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Synergistic *invitro* antimicrobial activity of polyherbal combination of *Morinda lucida* fruit and *Pterocarpus santalinoides* seed against multi-drug resistant clinical bacterial isolates

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Abstract:

Background: Synergistic drug combination has been shown to be a way of bypassing drug resistance and reducing the amounts of antimicrobials consumed. As infections caused by multi-drug resistant organisms (MDROs) continue to pose global threat, it is important to search for new antimicrobial combinations of plant origin that are safe and readily available. The objective of this study is to evaluate the synergistic antimicrobial activity of extracts of *Morinda lucida* fruit and *Pterocarpus santalinoides* seed against multi-drug resistant (MDR) bacterial isolates
Methodology: *Morinda lucida* and *P. santalinoides* fruits were plucked and washed clean, and the fruits of *P. santalinoides* were deseeded to remove the seeds. They were cut into smaller pieces and dried under shade before being milled into smooth powder and extracted with methanol. The clinical bacterial isolates used were MDR *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* The phytochemical constituents of the extracts were determined using the standard method. The *invitro* antimicrobial assay of the extracts was done at a concentration of 50 to 400 mg/ml using the agar well diffusion technique. The minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of each extract were determined using the Checker-board micro-titration method, and the FIC result obtained for each isolate was used to calculate the fractional inhibitory concentration index (FICI) of the combined extracts. The time kill assay was performed to confirm the synergistic and bactericidal activities from the MIC values obtained.
Results: The phytochemical analysis revealed that the extracts of both herbal plants contained alkaloids, phenol, tannin, saponin, glycosides, and terpenoids. The antimicrobial assay results showed that the polyherbal combination of the extracts was active against all the MDR bacterial isolates tested with varying zones of inhibition. The mean inhibition zone diameters of individual *M. lucida* and *P. santalinoides* ranged from 18.0±0.9mm to 26.0±1.4 mm and 17.0±0.1mm to 24.0±0.2mm respectively, while the MIC ranged from 6.25mg/ml to 12.5mg/ml for *M. lucida* and 12.5 mg/ml for *P. santalinoides*. The mean inhibition zone diameter of the polyherbal combination of the two plant extracts ranged from 25.00±00mm to 34.0±0.4mm, which compared favorably with that of levofloxacin control (28.0±0.0mm to 32.00±0.0mm), while their MIC ranged from 0.39mg/ml to 1.56mg/ml. The FIC of *M. lucida* extract ranged from 0.06mg/ml to 0.25mg/ml while that of *P. santalinoides* ranged from 0.03mg/ml to 0.13mg/ml. The FICI of combined extracts ranged from 0.09mg/ml to 0.38 mg/ml for all the MDR isolates, which is less than 0.5mg/ml, indicating synergism against all the isolates. The time of kill assay confirmed the synergistic and bactericidal activities with a 3log10 CFU/ml decrease in the number of viable cells within 6 hours of incubation.
Conclusion: Our findings showed synergistic antibacterial actions of extracts of *M. lucida* fruit and *P. santalinoides* seed against MDR clinical bacterial isolates, comparable to levofloxacin. Combination of these herbal plants may serve as alternative sources of antimicrobial agents for the treatment of infections caused by MDR bacterial pathogens.

Keywords: Multi-drug resistance, Fractional Inhibitory Concentration, Synergism, Checker-board, Time kill assay

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Activité antimicrobienne synergique in vitro d'une combinaison polyherbale de fruits de *Morinda lucida* et de graines de *Pterocarpus santalinoides* contre des isolats bactériens cliniques multirésistants aux médicaments

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Résumé:

Contexte: Il a été démontré que la combinaison synergique de médicaments est un moyen de contourner la résistance aux médicaments et de réduire les quantités d'antimicrobiens consommées. Alors que les infections causées par des organismes multirésistants aux médicaments (MDRO) continuent de constituer une menace mondiale, il est important de rechercher de nouvelles combinaisons d'antimicrobiens d'origine végétale qui soient sûres et facilement disponibles. L'objectif de cette étude est d'évaluer l'activité antimicrobienne synergique d'extraits de fruits de *Morinda lucida* et de graines de *Pterocarpus santalinoides* contre des isolats bactériens multirésistants aux médicaments (MDR).
Méthodologie: Les fruits de *Morinda lucida* et de *P. santalinoides* ont été cueillis et lavés, et les fruits de *P. santalinoides* ont été épépinés pour en retirer les graines. Ils ont été coupés en morceaux plus petits et séchés à l'ombre avant d'être broyés en poudre lisse et extraits au méthanol. Français Les isolats bactériens cliniques utilisés étaient MDR *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* et *Staphylococcus aureus*. Les constituants phytochimiques des extraits ont été déterminés à l'aide de la méthode standard. Le test antimicrobien in vitro des extraits a été effectué à une concentration de 50 à 400 mg/ml en utilisant la technique de diffusion en puits d'agar. La concentration minimale inhibitrice (CMI) et la concentration inhibitrice fractionnelle (CIF) de chaque extrait ont été déterminées à l'aide de la méthode de microtitration en damier, et le résultat de la CIF obtenu pour chaque isolat a été utilisé pour calculer l'indice de concentration inhibitrice fractionnelle (FICI) des extraits combinés. Le test de temps de destruction a été effectué pour confirmer les activités synergétiques et bactéricides à partir des valeurs de CMI obtenues.
Résultats: L'analyse phytochimique a révélé que les extraits des deux plantes médicinales contenaient des alcaloïdes, du phénol, du tanin, de la saponine, des glycosides et des terpénoïdes. Les résultats de l'essai antimicrobien ont montré que la combinaison polyherbale des extraits était active contre tous les isolats bactériens MDR testés avec différentes zones d'inhibition. Les diamètres moyens des zones d'inhibition de M. lucida et P. santalinoides individuels variaient respectivement de 18,0 ± 0,9mm à 26,0 ± 1,4mm et de 17,0 ± 0,1mm à 24,0 ± 0,2mm, tandis que la CMI variait de 6,25mg/ml à 12,5mg/ml pour *M. lucida* et de 12,5mg/ml pour *P. santalinoides*. Français Le diamètre moyen de la zone d'inhibition de la combinaison polyherbale des deux extraits de plantes variait de 25,00 ± 00mm à 34,0 ± 0,4mm, ce qui se compare favorablement à celui du témoin à la lévofloxacine (28,0 ± 0,0mm à 32,00 ± 0,0mm), tandis que leur CMI variait de 0,39mg/ml à 1,56mg/ml. Le FIC de l'extrait de *M. lucida* variait de 0,06mg/ml à 0,25mg/ml tandis que celui de *P. santalinoides* variait de 0,03mg/ml à 0,13mg/ml. Le FICI des extraits combinés variait de 0,09mg/ml à 0,38mg/ml pour tous les isolats MDR, ce qui est inférieur à 0,5mg/ml, indiquant une synergie contre tous les isolats. Le test de temps de destruction a confirmé les activités synergétiques et bactéricides avec une diminution de 3log10 UFC/ml du nombre de cellules viables dans les 6 heures suivant l'incubation.
Conclusion: Nos résultats ont montré des actions antibactériennes synergétiques d'extraits de fruits de *M. lucida* et de graines de *P. santalinoides* contre les isolats bactériens cliniques MDR, comparables à la lévofloxacine. La combinaison de ces plantes médicinales peut servir de sources alternatives d'agents antimicrobiens pour le traitement des infections causées par des agents pathogènes bactériens MDR.

Mots clés: Multirésistance aux médicaments, concentration inhibitrice fractionnelle, synergie, damier, test de temps de destruction

Introduction:

Infection caused by multi-drug-resistant organisms (MDROs) is a global concern, posing a health challenge to the general populace (1). This phenomenon has led to treatment failures from loss of efficacy of most orthodox drugs against the pathogens that were once susceptible to them (2). Antimicrobial resistance (AMR) occurs across all microorganisms including bacteria, fungi, viruses, protozoan and helminths, and infection caused by MDROs is associated

with high mortality and morbidity from failure of treatment, prolonged hospital stays and increased economic burden of the people infected (3).

Medicinal plants have gained recognition and patronage over the past two decades because of their proven efficacy as alternative to orthodox drugs which are expensive and not readily available, and to which microorganisms are becoming increasingly resistant (4). Herbal plants contain high amounts of secondary metabolites and many are non-toxic, readily available, and pocket-friendly with added nutritional values (5). Various parts such as leaf, stem-bark, roots, and fruits/seeds of herbal plants like *Morinda lucida* and *Pterocarpus santalinoides* have been employed in the management and treatment of different infections and diseases in folklore medicine (6).

The different parts of many plants have been evaluated for their efficacy as therapeutic agents. Different parts of *M. lucida* and *P. santalinoides* have been reported to possess inhibitory activity against multi-drug resistant (MDR) bacteria such as *Escherichia coli, Proteus* spp*, Salmonella* spp (8), *Staphylococcus* spp, *Klebsiella* spp (7), *Candida albicans* (8) and parasites (9).

Synergistic drug combination is one strategy for overcoming drug resistance and preventing or delaying emergence of drug resistance (10). However, there is paucity of information on the synergistic activity of combination of *M. lucida* and *P. santalinoides* against MDROs. Thus, the objective of this study is to evaluate the *invitro* antimicrobial activity of the combination of *M. lucida* fruit and *P. santalinoides* seed extracts against MDR clinical bacterial isolates.

Materials and method:

Study design and period:

This was an antimicrobial assay to determine the antibacterial activity of methanol extract of *M. lucida* fruit and *P. santalinoides* seed against selected MDR Gram-positive and Gram-negative bacteria isolated from the foot ulcers of diabetic patients. The clinical isolates were obtained from the Department of Microbiology, University of Nigeria Teaching hospital Ituku-Ozalla, Enugu State, Nigeria. The study was conducted between June 2023 and January 2024.

Ethical approval:

Ethical approval to conduct the study was obtained from the Ethics Committee of the College of Medicine, University of Nigeria Enugu Campus.

Plant collection and preparation:

Morinda lucida (Fig 1) and *P. santalinoides* (Fig 2) fruits were harvested, identified, authenticated, and given voucher specimen numbers by a botanist at the Department of Plant Science and Biotechnology, University of Nigeria Nsukka, and voucher specimens were deposited in the herbarium.

The fruits of *M. lucida* were thoroughly rinsed with multiple changes of clean water until completely clean. The fruits of *P. santalinoides* were deseeded and the seeds split opened to facilitate drying and grinding. They were then shade-dried before being pulverized into a fine powder, and the powder was stored in a sterile container until ready for use.

Fig 1: *Morinda lucida* fruit

Fig 2: *Pterocarpus santalinoides* seed

Extraction of plant and determination of extractive index values:

Cold maceration was used to extract compounds from *M. lucida* fruits and *P. santalinoides* seed. About 500g each of the pulverized fruits and seeds were soaked in 3 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 1000ml of 10% methanol and successfully partitioned into fractions using 4× 1000ml of n-hexane and 6×1000ml of ethyl acetate.

The purity of the extracts was assessed by plating the extracts on Mueller Hinton (MH) agar and incubated overnight. The formula used for calculating the extractive yield (%, w/w) is Weight of extract × 100/Weight of dried plant material.

Phytochemical screening of the plant extracts:

The phytochemical screening of each plant each was done for alkaloids tannins, steroids, flavonoids, saponins, quines, and terpenoids using the method described by the Association of Official Agricultural Chemists (11).

Determination of the antimicrobial activities of the plant extracts:

The MDR clinical bacterial pathogens used as test organisms were *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The antibacterial activities of *M. lucida* fruits and *P. santalinoides* seed extracts individually and in combination, were determined using the agar-well diffusion technique previously described by Achukwu et al., (12). The polyherbal was mixed in a 1:1 ratio at 400mg/ml concentration. A bacterial cell suspension of 5×10^5 was prepared and the turbidity standardized to 0.5 McFarland standard.

A sterile glass rod was inserted into the standardized cell suspension, allowing excess fluid to drain. The inoculum was evenly streaked over the entire surface of the MH agar plate and allowed to dry for 10 minutes. Wells were made with a 6mm sterile cork borer in the agar and the wells were filled with 0.5ml of each extract. Levofloxacin (0.05mg/ml) and MH broth without extract were filled into separate wells to serve as positive and negative controls respectively. The inoculated plates were incubated at 37ºC for 24 hrs. The antibacterial assay was performed in triplicates for all the bacterial isolates. The mean diameters of zone of inhibition of the extracts and controls, against each bacterial isolate were calculated and recorded.

Determination of synergistic antimicrobial activities of the plant extracts:

The checkerboard assay method was used to determine the minimum inhibitory concentration (MIC) of the extracts on the bacterial isolates and the fractional inhibitory concentration (FIC), which were then used to calculate the fractional inhibitory concentration index (FICI) to determine if there was synergistic effect of the combined extracts on the test bacterial isolates using the methods of Bellio et al., (13) and Samara et al., (14).

Briefly, a 96µL well was filled with 1.5 ml of MH broth, 100µl of *P. santalinoides* seed extract was added to the first row on the X-axis and serially diluted, and 100µl *of M. lucida* fruits extract was added to the first row on the Y-axis and serially diluted. 100µl of standardized bacteria inoculum was properly vortexed and added into the 96 wells. The microtitre plate was incubated for 24hours at 37^OC. The MIC was determined after checking for the presence of bacteria by adding 20µL of 2,3,5-triphenyl tetrazolium chloride (TTC) into the wells and then re-incubating for an extra one hour. The well with the lowest concentration of the extract that did not produce a red color, signifying absence of bacteria, was regarded as the MIC (15).

The FIC of each extract was calculated using the equation described by Bremmer et al., (16); FIC_A=MIC of A drug in combination/ MIC of drug A, and FIC_B=MIC of B drug in combination/MIC of drug B. The FICI is $FIC_A + FIC_B$ and is defined as synergism if FICI is ≤ 0.5, additive if FICI is 0.51-<1, indifferent if FICI is 1≤4.0, and antagonistic if FICI is >4.0 (16).

Time-kill assay:

The MIC concentrations obtained for each isolate against the polyherbal extract combination were used to determine the time of killing at the 2x and 3x MIC of the test bacterial isolates obtained using the plating method described by Achukwu et al., (12) and Bremmer et al., (16). One ml of combined extract at 2x and 3x MIC was added into 5ml MH broth and 0.1ml of the standardized bacterial cell suspension was added and properly mixed. The inoculated tubes were incubated at 37^0C over-

night. A 0.1ml of the mixture was removed from the broths every 2 hours to determine the time of bactericidal action, serially diluted and plated onto MH agar plate, which was incubated for 24 hours at 37^{O}C. Culture plates with bacteria colonies were counted and recorded as log10 CFU/ml according to the formula of Ay'ena et al., (22) and compared with the control.

The time kill assay graph was produced with the time of incubation plotted against the logarithm of the number of viable cells. A 3log10 fold decrease in the original bacterial population, corresponding to 99.9% killing, was regarded as bactericidal.

Statistical analysis of data:

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

Results:

The extractive value of *M. lucida* fruit and *P. santalinoides* seed as presented in Table 1, shows that methanol solvent for *M. lucida* fruit had a higher extractive value (37.56) than *P. santalinoides* seed (27.38). The phytochemical constituents of both plants showed that they contain valuable secondary metabolites such as tannin, phenols, terpenoids, alkaloids and flavonoids as shown in Table 2.

Table 1: Methanol extractive index value of *Morinda lucida* fruit and *Pterocarpus santalinoides* seed

Plant parts	Appearance of extract	Consistency	Extractive value (%) w/w
Morinda lucida	Green	Pasty	37.56
Pterocarpus santalinoides	Cream	Pasty	27.38

Table 2: Phytochemical constituents of extracts of *Morinda lucida* fruits and *Pterocarpus santalinoides* seed

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

Table 3: Mean inhibition zone diameters of different concentrations of *Morinda lucida* fruit and *Pterocarpus santalinoides* seed

Test isolates	*Morinda lucida* fruit extract concentration (mg/ml)				*Pterocarpus santalinoides* seed extract concentration (mg/ml)				Controls	
	400	200	100	50	400	200	100	50	Positive Control (levofloxacin 0.05 mg/ml)	Negative Control (DMSO)
S. aureus	26.00±0.2	23.00±0.7	22.00±0.5	20.00±0.3	24.00±0.0	23.00±1.4	21.00±0.0	20.00±0.0	32.00±0.0	0.00
K. pneumoniae	25.00±0.9	22.00±0.2	21.00±0.6	19.00±0.9	23.00±0.0	21.00±0.0	20.00±1.4	18.00±0.0	28.00±0.0	0.00
P. aeruginosa	24.00±0.2	21.00±0.0	20.00±0.6	18.00±0.9	22.00±0.0	20.00±0.0	18.00±1.4	17.00±0.4	28.00±0.0	0.00
E. coli	26.00±1.4	24.00±1.4	22.00±0.2	20.00±0.0	24.00±0.2	23.00±0.8	21.00±0.3	20.00±0.0	30.00±0.0	0.00
P. mirabilis	25.00±0.0	24.00±0.6	21.00±0.4	19.00±0.0	23.00±0.7	22.00±0.2	19.00±0.7	17.00±0.1	30.00±0.0	0.00

Antimicrobial activity of the plant extracts:

The antibacterial activity of the extracts was evaluated by measuring the zone of inhibition diameter as shown in Table 3. Both extracts were active against the test bacteria with varying degrees of zones of inhibitions. The mean diameters of zones of inhibition produced by *M. lucida* ranged from 18.0±0.9mm to 26.0±1.4mm. The highest zone of inhibition was produced against *E. coli* and *S. aureus,* followed by *K. pneumoniae* and *P. mirabilis* and the least was against *P. aeruginosa*. The mean diameters of zones of inhibition produced by *P. santalinoides* ranged from 17.0±0.1mm to 24.0±0.2mm, and the highest activity was against *S. aureus* and *E. coli* while the least was against *P. aeruginosa*.

The MIC of *M. lucida* against *P. mirabilis, S. aureus* and *E. coli* was 6.25mg/ml and MBC 3.125mg/ml while against *K. pneumoniae* and *P. aeruginosa*, the MIC was 12.5mg/ml and MBC 6.25mg/ml. The MIC of *P. santalinoides* against all the test bacterial isolates was 12.5mg/ml and the MBC 6.25mg/ml. Comparing the antimicrobial activity of extract from both plants, *M. lucida* had statistically significantly higher activity ($p<0.05$) than *P. santalinoides*.

The antibacterial activity of the methanol extracts of the polyherbal combination of *M. lucida* fruit and *P. santalinoides* seed was significantly higher ($p<0.05$) against all the bacterial isolates tested than the individual plant, signifying the synergistic activity of the extracts combination as shown in Table 4. The mean zone diameter of inhibition of the polyherbal combination against the test isolates ranged from 25.00±00 to 34.0±0.4mm, which compares favorably with the mean diameter of the inhibition zone of levofloxacin control that ranged from 28.0±0.0 to 32.00±0.0mm.

The zones of inhibition were highest against *S. aureus* and *E. coli* although there was no significant difference between the activities of the polyherbal combination against the MDR isolates. The MICs of the polyherbal combination as presented in Table 4, ranged from 0.39mg/ml to 1.56mg/ml against the isolates, and were significantly lower against the isolates when compared with their individual MIC.

Synergistic activity of the plant extracts:

The synergistic activity of the combination as shown in Table 5, showed that the FICI of the polyherbal combination against all the isolates were below 0.5, which signifies the synergistic activities of the combined extract against the MDR isolates.

Results of the time-kill assay of the extracts:

The time kill assay was expressed as 3log10 or 99.9% reduction in the number of viable cells from the initial inoculum and the result is presented in Fig 3. The time-kill assay activity at 2×MIC showed that there was close to 99.9% cell reduction after 6-8 hours of incubation while at 3×MIC there was a gross decrease in the number of viable cells and 99.9% of cell death occurred at 6 hours of incubation for all the isolates.

Table 4: Mean inhibition zone diameter (mm) and minimum inhibitory concentrations of methanol extracts of polyherbal combination of *Morinda lucida* fruits and *Pterocarpus santalinoides* seed against test bacterial isolates

Test isolates	Mean zone of inhibition diameter (mm) to extract concentration (mg/ml)				Minimum inhibitory concentration (mg/ml)		
	400	200	100	50	*Morinda lucida* fruit	*Pterocarpus santalinoides* seed	Combination of *Morinda lucida* fruits and *Pterocarpus santalinoides* seed
E. coli	34.00±0.0	32.00±0.9	31.00±0.4	28.00±1.4	6.25	12.5	0.39
K. pneumoniae	32.00±0.4	29.00±0.0	28.00±0.9	25.00±0.7	12.5	12.5	1.56
P. aeruginosa	32.00±0.4	30.00±0.6	28.00±1.4	25.00±0.0	12.5	12.5	1.56
P. mirabilis	33.00±0.2	30.00±0.8	28.00±1.5	27.00±0.4	6.25	12.5	0.39
S. aureus	34.00±0.4	33.00±1.4	31.00±1.4	29.00±0.0	6.25	12.5	0.39

Table 5: Evaluation of the synergistic activity of polyherbal combination of *Morinda lucida* fruits and *Pterocarpus santalinoides* seed extracts against multi-drug resistant bacterial isolates tested

Test isolates	FIC_A	FIC_B	FICI	Interpretation
Escherichia coli	0.06	0.03	0.09	Synergistic
Klebsiella pneumoniae	0.25	0.13	0.38	Synergistic
Pseudomonas aeruginosa	0.25	0.13	0.38	Synergistic
Proteus mirabilis	0.06	0.03	0.09	Synergistic
Staphylococcus aureus	0.06	0.03	0.09	Synergistic

A - *Morinda lucida*; B - *Pterocarpus santalinoides*; FICI ≤ 0.5 = synergism; 0.51 < FICI ≤ 4.0 = indifferent; FICI > 4.0 = antagonistic

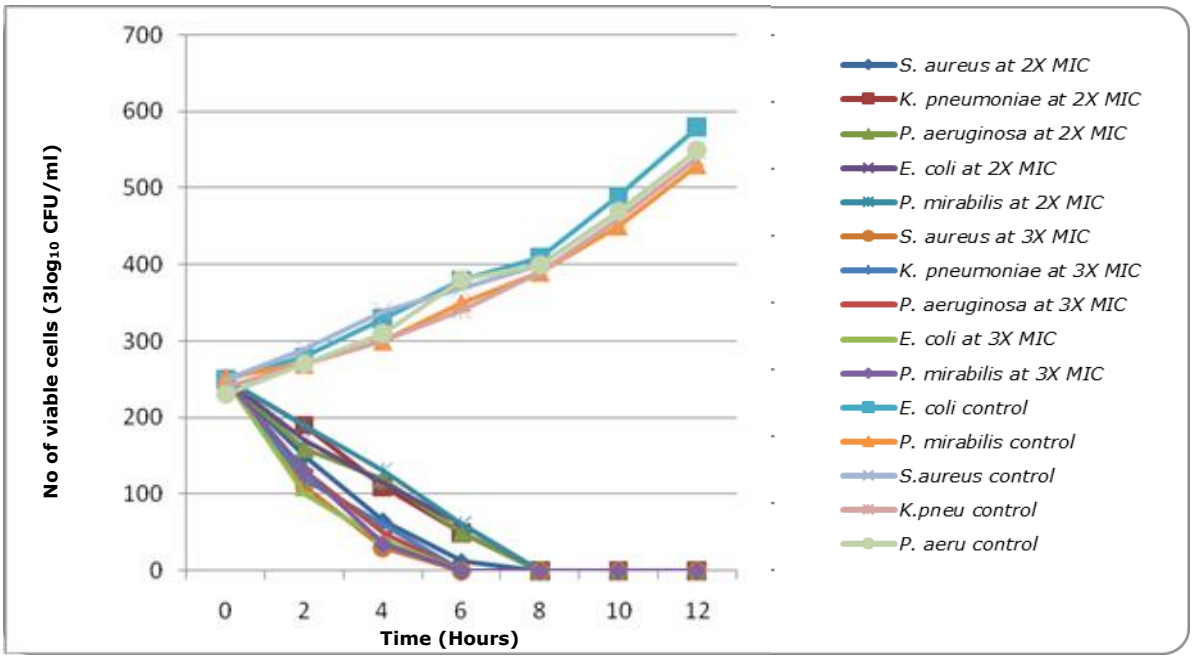

Fig 3: Time kill assay curve of the polyherbal combination of *Morinda lucida* fruits and *Pterocarpus santalinoides* seed against multi-drug resistant bacterial isolates at 2x and 3x MIC

Discussion:

The high extractive value of the extract using methanol could be the reason why alcohol is mostly used in the extraction of plant materials in traditional medicine. The various vital secondary metabolites found to be present in both extracts are known to be responsible for plants' antimicrobial, anti-inflammatory, and antioxidant properties. It accounts for the overall therapeutic potency of herbal plants (17). These phytochemicals are what are harnessed during drug production due to their efficacy in the treatment and management of infectious diseases. They help to revive orthodox antimicrobials by improving their potency and limiting drug resistance (18).

Saponins act by facilitating the entry of cytotoxic contents into cells, while tannins induce cell death through protein denaturation (19). Flavonoids inhibit cell walls, disrupt formation of biofilm, block population sensing, and impede adhesion and cell membrane formation (20). Secondary metabolites have promising effects in overcoming multiple drug resistance mechanisms and therefore useful in the management of infectious diseases caused by multi-drug-resistant organisms (18).

Both extracts in this study were active against the test bacteria with varying degrees

of zones of inhibitions. The highest zone of inhibition was produced by *M. lucida* fruit. The antimicrobial activity of *M. lucida* fruits demonstrated in our study agrees with the works of Kouamé et al., (21) where essential oil of *M. lucida* fruits was shown to be active against *P. aeruginosa, E. coli* and *S. aureus*. There are limited studies on antimicrobial activity of *M. lucida* fruits in the literature, making comparison difficult. The antimicrobial activity of *P. santalinoides* seeds against the test isolates in our study was similar to the works of Ay'ena et al., (22), who reported inhibitory activity of *P. santalinoides* against enteric organisms and that of Emencheta et al., (6) who reported *in vitro* efficacy against *S. aureus* and *E. coli*. There is also paucity of information in the for comparison with our current study, as most studies were done on the leaf and stem-bark of this plant. The MBCs of *M. lucida* fruit and *P. santalinoides* seed that ranged between 3.125 and 6.25mg/ml is an indication of good bactericidal effects of the extracts of the two plants.

The antimicrobial activity of the extract combination that produced larger zones of inhibition is a proof of higher antimicrobial activity in combination and suggests that the extracts have different mechanisms of action. The lower MIC of the combined extracts could be ascribed to the higher inhibitory action in combination and confirms synergistic activity of the plant combination. It may also suggest the presence of antimicrobial-resistant modifying compounds in the extracts, hence wider zones of inhibition towards the isolates. Mussarat et al., (23) reported that polyherbal combinations of plant are more effective than use of single extract as was observed in our study. There is paucity of information in the literature on extract-extract interaction to determine synergism of *M. lucida* fruit and *P. santalinoides* seed because most studies have concentrated on investigating the inhibitory activity of only the leaf and stem barks of the plants.

The main advantages of antimicrobial combination are synergistic effectiveness at lower concentrations which may help reduce the emergence of resistant strains, overcome resistant organisms and reduce the quantity of antimicrobials consumed. In the study by Adwan et al., (24), lower MICs in plant combination were reported, with synergistic effect against bacterial pathogens. Antimicrobials in combination could give boosted inhibition and synergistic activities against microorganisms and serve as effective antimicrobials.

Polyherbal combination is a common practice in traditional medicine as most of their preparations are in combination from inception (25). These combinations are acclaimed to be useful in the treatment of patients with severe infections caused by MDROs. The rationale for synergistic treatment is the reduction in doses of antimicrobials consumed, few or no toxicity, and prevention, delaying or overcoming resistance (25). In most scientific literatures, combination of drugs used in treatment is said to be synergistic the combination provides better therapeutic effect than when they are used individually for treatment (26). Interaction between the different components from individual phyto-compounds in combined extracts acting in unionism, as seen in our study, could be the reason for the synergistic effect observed, thereby enhancing elimination of microbes by combining different modes of action (27). The targeting of multiple pathways in the microbes could be the reason for the synergism (25).

The time-kill assay showed that polyherbal combination of extracts of *M. lucida* fruit and *P. santalinoides* seed were both time and concentration dependent in the inhibition of the bacterial isolates. The reduction in the number of viable colony counts within 2 and 8 hours of incubation showed that the combination had active bactericidal potentials and confirms their synergistic activity. This could be attributed to the antimicrobial properties of the phytochemical working in synergism to give the desired effect. Studies of synergistic activity of phytomedicine have been brought to limelight as the novel way to give scientific evidence of the higher therapeutic efficacy of polyherbal medicine combination to monotherapy. Compounds formed by synergistic combinations may be responsible for their enhanced efficacy as antimicrobial drugs (28).

Conclusion:

The combination of *M. lucida and P. santalinoides* extracts in our study demonstrated higher antimicrobial activities as shown by higher diameters of zones of inhibition and lower MICs against all the MDR isolates, when compared to those produced by extract of the individual herbal plant. The FICI of the combined *P. santalinoides* and *M. lucida* extracts also shows that the combinations produced synergistic effect. The results of our study give scientific credibility to the indigenous use of the extracts of these herbal plants for the treat-

ment of many infectious diseases in our environment

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Contribution of authors:

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

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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.

References:

1. Tang, K. W. K., Millar, B. C., and Moore, J. E. Antimicrobial Resistance (AMR). Br J Biomed Sci. 2023; 80: 1387 https://doi.org/10.3389/bjbs.2023.11387
2. Dadgostar, P. Antimicrobial Resistance: Implications and Costs. Infect Drug Resist. 2019; 12: 3903 - 3910. https://doi.org/10.2147/IDR.S234610
3. Pulingam, T., Parumasivam, T., Gazzali, A. M., et al. Antimicrobial resistance: Prevalence, economic burden, mechanisms of resistance and strategies to overcome. Eur J Pharm Sci. 2022;170:106103. https://doi.org/10.1016/j.ejps.2021.106103
4. Okaiyeto, K., and Oguntibeju, O. O. African Herbal Medicines: Adverse Effects and Cytotoxic Potentials with Different Therapeutic Applications. Int J Environ Res Publ Health. 2021; 18 (11): 5988 https://doi.org/10.3390/ijerph18115988
5. Emencheta, S. C., Enweani, I. B.., Oli, A. N., et al. Evaluation of Antimicrobial Activities of Fractions of Plant Parts of *Pterocarpus santalinoides.* Biotechnol J. 2019; 23 (1): 1-11. doi: 10.9734/bji/2019/v23i330081
6. Okafo, S. E., Anie, C.O., Alalor, C. A., and Nwankwo, L. U. Evaluation of physicochemical and antimicrobial properties of creams formulated using *Pterocarpus santalinoides* seeds methanol extract. J Appl Pharm Sci. 2023a; 13 (05): 126–135. doi: 10.7324/japs.2023.19934
7. Obaji, M., Enweani, I. B., and Oli, A. N., A unique combination of *Alchornea cordifolia* and *Pterocarpus santalinoides* in the management of multidrug resistant diarrhoeagenic bacterial infection. Asian J Biomed Pharm Sci. 2019; 10 (69): 1-18 doi: 10.35841/2249-622x.69.5059
8. Ochiabuto, O. M. T. B., Unaeze, B. C., Iyindu, M. E., and Obeagu, E. I. Evaluation of the phytochemical properties and antifungal effects of *Abrus sprecatorius* and *Morinda lucida* plant parts against clinical isolate of *Candida albicans.* Madonna University Journal of Medicine and Health Sciences. 2022 2 (1): 249-291.
9. Oladeji, O. S., Oluyori, A. P., and Dada, A. O. Anti-plasmodial activity of *Morinda lucida* Benth. Leaf and bark extracts against *Plasmodium berghei* infected mice. Saudi J Biol Sci. 2022; 29 (4): 2475 - 2482. https://doi.org/10.1016/j.sjbs.2021.12.017
10. Si, Z., Pethe, K., and Chan-Park, M. B. Chemical Basis of Combination Therapy to Combat Antibiotic Resistance. JACS Au. 2023; 3 (2): 276-292. https://doi.org/10.1021/jacsau.2c00532
11. Association of Official Agricultural Chemists (AOAC). Association of Official Analytical Chemists Method of Analysis. 21th ed. Washington D.C: AOAC International. 2023
12. Achukwu, N. O., Ebede, S. O., and Emenuga, V. N. Time Kill Assay Evaluation of *Piper guineense* Leaf and Seed Extracts against Enteric Pathogen. Trop J Nat Prod Res. 2023; 7 (12): 5684 - 5689. http://www.doi.org/10.26538/tjnpr/v7i12.47
13. Bellio, P., Fagnani, L., Nazzicone, L., and Celenza, G. New and simplified method for drug combination studies by checkerboard assay. Methods. 2021; 8: 101543. doi: 10.1016/j.mex.2021.101543
14. Samara, Y. K., Beniaic, H. G., El-abdali, Y., et al. Phytochemical Composition, Antibacterial and Antifungal Activities of Essential Oil of *Juniperus phoenicea* from Fez-Meknes Region, North Central Morocco. Trop J Natl Prod Res. 2023; 7(12):5633-5639.http://www.doi.org/10.26538/tjnpr/v7i12.39
15. El Kamari, F., Chlouchi, A., El Hamzaoui, N., et al. Chemical Composition, Antioxidant and Antimicrobial Activities of the Essential Oil of *Origanum majorana* Growing in Middle Atlas of Morocco. Trop J Natl Prod Res. 2023; 7 (10): 4232-4237. http://www.doi.org/10.26538/tjnpr/v7i10.16

16. Bremmer, D. N., Bauer, K. A., Pouch, S. M., et al. Correlations of checkerboard synergy testing with time kill analysis and clinical outcomes of extensively drug-resistant *Acinetobacter baumannii* respiratory infections. Antimicrob Agents Chemother. 2016; 60: 6892–689 doi: 10.1128/AAC.00981-16
17. Gonfa, Y. H., Tessema, F. B., Bachheti, A., et al. Anti-inflammatory activity of phytochemicals from medicinal plants and their nanoparticles: A review. CRBiot. 2023; 6: 100152. https://doi.org/10.1016/j.crbiot.2023.100152
18. Khameneh, B., Michael Eskin, N. A., Iranshahy, M., and Fazly Bazzaz, B. S. Phytochemicals: A Promising Weapon in the Arsenal against Antibiotic-Resistant Bacteria. Antibiotics. 2021; 10 (9): 1044 https://doi.org/10.3390/antibiotics10091044
19. Elekofehinti, O. O., Iwaloye, O., Olawale, F., and Ariyo, E. O. Saponins in Cancer Treatment: Current Progress and Future Prospects. Pathophysiol. 2021; 28 (2): 250-272. https://doi.org/10.3390/pathophysiology28020017
20. Zhou, M., Ma, J., Kang, M., et al. Flavonoids, gut microbiota, and host lipid metabolism. ELSC. 2024;24(5): https://doi.org/10.1002/elsc.202300065
21. Kouamé, B. K. F. P., Kablan, L., Djeda, R., et al. Chemical Composition and Evaluation of the Antimicrobial Activity of the Essential Oils from Fruits of *Morinda lucida*. Journal of Essential Oil-Bearing Plants, 2018; 21 (4): 905-912. doi: 10.1080/0972060x.2018.1517054
22. Ay´ena, A. C., Agassounon, D. T., Assogbadjo, G. A., Mensah, C., Agbangla, C., and Ahanhanzo, C. Usages et vuln´erabilit´e de *Pterocarpus santalinoides* L'Her ex DC. (Papilionoidae), une plante utilis´ee dans le traitement des gastroent´erites dans le Sud du Benin. Eur Sci J. 2016 ; 12(6) : 1857–7881. doi:10.19044/esj.2016.v12n6p218
23. Mussarat, S., Adnan, M., Begum, S., Rehman, S. U., and Hashem, A. Antimicrobial screening of polyherbal formulations traditionally used against gastrointestinal diseases. Saudi J Biol Sci. 2021;28(12): 6829-6843. doi: 10.1016/j.sjbs.2021.07.053
24. Adwan, G., Abu-Shanab, B., and Adwan, K. Antibacterial activities of some plant extracts alone and in combination with different antimicrobials against multidrug–resistant *Pseudomonas aeruginosa* strains. Asian Pac J Med. 2020; 3 (4): 266–269. doi: 10.1016/s1995-7645(10)60064-8
25. Donkor, M. N., Donkor, A. M., and Mosobil, R. Combination therapy: synergism among three plant extracts against selected pathogens. BMC Res Notes. 2023; 16(1): 83- 87. https://doi.org/10.1186/s13104-023-06354-7
26. Setiawan, P.Y.B., Kertia, N., Nurrochmad, A., and Wahyuono, S. Curcumin in combination: review of synergistic effects and mechanisms in the treatment of inflammation. J Appl Pharm Sci. 2021; 11 (2): 1–11 doi: 10.7324/japs.2021.110201
27. Suurbaar, J., Mosobil, R., and Donkor, A.M. Antibacterial and antifungal activities and phytochemical profile of leaf extract from different extractants of *Ricinus communis* against selected pathogens. BMC Res Notes.2017; 10(1):1–6. https://doi.org/10.1186/s13104-017-3001-2
28. Konaté, K., Mavoungou, J. F., Lepengué, A. N., et al. Antibacterial activity against β- lactamase producing Methicillin and Ampicillin-resistant *Staphylococcus aureus*: Fractional Inhibitory Concentration Index (FICI) determination. Ann Clin Microbiol Antimicrob. 2012; 11: 18. https://doi.org/10.1186/1476-0711-11-18