Achukwu et al. *Afr. J. Clin. Exper. Microbiol.* 2024; 25 (4): 438 – 445 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology. ISSN 1595-689X Oct 2024; Vol.25 No.4
AJCEM/2349. https://www.ajol.info/index.php/ajcem

Original Article **Open Access**

In vitro evaluation of *Morinda lucida* root extracts against multi-drug resistant bacterial pathogens isolated from diabetic foot ulcers

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Abstract:

Background: The continuous rise in microbial resistance to orthodox antimicrobial drugs has led to the search for alternative sources with proven efficacy to solve the challenges of antimicrobial resistance (AMR). The preferred alternatives are plant sources, and this has led to the evaluation of constituents and potency of medicinal plants to provide scientific justification for their use.
Methodology: The root of *Morinda lucida* plant was dug up from the ground, washed clean, and cut into smaller pieces and dried. The root was then ground into fine powder and extracted with water (aqueous), methanol, ethyl acetate and n-hexane. Multi-drug resistant (MDR) *Staphylococcus aureus, Escherichia coli Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* were isolated from wound swab samples collected from patients with diabetic foot ulcers using conventional cultures, biochemical identification, and antimicrobial susceptibility tests. The phytochemical and proximate contents of the extracts were assessed by standard technique. The *in vitro* antimicrobial activities of the extracts were determined at a concentration of 200mg/ml of the extract using the agar well diffusion technique. The minimum inhibitory and bactericidal concentrations were determined by serial doubling dilution. The methanol extract time-kill assay was performed to determine the time of kill of the bactericidal concentration.
Results: The phytochemical analysis showed that *M. lucida* root contains essential secondary metabolites such as flavonoids, alkaloids, tannin, saponin, glucosides, anthraquinone and quinine. The methanol and aqueous extracts showed higher *in vitro* antibacterial activity, producing the highest zone of inhibition of 27mm against *S. aureus* but a lower activity of 18 mm with n-hexane extract against all isolates except *S. aureus*. The MIC ranges from 3.125mg/ml and 25mg/ml. The time-kill assay of methanol extract at 2x and 3x MIC showed that bactericidal activity occurred within 0-8 hours of incubation, indicating high activity.
Conclusion: The antibacterial potency of *M. lucida* root extract and the phytochemical components from this study shows that it can serve as a source of alternative antimicrobial agent that may be effective in the treatment MDR bacterial infections.

Keywords: *Morinda lucida*, phytochemical, antibacterial activity, time kill, diabetic foot ulcer

Received Jun 7, 2024; Revised Jul 7, 2024; Accepted Jul 9, 2024

Évaluation *in vitro* d'extraits de racine de *Morinda lucida* contre des agents pathogènes bactériens multirésistants isolés d'ulcères du pied diabétique

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Résumé:

Contexte: L'augmentation continue de la résistance microbienne aux médicaments antimicrobiens orthodoxes a conduit à la recherche de sources alternatives ayant une efficacité prouvée pour résoudre les défis de la

résistance aux antimicrobiens (RAM). Les alternatives privilégiées sont les sources végétales, ce qui a conduit à l'évaluation des constituants et de la puissance des plantes médicinales afin de fournir une justification scientifique à leur utilisation.
Méthodologie: La racine de la plante *Morinda lucida* a été déterrée du sol, lavée, coupée en morceaux plus petits et séchée. La racine a ensuite été broyée en poudre fine et extraite avec de l'eau (aqueuse), du méthanol, de l'acétate d'éthyle et du n-hexane. *Staphylococcus aureus* multirésistant (MDR), *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* et *Pseudomonas aeruginosa* ont été isolés à partir d'échantillons d'écouvillons de plaies prélevés sur des patients atteints d'ulcères du pied diabétique à l'aide de cultures conventionnelles, d'identification biochimique et de tests de sensibilité aux antimicrobiens. Les contenus phytochimiques et immédiats des extraits ont été évalués par une technique standard. Les activités antimicrobiennes in vitro des extraits ont été déterminées à une concentration de 200mg/ml de l'extrait en utilisant la technique de diffusion sur puits d'agar. Les concentrations minimales inhibitrices et bactéricides ont été déterminées par double dilution en série. L'analyse de destruction temporelle de l'extrait de méthanol a été réalisée pour déterminer le moment de destruction de la concentration bactéricide.
Résultats: L'analyse phytochimique a montré que la racine de *M. lucida* contient des métabolites secondaires essentiels tels que des flavonoïdes, des alcaloïdes, du tanin, de la saponine, des glucosides, de l'anthraquinone et de la quinine. Le méthanol et les extraits aqueux ont montré une activité antibactérienne in vitro plus élevée, produisant la zone d'inhibition la plus élevée de 27mm contre *S. aureus* mais une activité plus faible de 18mm avec l'extrait de n-hexane contre tous les isolats à l'exception de *S. aureus*. La CMI varie de 3,125mg/ml à 25mg/ml. Le test de destruction temporelle de l'extrait de méthanol à 2x et 3x CMI a montré qu'une activité bactéricide s'est produite dans les 0 à 8 heures suivant l'incubation, indiquant une activité élevée.
Conclusion: Le pouvoir antibactérien de l'extrait de racine de *M. lucida* et des composants phytochimiques de cette étude montre qu'il peut servir de source d'agent antimicrobien alternatif susceptible d'être efficace dans le traitement des infections bactériennes MDR.

Mots clés: *Morinda lucida*, phytochimique, activité antibactérienne, le temps tue, ulcère du pied diabétique

Introduction:

The development of antimicrobial resistance by pathogens to the most valuable orthodox medicines has rendered the treatment of infections caused by them ineffective, driving humans back to nature in search of solutions. Herbal plants have been part of man's culture as most of them have essential therapeutic compounds, that could help in the treatment of animal and human diseases (1). It has been documented that the life span of therapeutic agents is limited (2), thus leading to the emergence of drug resistance. Moreover, most of conventional drugs are expensive and some have dangerous side effects. Hence, identifying new, safe and efficacious drugs that have little or no side effects, has turned out to be an important goal of research, which has created great interest in herbal plants. Medicinal plants are now being embraced by many scientists and research are aimed at screening herbal flora with the goal of obtaining secondary metabolites and minerals that could serve as alternatives to orthodox drugs (3).

Morinda lucida belongs to the family of Rubiacea which is Nigeria is known by local names such as 'Ezeogwu' in Igbo, 'Oruwo' in Yoruba and 'Idonzakara' in Hausa. The plant can grow as high as 18-25m (4). The stem-bark is usually rough, grey, and brittle, and the leaf is broadly elliptical. The roots of *M. lucida* are golden yellow in color with bitter taste, and are commonly used as chewing sticks in most of part of Nigeria but also as flavoring agent in food and alcohol-based beverages (5). Additionally, *M. lucida* is a highly valued herb in Nigeria by traditional healers where it is used in the treatment of malaria and various types of fever (6). It can also be used in the management of jaundice (6), as such, it is very essential in ethnomedicine (7). The stem-bark, root and leaf infusion and decoction are employed in the cure of wounds, stomachache and fever of all kinds including those ailments following child birth (8). The root alone can be used in the treatment of upper back pain, and menstrual disorders, increase sexual capacity, boost the immune system, and decrease disease resistance (9).

Studies have reported that *M. lucida* contains many secondary metabolites that possess antimicrobial, antioxidant and antiplasmodial activities (10). It is therefore important to search for new antibiotic source that can be readily available, cost-effective, and less toxic. Every part of *M. lucida* has been reported to possesses medicinal properties and is an all-year-round plant endowed with numerous nutrients (11). The entire plant parts such as stem, root, fruit, and leaves are used widely as multipurpose medicinal plants for the treatment of diseases and are believed to be rich in phytochemical components that have diverse effects on microbes, cancer, hypertension, diabetes, and antiplasmodial activities among other ailments (9).

Diabetic foot ulcers are wounds or open sores that occur in a significant number of diabetic patients. They typically develop in individuals with poorly managed diabetes mellitus (12). This makes it impossible for the ulcers to heal normally predisposing the patient to increased risk of infection and amputation by up to 84% (13). Some of the pathogens linked to diabetic foot ulcers inc-

lude *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus* species, *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus pyogenes, Streptococcus mutans* and *Bacillus subtilis* (14). Many studies have reported that *M. lucida* can be used to control diabetes mellitus and also possess antibacterial properties (15).

There is paucity of information on the *in vitro* effect of *M. lucida* on bacterial pathogens associated with diabetic foot ulcers. This study therefore aims to evaluate the *in vitro* antimicrobial activity of the root extracts of *M. lucida* against bacterial pathogens isolated from patients with diabetic foot ulcers.

Materials and method:

Study setting and ethical approval:

The study was conducted in the University of Nigeria (UNN) Enugu, Nigeria. Ethical approval to conduct the study was obtained from the Ethics Committee of the College of Medicine, University of Nigeria Enugu Campus.

Plant collection:

The roots of *M. lucida* were harvested, identified, authenticated, and given specimen voucher number by a Botanist in the Department of Plant Science and Biotechnology, University of Nigeria Nsukka, and deposited in the herbarium (Fig 1). The root was thoroughly rinsed with multiple changes of clean water until it was completely clean. This was then cut into small pieces to facilitate drying and grinding, and shade-dried until fully dried before being pulverized into fine powder. Finally, the powder was stored in a sterile container until ready for use.

Fig 1: Freshly harvested *Morinda lucida* root

Preparation of plant for extraction:

Cold maceration was employed to extract compounds from the *M. lucida* roots. One thousand gram (1000g) of the pulverized roots were soaked in 6 liters of 90% methanol for 48 hours with intermittent shaking. The resulting filtrate was dried under reduced pressure at low temperature to obtain the dried extract after which it was then reconstituted in 1000 ml of 10% methanol and successfully partitioned into fractions using 4× 1000 ml of n-hexane and 6×1000 ml of ethyl acetate. The purity of the extracts was assessed by plating the extracts on Mueller Hinton agar and incubated overnight.

Phytochemical screening and proximate analysis:

The phytochemicals assessed were alkaloids tannins, steroids, flavonoids, saponins, quines, and terpenoids and this was done using the method of El-akhal et al., (16). The pulverized root extracts were analyzed for moisture, crude ash, crude fiber, dry matter, crude fat, protein, and mineral composition according to the methods of Association of Official Agricultural Chemists (17) and Satriadi et al., (18).

Antimicrobial activity of the root extracts of *Morinda lucida*:

Antimicrobial activity of the root extracts was tested against multi-drug resistant *K. pneumoniae, P. mirabilis, S. aureus, P aeruginosa* and *E. coli* isolated from wounds of patients with diabetic foot ulcers using conventional cultures, biochemical identification, and antimicrobial susceptibility test.

The antimicrobial activity of the root extracts was assessed using the agar-well diffusion technique as previously described by Achukwu (19) and Hossain (20). Cell suspension of 5×10^5 of each bacterial isolate was prepared and the turbidity standardized to 0.5 McFarland standards. A sterile glass rod was dipped into the standardized cell suspension, allowing excess fluid to drain. The inoculum was evenly streaked over the entire surface of Mueller-Hinton (MH) agar plate and allowed to dry for 10 minutes. Wells were then made with a 6 mm sterile cork-borer in the agar plate and filled with 0.5 ml of each extract. Levofloxacin (0.05mg/ml) and MH broth without extract were used as positive and negative control respectively.

The plates were incubated at 37ºC for 24 hours, following which the inhibition zone diameters were measured. The test was performed in triplicate and the mean zone diameters of inhibition were calculated for each isolate and extract.

Determination of MIC and MBC:

The minimum inhibitory and bacteri-

cidal concentrations were determined by doubling dilution as described by Ehaimir (21). The exacts were prepared into different concentrations ranging from 200 mg/ml to 0.78 mg/ml. The extract concentrations were incorporated into 5ml MH broth and 100 µl of the test inoculum added to each tube. These were incubated for 24 hours at 37°C. The tube with the lowest concentration (without turbidity) is the MIC. The non-turbid tubes were sub-cultured on solid MH agar and the least concentration that produce no growth is regarded as the minimum bactericidal concentration (MBC).

***Time-kill assay*:**

The MIC obtained was used to assess the time of killing by the methanol crude extract at 1×, 2×, and 3× MIC of the test isolates obtained using plating method previously described by Achukwu (19). One millimeter of methanol extract at 1×, 2×, and 3× MIC were added into 5 ml MH broth, and 0.1 ml of the standardized bacterial cell suspension added and properly mixed. The tubes were incubated at 37°C overnight. A 0.1ml of the mixture was removed from the broths every 2 hours for 12 hours (to determine the time of bactericidal action), serially diluted, plated on MH agar, and incubated for 24 hours at 37°C.

Culture plate with significant bacteria colonies were counted and recorded as log10 CFU/ml according to the formula of Bremmer et al., (22), and then compared with the control. The time-kill curve of the time of incubation was plotted against the logarithmic of the number of viable cells. A 3log10 fold decline in the original cell population which is equivalent to 99.9% death of viable cells is regarded as bactericidal concentration.

Statistical analysis:

The data were presented as mean of three replicates and expressed as mean ± SD and analysed using the Statistical Packages for the Social Sciences (SPSS) version 17.0. The level of significance was set at $p \leq 0.05$.

Results:

The extract yield showed that aqueous extract gave a highest yield of 30.3%, followed by methanol extract at 26.4%, ethyl acetate fraction at 21.5%, and the least is n-hexane at 17.3% (Table 1). The phytochemical screening revealed that it contains essential secondary metabolites such as flavonoids, alkaloids, phenols, tannins, quines, and anthraquinones (Table 2). The proximate screening of the *M. lucida* root extract as presented in Table 3 showed that it contains significant amount of crude fiber (11.46%), carbohydrates (44.32%), fat (15.15%), and protein (7.68%).

Table 1: Percentage yield of extracts and fractions

Extract	**Yield %**
Methanol	26.4
Aqueous	30.3
Ethyl acetate fraction	21.5
N-hexane fraction	17.3

Table 2: Phytochemical constituents of *Morinda lucida* root extracts

Constituents	***Morinda lucida* root**
Anthraquinones	+
Saponins	+
Tannins	+
Alkaloids	+
Flavonoids	+
Cardiac glucosides	-
Phenols	+
Steroids	+
Terpenoids	+
Phylobatanins	-
Quinines	+
Quinones	+

+ = present; - = Absent

Table 3: Proximate analysis of *Morinda lucida* root

Sample	**Percentage composition (%)**
Crude fiber	11.46
Ash	11.23
Moisture	9.87
Carbohydrates	44.32
Fat	15.15
Protein	7.68

The *in vitro* susceptibility testing showed antibacterial activity with varied zones of inhibition as presented in Table 4. Methanol extract produced the highest mean inhibition zone diameter (27.00±0.5mm) against *S. aureus,* followed by mean zone diameter of 26.00±0.3mm against other bacterial pathogens, and the least mean inhibition zone diameter of 25.00±0.3mm against *K. pneumoniae* ($p>0.05$). The aqueous extract produced the highest mean inhibition zone diameter of 27.00±0.3mm against *S. aureus*, follo-

wed by 26.00±0.3mm against *K. pneumoniae* and the least (25.00±0.0mm) against *P. mirabilis*, *P. aeruginosa* and *E. coli* (p>0.05). The ethyl acetate extract produced the highest inhibition zone diameter of 23.00±0.2mm against *E. coli* and *S. aureus*, followed by 22.00±0.3mm against *P. mirabilis* and the least (20.00±0.3 mm) against *P. aeruginosa* (p>0.05). The hexane extract produced the highest inhibition zone diameter of 19.00± 0.3mm against *S. aureus*, followed by 21.00±0.4 mm against *S. aureus* and least mean inhibition zone diameter of 18.00±0.1 mm against other bacterial pathogens.

The root extracts of *M. lucida* exhibited bacteriostatic activities at different concentrations ranging from 3.125 to 12.5mg/ml and bactericidal activities ranging from 6.25 mg/ml to 25.00mg/ml as shown in Table 5.

Table 4: Zone of inhibition of *Morinda lucida* roots extracts at 200 mg/ml

Bacteria strain	Methanol	Aqueous	Ethyl acetate	Hexane	Positive control (levofloxacin 0.05mg/ml)	Negative control (DMSO)
Proteus mirabilis	26.00±0.3	25.00±0.2	22.00±0.3	18.00±0.3	30.00±0.0	0.00
Staphylococcus aureus	27.00±0.5	27.00±0.3	23.00±0.1	19.00±0.4	32.00±0.0	0.00
Klebsiella pneumonia	25.00±0.3	26.00±0.3	21.00±0.4	18.00±0.1	28.00±0.0	0.00
Escherichia coli	26.00±0.3	25.00±0.3	23.00±0.3	18.00±0.6	28.00±0.0	0.00
Pseudomonas aeruginosa	26.00±0.3	25.00±0.2	20.00±0.2	18.00±0.3	30.00±0.0	0.00

Table 5: Minimum inhibitory and bactericidal concentrations of *Morinda lucida* roots extracts

Bacteria strain	Methanol		Aqueous		Ethyl acetate		Hexane	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Proteus mirabilis	3.125	6.25	3.125	6.25	3.125	6.25	12.50	25.00
Staphylococcus aureus	3.125	6.25	3.125	6.25	6.25	12.50	12.50	25.00
Klebsiella pneumonia	3.125	6.25	3.125	6.25	6.25	12.50	12.50	25.00
Escherichia coli	3.125	6.25	3.125	6.25	6.25	12.50	12.50	25.00
Pseudomonas aeruginosa	6.25	12.50	6.25	12.50	6.25	12.50	12.50	25.00

MIC = Minimum inhibitory concentration; MBC = Minimum bactericidal concentration

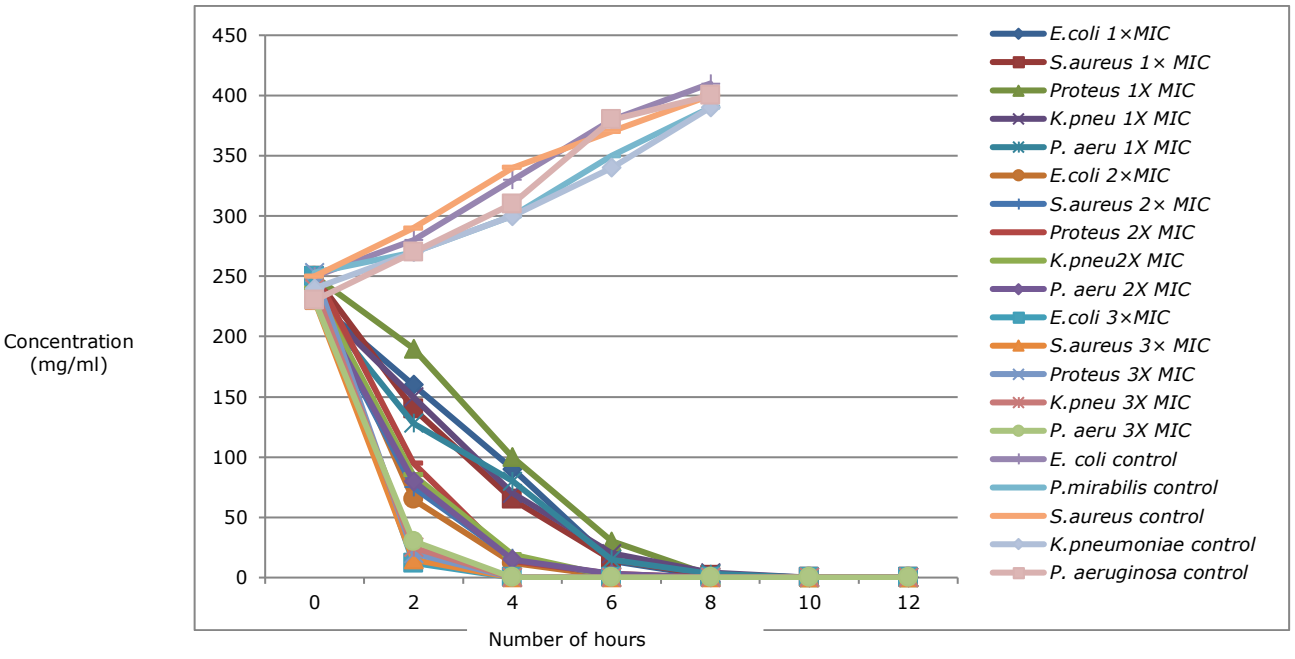

Fig 1: The time kill curve of methanol root extract of *Morinda lucida* at MIC, 2×MIC and 3×MIC

The time-kill assay was expressed as the 3log10 or 99.9% reduction of the number of viable cells from the initial inoculum (Fig 2). The time-kill assay at 1×MIC showed that there was close to 99.9% cell reduction after 6-8 hours of incubation. At 2×MIC, 99.9% cell death occurred after 4 hours of incubation while at 3×MIC, a gross reduction in the number of viable cells was seen and 99.9% cell death occurred before 4 hours of incubation for all the isolates.

Discussion:

In the study, the extract yield showed that aqueous extract gave a highest yield, followed by methanol extract, ethyl acetate and the least was n-hexane. This may explain why indigenous use in ethnomedicine is mainly aqueous or alcohol-based extraction. The phytochemical screening revealed that the root extract of *M. lucida* contains essential secondary metabolites such as flavonoids, alkaloids, phenols, tannins, quines, and anthraquinones. These compounds are responsible for the therapeutic usage of medicinal plants (23). These phytochemicals are important in drug production due to their effectiveness in elimination of infectious diseases (19). Saponins act by facilitating the entry of cytotoxic contents into cells, while tannins induce cell death through protein denaturation (23). Flavonoids inhibits bacterial cell wall and cell membrane formation (24).

Comparing the extract solvent, methanol extract produced the highest diameter of zone of inhibition, which was comparable to the inhibition zones produced by the standard antibiotic (levofloxacin) used, followed by ethyl acetate and aqueous extracts and the least was n-hexane extract. The high zone of inhibition obtained from the standard antibiotic could be attributed to the use of more refined and pure compounds in its preparation judging by the inhibition zone diameters. This finding aligns with those of similar studies (2, 26), where plant extracts inhibited bacterial pathogens, which gives scientific credibility to the native use of the plant in folklore medicine and could serve as promising novel antibacterial agents. The highest diameters of inhibition zones were observed in both aqueous and methanol extracts which could also explain why traditional medicine practitioners soak the roots of *M. lucida* in water or alcohol (locally called "kaikai" or "akpuru achia") for the treatment of wounds and other infections.

The root extracts of *M. lucida* exhibited bacteriostatic action at different concentrations ranging from 3.125 to 12.5 mg/ml and bactericidal action at 6.25 to 25 mg/ml. This gives scientific credibility of its indigenous use in the management of infections because it produced the desired effect at low concentrations. Fakoya et al., (2) in 2014 reported *in vitro* inhibitory actions of methanol and aqueous extracts of leaf, root and stem bark of *M. lucida* against *Salmonella* Paratyphi*, E. coli* and *Salmonella* Typhi.

The bacteriostatic and bactericidal actions of *M. lucida* roots can be attributed to the potentially active secondary metabolites present in generous amounts in the roots. They include glycosides, alkaloids, tannins, saponins, steroids phenols, flavonoids, hydrogen cyanide and terpenoids. Alkaloids have antibacterial, antiviral, and anti-inflammatory properties, and help in the production of the end product of metabolism. Tannin has been reported to hasten the rate of wound healing and possess antibacterial and antioxidant properties. The presence of these phytochemicals in *M. lucida* gives scientific credibility to its indigenous use in ethnomedicine.

The time-kill assay shows that methanol root extract of *M. lucida* is both concentration and time-dependent in the elimination of bacterial pathogens isolated from diabetic foot wound ulcers in this study. The reduction in the number of viable colony count within 2 and 6 hours of incubation shows that it has high bactericidal property, and this may be attributed to the antimicrobial properties of flavonoids, working in synergy with other phytochemicals present, to give the desired effect. The report of antimicrobial activity of *M. lucida* root in ethnomedicine is of great importance because it may serve as credible alternative to conventional medicine in the management of various infectious diseases that are difficult to treat. This can enable its use in the synthesis of novel, less toxic, cost-effective, and efficacious antibiotics.

Conclusion:

In our study, the root extracts of *M. lucida* demonstrated excellent antibacterial activities against MDR Gram-positive and Gram-negative bacterial pathogens isolated from wound ulcers of diabetic patients. The presence of secondary metabolites in the root extracts are responsible for the antibacterial potency, and *M. lucida* may therefore serve as a robust source of antimicrobials that are effective in treatment of infections caused by drug-resistant microbial pathogens.

Acknowledgments:

The authors wish to acknowledge the staff of the Department of Medical Laboratory Science, University of Nigeria for the use of of their laboratory facilities. The assistance of the staff and management of Central Science Laboratory, University of Nigeria (UNN) Nsukka Nigeria in the molecular identification of our bacterial isolates and detection of resistant

genes, is acknowledged. The patients who gave informed consent for sample collection are well appreciated.

Contribution of authors:

ANO designed the study, performed statistical analysis of data, wrote the initial manuscript draft and the final manuscript; UEU wrote the protocol and contributed to literature search; ANO, EIB and UEU performed the laboratory works; ANO and UEU contributed to the discussions; EIB supervised the study and proof read the final manuscript for publication. All authors approved the final manuscript submitted for publication.

Source of funds:

The study was funded by the Research Grant of the University of Nigeria Enugu.

Conflict of interest:

Authors declare no conflict of interest

Declaration of authors:

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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