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Fractional Inhibitory Concentration Index of Poly-Herbal *Combination of* Morinda *lucida* and *Pterocarpus santalinoides Methanol* Root Extracts Against Multiple Drug Resistant Bacterial Isolated From Diabetic Foot Ulcers.

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Abstract

The antimicrobial resistant bacteria make treatment of microbial infections with common antibiotics difficult and expensive. This has led to a serious scientific search for combination therapy antimicrobial agents that will be safe, effective, less toxic, and affordable. The aim of this study was to evaluate the in-vitro action of combining the methanol root extracts of *Morinda lucida and Pterocarpus santalinoides* against multiple drug-resistant organisms isolated from diabetic foot ulcers. The phytochemical constituents were determined using standard methods. The antimicrobial susceptibility profile and antibacterial activity of the extracts was tested using agar well diffusion test. The minimum inhibitory concentration and fractional inhibitory concentration were done using Checker-board micro-titration method, and the fractional inhibitory concentration index (FICI) calculated, and time-kill assay was done to confirm the synergistic and bactericidal activity. The antimicrobial testing of the combination showed high zones of inhibition diameter ranging between 29-40mm against the isolates. The FICI of the methanol roots extract combination demonstrated synergy against the test isolates ranging between 0.12 -0.19. The time of kill showed a huge decrease in the quantity of viable cells. This study suggests that this plant combination could serve as an alternative antimicrobial agent to treat multiple drug-resistant organisms.

Keywords: Antibacterial, Synergy, Checkerboard, Multiple Drug Resistant, Time kill Assay, Crude extract

Introduction

It is estimated that in the next 30 years, resistance of bacteria to antibiotics will be more fatal than cancer and may cause the death of over 10 million of the population each year by 2050 (Murray,2022). Antibiotics are regarded as revolutionized medicines' used in controlling microorganisms and their therapeutic exploit is regarded a miracle in medical olden times (Atta, *et al.,* 2023). With continuous rise in the cases of drug resistance, treatment, prevention and control of these microbes becomes extremely difficult. Regardless of their numerous advantages, the uncontrolled use of antibiotics, the chief drivers of drug-resistant development remains abuse of antibiotics (Hutchings, *et al.,* 2019). The achievement of contemporary medicine cancer chemotherapy, surgeries and in the treating of infections and diseases would be compromised (WHO, 2023).

Herbal plants are known to be rich in phytochemicals and can be produced as antimicrobials that are effective and safe. Antimicrobial combination is the use of two, three, or more antimicrobial agents simultaneously. It helps in the treatment of resistant organisms, reducing the ability of an organism to develop resistance and use of low doses of the drug for treatment giving the desired effect than single drugs (Guan, *et al.,* 2016; Zhou, *et al.,* 2020). Several studies have reported that combinational therapy is better than monotherapy (Bassetti, *et al.,* 2016; Trecarichi, and Tumbarello, 2017; Wang, *et al.,* 2019). The use of multiple plant part combinations is widespread among traditional healers. Mixture of crude plant extracts may help

overcome drug resistance and serve as an alternative antibiotic (Ayaz, *et al.,* 2019) It is believed that secondary metabolites in crude extract help to synergize its bacteriostatic or bactericidal actions when combined and there is a tendency for bacteria resistance to a single plant (Vadhana, *et al.,* 2015). Synergism is, therefore, a better option in the treatment of multiple drug-resistant organisms.

Morinda lucida and *P. santalinoides* are medicinal plants commonly used in ethnomedicine in the management of different infections and diseases like diabetes, dysentery, all kinds of fever, skin diseases, diarrhoea, and menstrual disorders. The whole parts of both plants contain essential phytochemicals that account for their antimicrobial, anti-oxidant, anti-inflammation properties and various vitamins and minerals.

Diabetic foot ulcers occur as a result of uncontrolled diabetes mellitus. The foot ulcers are prone to contamination if proper hygiene is not maintained. Most times the contamination is polymicrobial and this will make it very difficult to heal, predisposing the infecting microorganisms to mutation leading to drug resistance and if not properly managed will lead to amputation (Atlaw *et al.,* 2022). It can be infected by fungi, bacteria both positive and negative. The most implicated organism includes *Proteus mirabilis, Klebsiella pneumonia Escherichia coli, Staphylococcus species, Pseudomonas aeruginosa, Streptococcus species amongst others*

The aim of this study was to evaluate the antimicrobial action of combining crude extracts on the multiple drug resistant pathogens from diabetic foot ulcers. The plants were chosen on the basis of their antimicrobial, anti-inflammatory, and antioxidant activities reported.

Materials and methods

The roots of *Morinda lucida* and *Pterocarpus santalinoides* were harvested from a family garden at the University of Nigeria, Enugu Campus senior staff quarters. The plants were identified, authenticated, and given a voucher specimen number by an expert Botanist Mr. Felix Nwafor in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, and a voucher specimen was deposited at the herbarium. They were properly washed with several changes of clean water until clean and were cut into small pieces for easy drying and grinding. They were dried under shade until completely dried then milled into fine powder and stored in a sterile clean containers till ready to use.

Plate 1: Pterocarpus santalinoides roots

Plate 2 : Morinda lucida roots

Preparation of plant for extraction

The crushed roots of *Morinda lucida* and *Pterocarpus santalinoides* were extracted by adding one thousand grams of powder in 5000ml of 95% methanol and were extracted using cold maceration for 48 hours. Evaporation of the solvent was done using a rotary vacuum evaporator (R-200, Büchi Rotavap Germany). The plant extract was tested for sterility by plating out on Mueller Hinton agar and

incubating it for 24hours before storing in the refrigerator at 4°C

Determination of percentage yield
The determination of the percentage (%) yield from the extracts was done using the formula as previously described (Achukwu, *et al.,* 2022)

Phytochemical Screening
The phytochemical constituents of the roots were assessed using standard methods (*Okafo, et al.,2024*).

Isolation of Test Organisms
The tested microorganisms isolated from diabetic foot ulcers were Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis and Staphylococcus aureus. The multiple drug resistant profile and presence of genes were determined. Standardized cell suspensions of 5×10^5 were prepared from the test isolates before use.

Preparation of extract concentrations
Crude root extract of *Morinda lucida* and *Pterocarpus santalinoides* stock solutions were prepared by dissolving 2g of each root extract in 5ml Dimethyl Sulfoxide (DMSO) to give a working stock solution of 400mg/mL respectively.

Antimicrobial properties of all extract
The antimicrobial activities of the poly-herbal crude extracts' combination crude were determined using agar-well diffusion as was described by (Nwosu, *et al.,* 2022) the cell suspension was evenly spread on the agar surfaces. A sterile cork borer was used to make wells on the agar surface. The extract at combination of 1:1 at 400 mg/mL concentration was added to the wells and allows for 15 minutes to diffuse into the agar. Before incubating at 37°C. Levofloxacine (0.005mg/mL) and Dimethyl Sulphur oxide served as positive and negative controls. The test was done in duplicates and the zones of inhibition diameter recorded to the nearest millimeter.

Minimum Inhibitory Concentration and synergistic In-vitro interaction of combined antimicrobial activities of the methanol extract of *Pterocarpus santalinoides* and *Morinda lucida*
Checkerboard was used to appraise the synergistic and the minimum inhibitory concentration of the antimicrobial combinations of crude methanol extracts of both plant parts against the test isolates using the agar diffusion method by calculating the fractional inhibitory concentration as described by (CLSI, 2023; Samara, *et al.,* 2023). This was dome in a 96 micro-titration well. All the wells were filled with 1.5mL of Muller Hilton broth, and 100µL of *M. lucida* extract added to the first row on the X-axis and serially diluted and 100µL *of Pterocarpus santalinoides* extract added to first row on the Y-axis and it was serially diluted and100µL of standardized bacterial inoculum that were properly vortexed and added into the 96 wells. The plate was incubated for 24 hours at 37°C (Bello, *et al.,* 2021). The presence of bacteria was determined by adding 20µL of 2, 3, 5-triphenyltetrazolium chloride (TTC) into the wells to establish the minimum inhibitory concentration, then incubated for extra one hour. The least concentration that did not produce red colour indicating no bacterial growth is regarded as the MIC (El Kamari *et al.,* 2023).

The fractional inhibitory concentration index (FICI) is used to interoperate antimicrobial combination as ≤ 0.5= synergism; $0.51 < FICI \leq 4.0$ = indifferent FICI > 4.0 = antagonistic and was calculated using the equation described by (20)

FICA= (MIC of *Pterocarpus santalinoides* and *Morinda lucida* in combination)
(MIC *Pterocarpus santalinoides* alone)
FICB= (MIC of *Pterocarpus santalinoides* and *Morinda lucida* in combination)
(MIC *Morinda lucida* alone)
FICI = FICA + FICB

Time-kill assay for bactericidal and synergistic testing
The bacteriostatic concentration obtained was used to verify the time of bactericidal action. This was done using the methods of (Bremmer, *et al.,* 2016; Achukwu, *et al.,* 2023). This was assessed at 2 xMIC and 3xMIC. Eighty micro liters of the

extract was added in the test tube containing 1000 µL of Muller Hilton broth and 20 µL of each standardized bacteria inoculum. A 100 µL of the mixture was removed every 2 hours for 12hours and a serial dilution of it made and10 µL of it was then plated out. The plate count was done after overnight incubation and the graph of logarithm of number of colonies in cfu/mL and time of incubation was plotted. Bactericidal activity is said to occur when the activity is greater than ≥ 2 log10 CFU/ mL fold reduction in the initial inoculums (Ju, *et al.,* 2022)

Ethical permission

Ethical permission to carry out this work was obtained from the Ethics Committee College of Medicine University of Nigeria.

Statistical Analysis

The results were expressed as the mean of three replicates. The significance level was set at $p \leq 0.05$. Data obtained were statistically analyzed using one-way Analysis of Variance ANOVA.

Results and Discussion

Table 1: Phytochemical Constituents of *Morinda lucida* and *Pterocarpus santalinoides*

Plant	Saponins	Tannins	Alkaloids	Flavonoids	Phenols	Steroids	Terpenoids	Glycosides
Pterocarpus santalinoide	+	+	+	+	+	+	+	+
Morinda lucida	+	+	+	+	+	+	+	+

Table 2: **Mean Inhibition Zone Diameter (mm) and Minimum Inhibitory Concentration of *Pterocarpus santalinoides* and *Morinda lucida Root Extracts* respectively**

Bacterial Isolates	***Morinda lucida*** Zone Of Inhibition Diameter (mm)					**MIC mg/mL**	***Pterocarpus santalinoides*** Zone Of Inhibition Diameter (mm)					**MIC mg/mL**
	400	200	100	50	25		400	200	100	50	25	
S. aureus	27.00±0.2	26.00±0.7	23.00±0.5	21.00±0.3	20.00±0.0	6.25	24.00±0.1	23.00±0.9	21.00±0.6	20.00±0.8	18.00±0.6	12.5
K. pneumoniae	25.00±0.9	23.00±0.2	21.00±0.6	20.00±0.9	18.00±0.1	12.5	23.00±0.6	21.00±0.5	20.00±0.4	19.00±0.6	17.00±0.5	12.5
P. aeruginosa	23.00±0.2	21.00±0.8	20.00±0.6	18.00±0.9	17.00±0.5	12.5	22.00±0.2	20.00±0.7	19.00±0.9	17.00±0.3	15.00±0.2	12.5
E. coli	25.00±0.4	24.00±0.8	22.00±0.2	20.00±0.7	20.00±0.5	6.25	24.00±0.2	23.00±0.8	21.00±0.1	20.00±0.0	18.00±0.3	12.5
P. mirabilis	25.00±0.0	24.00±0.6	22.00±0.4	20.00±0.8	19.00±0.6	6.25	24.00±0.7	23.00±0.2	21.00±0.7	20.00±0.1	18.00±0.0	12.5

Table 3: Mean Inhibition Zone Diameter (mm) of Polyherbal Combinations of *Morindalucida* and *Pterocarpus santalinoides Root Extracts* (mm)

Concentration of Plant extract combinations at 1:1 ratio (mg/mL)	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Proteus mirabilis*
400	39.00±0.4	39.00±0.6	38.00±0.0	39.00±0.3	40.00±0.5
200	35.00±0.5	33.00±0.2	34.00±0.2	36. 00±1.2	38. 00±2.0
100	33.00±0.9	30.00±0.0	30.00±0.1	33. 00±0.8	35. 00±1.3
50	29.00±0.2	27.00±0.8	28.00±0.8	31. 00±0.7	31.00±0.0
25	25.00±0.3	24.00±0.5	24. 00±0.5	26. 00±0.6	28. 00±0.0
Levofloxacin 0.005mg/ml	35.00±0.0	37.00±0.0	37.00±0.0	37.00±0.0	37.00±0.0

Table 4: Fractional Inhibitory Concentration Index of the Polyherbal Combination of *Morinda lucida* and *Pterocarpus santalinoides* Roots Extracts against the Multiple Drug Resistant Bacterial Isolates Tested

Bacterial Isolates	MIC of Combinations mg/Ml	FIC_A	FIC_B	FICI	Interpretation
P. mirab ilis	0.79	0.13	0.06	0.19	Synergy
S. aureus	0.39	0.06	0.06	0.12	Synergy
K. pneumonia	0.78	0.06	0.06	0.12	Synergy
Escherichia coli	0.39	0.06	0.03	0.09	Synergy
P. aeruginosa	0.78	0.13	0.06	0.19	Synergy

A- Pterocarpus santalinoides, B- Morinda lucida

The FICI of the poly-herbal combination of Pterocarpus santalinoides and Morinda lucida all showed synergistic activities against the MDR isolates from diabetic foot ulcers.

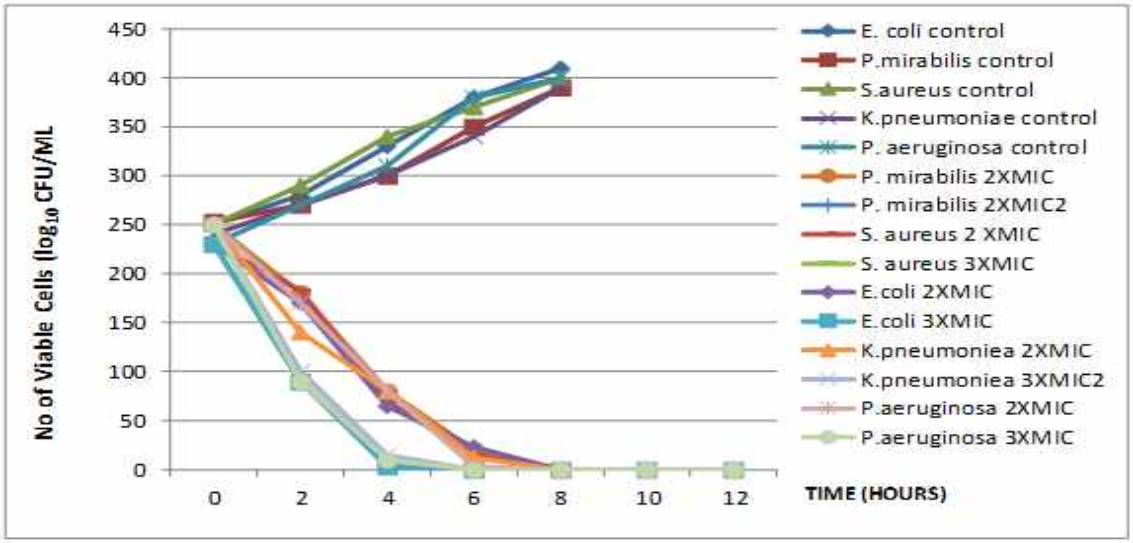

Figure 1: *Morinda lucida* and *Pterocarpus santalinoides* Methanol root Extracts Poly-herbal Combinations at 2 X MIC and 3X MIC.

Results and Discussion

The increase in cases of multiple drug resistance is very crucial because it accounts for failure in treatment regimens and overstay in hospitals. The isolates used in the study were multidrug resistant bacterial isolates from wound ulcers. The status of any antimicrobial agent in a single or combination must be established against a bacteria species before it can be used. The primary metabolites are vital for development and plant growth, while phytochemicals play a role in physiology, environmental communication and plant defense (Basavegowda, and Baek, 2022).

The root extract of M. lucida yielded 16.83g while P. santalinoides gave a yield of 17.79g. P. santalinoides gave a higher yield than M. lucida. The phytochemical components of both plants showed that they both contain essential phytochemicals like tannin, alkaloids, phenols, Terpenoids, saponin, flavonoids, and glycosides. As shown in table 1 which are believed to be responsible for their antimicrobial activities (Ezeagha, *et al.,* 2021)

This study evaluated the methanol root extracts poly-herbal combination of M. lucida and P. santalinoides combination and was tested against multiple drug resistance bacterial isolates from diabetic foot ulcers and was found to be highly sensitive to the isolates. The mean inhibition zone diameter of the single extracts as shown in table 2 indicated that respective plant root extracts was effective against the test bacteria isolates with IZD ranging between 27mm diameter and MIC between 6. 25 12.5mg/mL for M. lucida and 15.00- 24mm IZD with MIC 12.5 mg/mL for P. santalinodes.

The mean inhibition zone diameter of the poly-herbal combinations of M. lucida and P. santalinoides at the concentration range of 25 - 400mg/ml at a ratio of 1:1 as seen in table 3, reveals that it has a high mean inhibition zone diameter. The sensitivity of the extract is concentration-dependent. The mean IZD at 25mg/mL is still greater than the CLSI recommended diameter. There is no significant difference $p>0.05$ between the activity of levofloxacin and the crude extract combination at 200mg/mL and above. The poly-herbal combination had antimicrobial activity against the multiple drug-resistant isolates from diabetic foot ulcers.

The antimicrobial activity of the plant's combination might be credited to the phytochemical constituents present in the plant's roots. They are found to contain tannin, alkaloids, phenol and terpenoids. These phytochemicals have been proven to cause apoptosis, bacteria transduction signal interference alteration of protein-protein interaction in the bacteria cell wall, and interfering with the host immunological response (Ayaz, *et al* 2019; Gupta, and Birdi, 2022). Therefore, the antimicrobial activity of the poly-herbal extract can be attributed to the number of phytochemicals present. Record has shown that presence of phytochemicals like alkaloids, tannins, phenols among others help in overcoming resistance. (Elsohly *et al.,* 2017).

Antimicrobials from plant extract are the alternative to explore as the cases of multiple drug resistant isolates are on the rise. The activities of plant chemicals could be indirect or direct antibacterial resistant overcoming agent (Alam *et al.,* 2022). A single plant part may be able to inhibit or kill a bacteria pathogen but when plant that work in synergism is combined it will kill as a broad spectrum (Obaji *et al.,*2019). However not all combinations can produce synergy, so all plant combinations must be tested for synergy before use. The methanol root extract of M. lucida and P. santalinoides showed a high IZD ranging between 29 and 40mm showing high sensitivity of the combined antimicrobial to the multiple drug resistance bacterial isolates from diabetic foot ulcers. The MICs of the individual extracts showed that they are both sensitive to the multidrug resistant isolates used. The individual MIC is 6.25 and 12.5 mg/ml for Morinda lucida and Pterocarpus santalinoides respectively against the entire test isolates, this is in agreement with the report of Donkor *et al.* (2023) who studied Pterocarpus santalinoides activity against multiple drug resistant

diarrheagenic bacterial infection. The mean IZD of the combined root extract showed that they were very sensitive to the multiple drug-resistant isolates from diabetic foot ulcers. The MICs of the combined extract ranged from 0.39 to 0.79mg/mL. The mean IZD of the plant extract combination and their MIC were significantly high compared to their antimicrobial activity. This is an indication that it could work better in combination than when used alone (Tang *et al.,*2018).

Reports on the antimicrobial activities of herbal plant clinical isolates revealed that crude plant extracts are important source of resistance-modifying compounds (Atta *et al.,*2023) and crude extracts are believed to be a very good reservoir of antibacterial adjuvants for multiple drug resistance organisms (Ayaz *et al.,*2019). This study revealed that combination of Morinda lucida and Pterocarpus santalinoides methanol root extract could act synergistically in vitro against multidrug-resistant bacteria. The high rate of multiple drug-resistant organisms' emergence in the past decades has become alarming in different parts of the world and this has stirred the need to search for antimicrobials with synergistic combinations (Musa *et al.,*2021). A checkerboard technique was used to calculate the fractional inhibitory concentration index to determine the synergistic status of the combination. It is important that before any combination is made the FICI must be established before use. The fractional inhibitory concentration index of the plant extract combination showed that they produced synergistic activities against the multiple drug resistance isolates tested. The FiCI synergism ranged between 0.09 – 0.19 as shown in table 4 It has a more pronounced synergism against E. coli and K. pnuemoniae followed by P. mirabilis, S. aureus and P. aeruginosa. An antimicrobial combination that exhibits synergism is indicative that they both have a different mechanism of action (Tang, *et al.,*2018). The main advantage of an antimicrobial combination that is synergistic is that it is effective at a lower concentration (Musa, *et al.,*2021), help reduce the emergence of the resistant strain (Uddin, *et al.,*2022) help to overcome resistant organisms (Zhangyong, *et al.,*2023) and reduces the quantity of antimicrobial that is pumped into the body and eliminating microorganism using multiple ways (Xuan, *et al.,*2023). When antimicrobials, either plant extract or conventional antibiotics with different modes of action are combined, each drug can be used at its appropriate dose, without undesirable side effects. Synergistic interaction helps cell mediators to improve antimicrobial activity, improve the biochemical pathways, and adjust microbial enzymes (Haroun and Al-Kayali, 2016). Synergy might be linked to the presence of phenols in the plant extracts and has been documented to help in the reduction of antimicrobial MIC of antibiotics (Álvarez-Martínez, *et al.,*2020). There is paucity of information on the synergistic studies of plant poly-herbal combinations hence making it highly difficult to compare results.

The time-kill assay of the antimicrobial combination (fig 1) showed the bactericidal activities of the plant combination at 2×MIC and 3×MIC. At the 2×MIC it showed a $\geq$ 3log10 reduction in the amount of viable cells within 6 hours of incubation while at 3×MIC the number was able to reduce the number of viable cells before 4 hours of incubation against all the test isolates and there was no re-growth observed signifying a powerful bactericidal synergy between them. In the control we noticed a steady increase in the number of viable cells. Time kill is used to confirm bactericidal activity and is achieved at $3Log_{10}$ decrease from initial inoculum (Panthong, *et al.,*2020). This indicate that the combination is a good broad-spectrum antimicrobial.

Conclusion

Antimicrobial synergy was observed in the poly-herbal combination against multiple drug-resistant pathogenic bacteria isolates from diabetic foot ulcers. The poly-herbal extract combinations resulted in sufficient MIC reductions, suggesting that the poly-herbal combinations may be useful in the treatment of infectious diseases with multidrug-resistant as an alternative and/or complementary to antibiotic therapy.

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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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