *Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumoniae*, *Pseudomonas aeruginosa* et *Staphylococcus aureus*. Français Le rendement de l'extrait de plante a montré que la fraction aqueuse de l'extrait de feuille donnait un rendement plus élevé de 37,3%, suivie de la fraction d'acétate d'éthyle de l'extrait de feuille à 26,8%, de la fraction d'hexane de l'extrait de feuille à 22,3%, et le moins élevé était l'extrait de feuille au méthanol brut à 13,4%. L'analyse phytochimique a montré que les extraits de feuille contenaient des phénols, des flavonoïdes, des tanins, des glycosides, des alcaloïdes, de la saponine, des terpénoïdes et des triterpènes. L'extrait au méthanol a produit le diamètre moyen de zone d'inhibition le plus élevé de 19,7±00mm contre *E. coli*, 19,3±1,2mm contre *P. aeruginosa*, 19,3±1,2mm contre *S. pneumoniae*, 18,7±1,2mm contre *S. aureus* et 17,7±1,5mm contre *K. pneumoniae*. Les activités bactériostatiques (CMI) de cet extrait au méthanol à différentes concentrations variaient de 3,125 à 25,00mg/ml, et les activités bactéricides (CBM) variaient de 6,25 à 50,00mg/ml. Le test de temps de destruction de l'extrait de feuille au méthanol brut à 1xCMI, 2xCMI et 3x CMI a montré une diminution du nombre de cellules viables de l'inoculum initial dans les 2 à 8 heures suivant l'incubation, indiquant une activité élevée.
Conclusion: Les extraits de feuilles au méthanol de *N. imperialis* pourraient être utilisés comme source complémentaire d'antimicrobiens à l'antibiotique conventionnel dans le traitement des infections des plaies causées par des bactéries MDR en raison de la teneur en métabolites secondaires essentiels de l'extrait de plante et des activités antibactériennes observées.

Mots clés: *Napoleona imperialis*, extrait, bactériostatique, bactéricide, test de temps de destruction

Introduction:

The emergence of multi-drug resistant organisms (MDROs) in health care setting calls for continuous surveillance of antibiotic usage and identification of new and efficient compounds with prospect for developing new antimicrobial drugs (1). The World Health Organization (WHO) considers antimicrobial resistance (AMR) as an urgent crisis facing public health sectors (2).

Native communities in Africa have long practiced the use of herbal extracts in the treatment of various illnesses with proven efficacy (2). Medicinal plants contain rich bioactive compounds that are believed to aid the antimicrobial activities (3). There is an urgent need to discover new and effective antimicrobial candidate molecules that would be effective in treating infections caused by MDROs.

Napoleona imperialis belong to the family of *Lecythidiaceae* and is commonly known in different parts of Nigeria as Ukpakonrisa in Edo State, Akpodo/ukpodu in Anambra State, Ntum in Ikwuano, Obu-anagbo and Isi efe in Umuahia, Udure in Enugu-Ezike and Otukuche in Igala (4). The plant is an evergreen West African tropical tree and has a crown with very low dense branching. It can grow up to 6mm tall and is found mostly in Eastern Nigeria (5). There are various species which include *N. volgeri* Hook, *N. imperialis* (NI) P. Beau, and *N. gossweilien* Baker F (6), but its economic importance is not widely documented and *N. imperialis* is the least known specie (7).

The antimicrobial activities of *N. imperialis* leaves have been reported, the pulp of the fruit and stem bark help in alleviating pulmonary symptoms when chewed, and the leaf is an effective antibacterial agent (5). The report of Idu, et al., (8) showed that the leaf extract of *N. imperialis* is a broad-spectrum antibiotic with proven activity against fungal and bacterial isolates (9). Infections of the respiratory tract are treated using *N. imperialis* fruit and bark. Pulp of the fruit serves as food among the local indigenous Easterners in Nigeria. The use of the twig as chewing stick

helps to maintain oral hygiene (1).

Vital phytochemicals contained in the plant, which include tannins, alkaloids, steroids, flavonoids, glycoside, carbohydrate, saponins, and proteins are found in the roots (10). *Napoleona imperialis* is found to contain free-radical scavenging properties and antioxidant (6). Esimone et al., (11) have reported its ability to enhance wound healing, and its anti-hypertensive action. The leaves contained saponins, cyanide, proteins, tannins and glycosides (12). Ndukwe et al., (13) reported antibacterial activity of the seed and rind against Gram-positive and Gram-negative bacteria, and the leaf extract was safe at concentration of up to 5000mg/kg.

Among the chief complication of uncontrolled diabetes is foot ulcers which occurs as a result of poor foot care, underlying neuropathy, poor glycemic control, peripheral vascular disease amongst others (14). It is usually implicated as a risk factor for lower limb amputation and osteomyelitis and develops in areas that comes in contact with repeated sensational pressure and trauma (15). Microorganisms mostly associated with diabetic foot ulcers include *Escherichia coli, Streptococcus* species*, Proteus* species*, Pseudomonas aeruginosa* and *Staphylococcus aureus* (13). Increased contamination of diabetic ulcers is seen mostly in the skin and nares, with *S. aureus* having the highest chance of contamination of the foot ulcers (16). Previous studies have shown that *S. aureus* was the most predominant bacteria isolate from wound ulcers (17).

There is paucity of information on the *invitro* evaluation of antimicrobial activities of *N. imperialis* leaf against the bacterial isolates associated with diabetic foot ulcers. This study aimed at evaluating *invitro* antimicrobial activities of leaf extracts of *N. imperialis* against MDR bacteria isolated from diabetic foot ulcer.

Materials and method:

Study setting and ethical approval:

This study was carried out at Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi Nigeria, and wound swabs were collected from diabetic patients with foot ulcers. Approval was obtained from the Ethics Committee of the hospital and signed informed consent was also obtained from individuals who participated in the study.

Source of bacterial isolates:

Bacterial isolates were recovered from diabetic foot ulcer patients. Wound swabs collected from the patients were cultured using standard method and incubated aerobically for bacteria isolation. Phenotypic and genotypic methods were used for bacteria species identification and detection of drug-resistant genes.

Plant collection:

Fresh leaves of *N. imperialis* (Fig 1) were harvested, identified, and authenticated by a plant taxonomist at the Department of Plant Biology and Biotechnology, Nnamdi Azikiwe University Awka, and deposited in the herbarium with a voucher number NAUH-19F. The leaves were washed thoroughly and spread out on a newspaper where they were allowed to dry in a well-ventilated room. The dried sample was ground into a fine powder with a mechanical milling machine and stored in an airtight container for further studies.

Fig:1. Fresh *Napoleona imperialis* leaves in the forest

Preparation of plant for extraction:

One kg of pulverized leaf extract of *N. imperialis* was extracted by cold maceration technique. This was soaked in 6 liters of 90% methanol for 48 hours with intermittent shaking. The filtrate was dried under reduced pressure at a low temperature to recover the extract which was further reconstituted in 1000ml of 10% methanol and partitioned into various fractions of 4x1000ml of hexane, and 6x1000 ml of ethyl-acetate. The methanol extract and the fractions were dried in an evaporator at 40^{o}C and stored at 4^{o}C till ready for use. The purity of the extracts was assessed by plating the extract on Mueller Hinton (MH) agar and incubated at 37^{o}C for 24 hours.

Phytochemical screening:

Qualitative phytochemical screening of leaf extract was performed using standard methods as described by Onah et al., (15). The phytochemicals assessed include phenol, flavonoids, tannins, glucosides, alkaloids, anthrocyanin, terpenoids, aminoacids and triterpenes.

Antibacterial activity of methanol crude and fraction leaf extracts of *Napoleona imperialis*:

Antimicrobial activity of the methanol crude extract and the fractions were tested against MDR bacteria (*P. aeruginosa, Klebs-*

iella pneumoniae, S. aureus, E. coli, and *Streptococcus pneumoniae*) using the agar well diffusion method as previously described (16, 18). Briefly, two grams of each of the plant extracts were reconstituted in 5ml dimethyl sulphoxide (DMSO) to obtain a working stock concentration of 400 mg/ml. Muller-Hinton agar (Oxoid, USA) and Chocolate agar (for *S. pneumoniae*) were prepared in sterile Petri dishes and allowed to set. Standard turbidity (0.5 McFarland) of overnight cultures of the test isolates was inoculated aseptically on the agar plate using a sterile swab and, a 6 mm sterile metal cork-borer was used to make wells in the agar plates. Five hundred micro liter (500µl) of the methanol extract and the fractions as well as controls were aseptically used to fill each well and allowed to stand for 15 minutes to enable the extracts disperse into the agar. Levofloxacin (0.05mg/ml) served as the positive control while MH broth without extract served as the negative control.

The plates were incubated at 37°C for 24 hours, and the zone diameter of inhibition was measured with a ruler. The assay procedure was performed in triplicate and the mean inhibition zone diameter was recorded to the nearest mm. The zone diameter of inhibition produced by the extracts was compared to the zone diameter breakpoints for levofloxacin using the CLSI guideline (18) to determine sensitivity, intermediate or resistance (Table 1).

Determination of the minimum inhibitory and bactericidal concentrations:

The serial doubling dilution was used to determine the MIC and MBC as described by Achukwu et al., (19). Five milliliters of MH broth were added into test tubes and 1ml of each methanol crude extract and the fractions of the stock solutions was added into the first tubes. A two-fold dilution of the methanol extracts and the fractions in test tubes were carried out to obtain various concentrations at 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.781--mg/ml.

A standardized inoculum (McFarland) of 0.1ml of the test organisms was added into each test tube and incubated at 37°C for 24 hours. The first test tube that showed no turbidity (least concentration of extract) was taken to be the MIC. All the tubes that showed no turbidity were subsequently inoculated onto a MH agar plate and incubated for further 24 hours at 37°C. The MBC was the minimum concentration of the extracts that did not produce visible growth on the MH agar plate.

Time kill assay:

The percentage and log reductions from the initial microbial population (time kill) for each time point of methanol extract was determined using the method described by Ndukwe et al., (1) at concentrations of 1xMIC, 2xMIC, and 3xMIC using the plating method. Approximately 1ml of the methanol extract at 1xMIC, 2xMIC, and x3MIC was added to 5ml MH broth and 0.1ml of the standardized bacterial cell suspension was added and properly vortexed. The tubes were incubated at 37°C for 24 hours.

An aliquot of 0.1 ml of the mixture was removed from the tube every 2 hours for a minimum of 12 hours. This was serially diluted and plated on MH agar, and incubated for 24 hours at 37°C. The MH plates with significant bacteria colonies were counted and recorded as log 10 CFU/ml according to the formula of Bremmer et al., (20) and compared with the control.

Table 1: CLSI mean inhibition zone diameter (in mm) break points for the test bacterial isolates to levofloxacin (18)

Isolates/Levofloxacin	Sensitive	Intermediate	Resistance	Zone inhibition diameter of control
Escherichia coli	>21	17-21	≤16	25
Staphylococcus aureus	>19	16-18	≤15	23
Klebsiella Pneumoniae	>21	15-21	≤16	23
Pseudomonas aeruginosa	>22	15-21	≤14	25
Streptococcus pneumoniae	>17	14-16	≤13	22

The time-kill curve was produced by plotting the time of incubation against the logarithm of the number of viable cells. A 3log10 fold reduction in the original cell population which corresponds to 99.9% death of viable cells was regarded as the bactericidal concentration.

Statistical analysis:

The data obtained were presented as the mean of three replicates and expressed as mean± SD and analyzed using the statistical packages for the social (SPSS) version 27.0. The level of significance was set at $p \leq 0.05$.

Results:

Extract yield and phytochemical constituents:

The result of leaf extract yield indicated that aqueous fraction of leaf extract had the highest extract yield of 37.3%, followed by ethyl acetate fraction of leaf extract that produced yield of 26.8%, hexane fraction of leaf extract produced yield of 22.3%, and the least yiel d extracts was produced from methanol crude leaf extract at 13.4%.

The qualitative phytochemical analysis showed that methanol leaf extracts contain a number of secondary metabolites such as phenols, flavonoids, tannins, glycosides, alkaloids, saponins, terpenoids, and triterpenes.

Antimicrobial activities of the extracts:

The results of the *invitro* antimicrobial activity of the extract showed different zones of inhibition as presented in Table 2. Methanol crude extract produced a higher mean inhibition zone diameter of 19.7±0.3mm against *E. coli,* followed by a mean inhibition zone diameter of 19.3±1.2mm against *P. aeruginosa* and *S. pneumoniae* respectively, 18.7±1.2mm against *S. aureus*, and the least mean inhibition diameter of 17.7±1.5mm against *K. pneumoniae* ($p \leq 0.05$). Ethyl acetate extract produced higher mean inhibition zone diameter of 19.3± 1.2mm against *E. coli,* 18.7±12mm against *S. aureus,* 17.6±0.6mm against *K. pneumoniae,* 14.00±0.6 against *P. aeruginosa* and the least mean inhibition zone diameter of 12.7±1.2mm against *S. pneumoniae* ($p<0.05$).

The aqueous extract produced inhibition zone diameter of 19.00±0.0mm against *E. coli,* 17.7±0.6mm against *P. aeruginosa,* 14.0±0.3mm against *K. pneumoniae,* 14.0± 0.2mm against *S. pneumoniae* and the least, 12.7±1.2m against *S. aureus* ($p<0.05$). The hexane extract produced the highest mean inhibition zone diameter of 18.0±0.0mm against *K. pneumoniae*, 16.00±0.6mm against *P. aeruginosa*, 16.0±0.0mm against *S. aureus* and *S. pneumoniae*, and 11.7±2.5mm against *E. coli* ($p<0.05$).

The positive control antibiotic (levofloxacin) showed good inhibitory activity against *K. pneumoniae*, *S. aureus P. aeruginosa, E. coli,* and *S. pneumoniae* at inhibition zone diameters of 23.0mm, 23.0mm, 25.0mm, 25.0 mm and 22.0mm respectively while the negative control (DMSO) showed no activity (Table 3).

Minimum inhibitory concentrations and time kill assay of the extracts:

The bacteriostatic activities of the methanol leaf extracts of *N. imperialis* ranged from 3.125 to 25.00 mg/ml and the bactericidal activities ranged from 6.25 to 50.00 mg/ml as shown in Table 4. The time-kill assay represented a 3log10 or 99.9% reduction in the number of viable cells from the initial inoculum (Table 2).

The result of the kill-time assay showed that at 1xMIC, a reduction from initial inoculum to about 99.9% was observed at 6 to 8 hours. At 2xMIC it was observed at about 4 hours and at 3xMIC, a huge decrease in the quantity of viable cell count was observed at about 2 hours of incubation for all test isolates (Fig 2).

Table 2: Zone of inhibition diameter of *Napoleona imperialis* leaf extracts at 400mg/ml on test bacterial isolates

Bacterial isolates/solvent	Methanol	Aqueous	Ethyl acetate	Hexane	Positive control (levofloxacin 0.05mg/ml)	Negative control (DMSO)
Escherichia coli	19.7 ±03	19.0±0.0	19.3 ±1.2	11.7±2.5	25.0±0.0	0
Staphylococcus aureus	18.7±1.2	12.7±1.2	18.7±1.2	16.0±0.0	23±0.0.0	0
Klebsiella pneumoniae	17.7±1.5	14.0±0.3	17.6±0.6	18.0±0.0	23.0±0.0	0
Pseudomonas aeruginosa	19.3±1.2	17.7±0.6	14.0±0.3	16.0±0.6	25.0±0.0	0
Streptococcus pneumoniae	19.3±1.2	14.0±2.0	12.7±1.2	16.0±00	22.0±0.00	0

Table 3: Minimum inhibitory and bactericidal concentrations of *Napoleona imperialis* leaf extracts on test bacterial isolates

Test isolates/solvents	**Methanol**		**Aqueous**		**Ethyl acetate**		**Hexane**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	6.25	12.5	6.25	12.5	6.25	12.5	6.25	12.5
Staphylococcus aureus	6.25	12.5	25.0	50.0	6.25	12.5	12.5	25.0
Klebsiella pneumoniae	6.25	6.25	12.5	25.0	25.0	50.0	6.25	12.5
Pseudomonas aeruginosa	3.125	6.25	6.25	12.5	12.5	25.0	12.5	25.0
Streptococcus pneumoniae	6.25	12.5	6.25	12.5	12.5	25.0	6.25	12.5

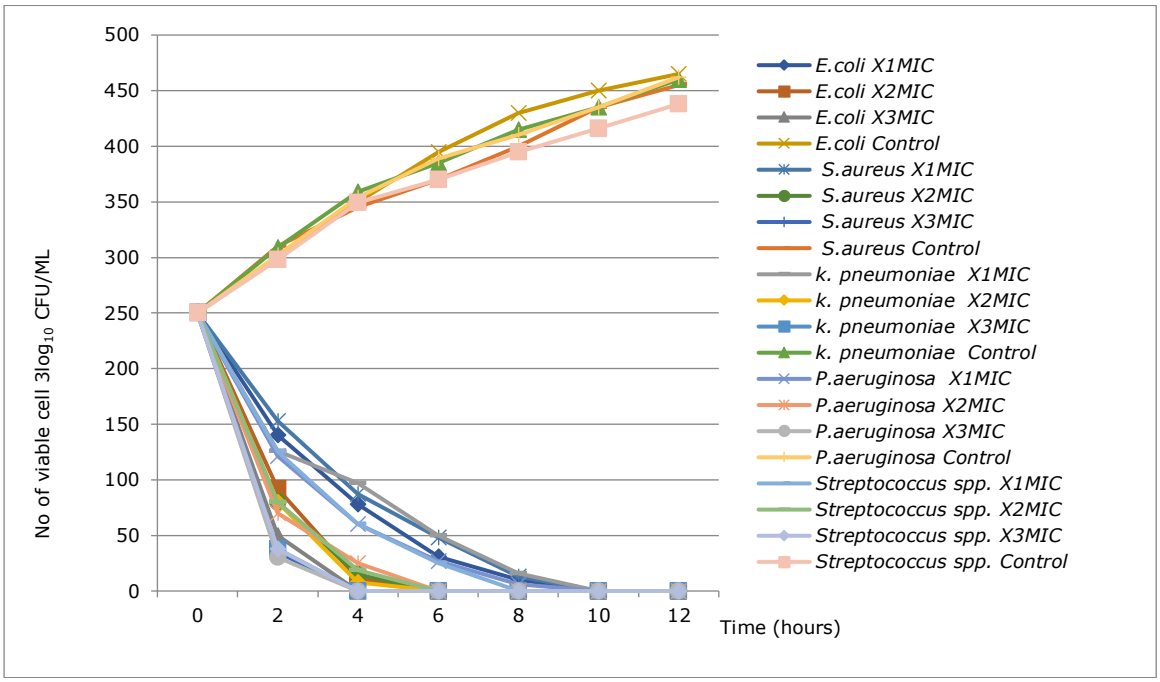

Fig 2: The time- kill curve of *Napoleona imperialis* methanol leaf extracts at 1xMIC, 2xMIC and 3xMIC on test bacterial isolates

Discussion:

The leaf extract yield revealed that aqueous extract had the highest yield, followed by ethyl acetate, hexane and the least was methanol extract. This supports the rationale for the indigenous use of mainly aqueous or alcohol-based extraction method in ethno-medicine. The phytochemical screening showed that the leaf extract of *N. imperialis* contained essential secondary metabolites such as phenols, flavonoids, tannins, glycosides, alkaloids, saponin, terpenoids, and triterpenes in varying degrees in all the extracts. These compounds are responsible for the therapeutic usage of medicinal plants (21). Flavonoids inhibit bacterial cell wall and cell membrane formation (16). This is similar to the reports of many studies (6,8,11) where the root and leaves extracts of *N. imperialis* produces enormous phytochemical constituents.

Comparing the various solvents used for extraction, methanol extract produced inhibition zone diameter for the test bacterial isolates that best compares with the inhibition zone diameters produced by the control antibiotic (levofloxacin) used, followed by ethyl acetate extract, aqueous extract, and n-hexane extract produced the least mean inhibition zone diameter. The observation regarding the antimicrobial action of plant extracts may be connected to the age of the plant used, the freshness of the plant material, physical factors, the timing of the plant material harvesting, and the drying technique used before the extraction process (3).

Comparing the mean inhibition zone diameters produced by the test isolates against the extracts with levofloxacin zone diameter breakpoints that determine sensitivity, intermediate or resistance by the CLSI guideline, the mean zone diameter range produced by *E. coli* isolates (11.7-19.7 mm) in our study indicates that they are intermediate to the methanol, aqueous and ethyl acetate extracts, and resistant to n-hexane fraction of the leaf ext-

ract.

Staphylococcus aureus isolates with mean inhibition zone diameter range of 16.0-18.7mm shows that they are susceptible to ethyl acetate fraction of the leaf extract, intermediate to methanol crude and resistant to aqueous and hexane fractions extracts. *Klebsiella pneumoniae* with mean inhibition zone diameter range of 14.0-18.0 mm shows that they are susceptible to the hexane fraction leaf extract, intermediate to methanol and ethyl-acetate extracts, and resistant to the aqueous extract. *Pseudomonas aeruginosa* with mean inhibition zone diameter range of 14.0-19.3mm indicates that they are susceptible to methanol and aqueous extracts, intermediate to hexane and resistant to ethyl acetate extract. *Streptococcus pneumoniae* with mean inhibition zone diameter range of 12.7-19.3mm are susceptible to the methanol extract. Although, the antibacterial activities of the leaf extracts with the different solvents appeared lower compared to levofloxacin control, these extracts nevertheless possess promising antibacterial activities that may become pronounced if the bioactive components are isolated.

The leaf extracts of *N. imperialis* produced bacteriostatic action at different concentrations that ranged from 3.165-12.5 mg/ml and bactericidal action from 6.125-50 mg/ml against the test isolates. This provide scientific evidence in support of the indigenous use of this leaf in the treatment of infected diabetic foot ulcers since the extracts produced their antibacterial effects at relatively low concentrations. Our finding is similar to the study of Ndukwe et al., (1), who reported that the leaf extracts exhibited antimicrobial activities against both Gram-negative and Gram-positive bacterial isolates**.** Our result also agrees with another study that reported broad-spectrum antimicrobial activities of ethyl acetate extracts of stem bark and leaf of *N. imperialis* against bacterial isolates (8).

The bacteriostatic and bactericidal activities of *N. imperialis* leaf extract can be attributed to the enormous secondary metabolites contained in the leaf extract which include phenols, flavonoids, tannins, glycosides, alkaloids, saponin, terpenoids, and triterpenes (21). Alkaloids have been shown to possess antibacterial, antiviral, and anti-inflammatory properties, and also aid in the production of other end products of metabolism. Tannin has been reported to hasten the rate of wound healing and possesses antibacterial and antioxidant properties. Our study agrees with a previous study by Ihekwereme et al., (12), who reported the antibacterial activity of *N. imperialis* leaf extracts against both Gram-negative and Gam-positive bacterial isolates.

The time-kill assay showed that methanol leaf extract of *N. imperialis* was time and concentration-dependent against the bacterial test isolates. This was observed when the test isolates were exposed to 1xMIC concentration of the leaf extract, which took about 6-8 hours to initiate a reduction in viable cell count but when the concentration was increased to 3x MIC, it took only about 2 hours for a reduction in viable cells to be initiated. The reduction in the number of viable colonies counts within 2 and 8 hours of incubation suggested enormous bactericidal properties and may be ascribed to the presence of flavonoids, tannins and alkaloids in conjunction with other phytochemicals that may be present.

Conclusion:

Evaluation of the antimicrobial activity of the methanol leaf extract of *N. imperialis* is of great importance to traditional medicine because it can increase its acceptability to the populace once backed by scientific evidence of efficacy as shown in the current study. Therefore, this extract could be recommended as a complementary antibacterial candidate to conventional medicine in the treatment of infections caused by MDR microbial isolates. Further studies are required to isolate and elucidate the bioactive components in the extract.

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Contribution of authors:

UEA and EIB designed the study; ANO wrote the protocol; and contributed to the literature search; UEA EIB and ANO, performed the laboratory analysis; ANO performed statistical analysis of data; UEA and EIB contributed in discussions; UEA produced the initial manuscript draft; EIB supervised the study; UEA wrote the final manuscript; EIB proofread the manuscript and all authors approved the final manuscript submitted for publication.

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Conflict of interest:

Authors declare no conflict of interest

Authors' declaration:

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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