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Research Article

Synergistic Antibacterial Effect of *Mangifera Indica* and *Annona Muricata* on Antibiotic-Resistant Wound Isolates

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ABSTRACT

Mangifera indica and *Annona muricata* well-known economic and traditional plants in Nigeria were investigated for their phytochemicals and anti-microbial activities. This study presents the investigation of antimicrobial potential of two medicinal plant of *Mangifera indica* and *Annona muricata* leaf extracts alone and in combination against antibiotic-resistant bacteria associated with wound infections. A total of 40 samples collected from students of Caleb University Lagos. Morphological and biochemical characterization of bacteria were determined using standard microbiological methods. The *M. indica* and *A. muricata* leaves were subjected to ethanolic extraction method. Synergistic antibacterial effect of the combined extracts was determined using Fractional Inhibitory Concentration (Σ FIC). The percentage recovery of plant leaf extracts showed *A. muricata* with a higher yield (22.16%) compared to *M. indica* (8.48%). Qualitative phytochemical screening indicated the presence of Tannins, Steroids, Alkaloids, Reducing sugar, Flavonoids, and Glycoside in both extracts, while Phlobatannins was absent. Steroid was found to be the least abundant (18.13 – 22.61 mg/100g) in both plants. Antibiotic susceptibility testing of the isolates demonstrated varied sensitivity patterns, with the isolates showing resistance to multiple antibiotics. The MIC and MBC values for the extracts alone and in combination were determined, revealing significant antibacterial activity. Combined extracts exhibited synergistic effects (Σ FIC $\leq$ 0.5) against the tested isolates with *Staphylococcus aureus* (Σ FIC=0.24), *Escherichia coli* (Σ FIC=0.40), *Klebsiella pneumoniae* (Σ FIC=0.06), and *Pseudomonas aeruginosa* (Σ FIC=0.12). The antibacterial efficacy of leaf extracts of *M. indica* and *A. muricata* has been evidently proven against resistant bacterial isolates associated with wound infections.

Keywords: *Mangifera indica*, *Annona muricata*, Antibiotic resistance, Synergy, Wound infection

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INTRODUCTION

Antimicrobial resistance represents a major global health threat across the Globe (World Health Organization, 2022). Misuse of antibiotic leads to an emergence of multidrug-resistant bacteria and this has rendered many traditional antibiotics ineffective, limiting treatment options for infectious diseases (CDC, 2019). The issue of multidrug resistance can be controlled by exploring the synergism of antimicrobial agents that is combine therapy with using by-products of plants. Bacteria develop various mechanism of resistance due to changes on the chromosomal genes which evolved from horizontal gene transfer. This common phenomenon among disease causing organisms known as selective pressure (Bassetti et al., 2022; Hryniewicz, 2023). Severe wound infection develops when healing process failed to be achieved. Damage on skin requires a complex and regulated steps before achieving a total healing (Han, 2017). Rapid growth of microorganisms at the wound site can result into death of the cells or tissues around the affected region (International Wound Infection Institute, 2022). It has been shown that *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus mirabilis* are mostly the predominant pathogen associated with wound infection (Maddocks, 2017).

Mango scientifically called *Mangifera indica* has been known to possess therapeutic function and phytochemical constituent. *M. indica* has been locally used as medicine in the treatment of various conditions (Ediriweera et al., 2017). Various phytochemicals present in *M. indica* include alkaloids, phenols, flavonoids, tannins, and saponins, anthraquinones in its leaf extracts (Sarker et al., 2017; Okareh et al., 2019; Ogidi et al., 2021). *Annona muricata* L. (*A. muricata*) commonly called Soursop is a medicinal plant in the Annonaceae family and is known for its health benefits (Coria-Téllez et al., 2018). The pharmacological activities of *A. muricata*, denotes therapeutic functions in cancer, diabetes, hypertension, respiratory illnesses, fever, and bacterial infections (Kasole et al., 2019; Mutakin et al., 2022). The leaf and bark extracts of the plant have been known to possess promising wound-healing activity (Moghadamtousi et al., 2015). Findings revealed that ethanol showed stronger extraction capacity consequently resulting in greater number of active constituents responsible for its antibacterial activity compared other extraction solvents (Lambe and Oladunmoye, 2023). Synergistic study on ethanol leaf extracts of *A. muricata* have been reported with conventional antibiotics and not with other plant extracts. It was found to possess significant antibacterial potency with standard antibiotics such as streptomycin and tetracycline when used as a positive control (Solomon-Wisdom et al., 2014). When the ethanol leaf extracts of *A. muricata* was combined with streptomycin

and tetracycline, it produced greater effects compared to singly used and this could be attributed to the synergistic effects of its phytochemicals (Neglo et al., 2021). Unfortunately, there is paucity of information on synergism of *M. indica*, and synergistic study on plant extracts in combination against antibiotic-resistant wound isolates. This is mostly underrated thus have received limited attention. Consequently, the current study aimed to determine synergistic antibacterial effect of *M. indica* and *A. muricata* on antibiotic-resistant wound isolates.

MATERIALS AND METHODS

Study area and population

Caleb University is a private institution located in Imota, Lagos State, Nigeria with Longitude 6.6674° N and Latitude 3.6408° E. The university has a student population of over 4,000 comprising both male and female students.

Ethical Consideration

Ethical approval for the study was obtained from the Caleb University Lagos Research Ethics Committee according to the declaration of Helsinki.

Selection of participants

Inclusion Criteria

Male students with 5-days wound and students with open wounds only were included in the study.

Exclusion Criteria

Students on antibiotics therapy 3 months prior to the time of the study were excluded.

Collection and Isolation of samples

A total of 40 swab samples were obtained from students of Caleb University Lagos, Nigeria using a sterile swab stick. Within 30 minutes following sample collection, ice packs were used to transport the samples to the Microbiology laboratory of Caleb University Lagos. Over the course of five months (January, 2024 to May, 2024) samples were collected. Samples were streaked in aseptic petri plates containing Blood agar, Mannitol salt agar, Eosin methylene blue agar, and MacConkey agar, and growth was noted after a 24 hour incubation period at 37 °C. Standard gram staining and biochemical tests were further conducted.

Antibiotic Susceptibility Testing of the Wound Isolates

A 0.5 McFarland standard of the test organisms were prepared and inoculation of the bacterial isolates with positive phenotypic testing onto Mueller-Hinton agar plates was done as recommended by the Clinical Laboratory Standard Institute (CLSI, 2019). Antibiotic discs used: Amoxicillin (30 µg), Ciprofloxacin (30 µg),

Erythromycin (10 µg), Perfloxacin (30 µg), Levofloxacin (20µg), Streptomycin (30 µg), Rocephin (25 µg), Augmentin (10 µg), Gentamycin (30µg), Amplicox (30 µg), Chloramphenicol (30 µg), and Zinnacef (20 µg). The plates were then incubated at 37°C for 18 – 24 hours and observed for zones of inhibition around antibiotic disc. Appropriate controls were used.

Collection of *M. indica* and *A. muricata* Leaves

The *M. indica* leaves were obtained from Caleb University, Imota Lagos and the *A. muricata* leaves were collected from Ogudu market, Lagos. The plants were identified and authenticated at the Department of Biological Sciences and Biotechnology, Caleb University, Imota, Lagos. The plants were cleansed with sterile distilled water and air dried at 28 °C ±2 for 30 days before grinding.

Extract Preparation

The leaves of *M. indica* and *A. muricata* leaves were air-dried for thirty (30) days. They were weighed consequently on different days. They were both pulverized using a laboratory blender and stored in a cool dry place. Ethanol extraction method was employed with, 400g of each solute (*M. indica* and *A. muricata*) added to 800ml of ethanol (1:2). The mixture was placed on the mechanical shaker for 72 hours at 25°C. Following filtration, each of the mixture was further concentrated using a rotary evaporator at 56°C for 30 minutes.

The percentage yield of the *M. indica* and *A. muricata* leaf extract was determined thus:

$$X = \frac{Y}{Z} \times 100$$

X= Percentage yield of extract (%); Y = Weight of extract recovered after extraction; Z = Initial weight of plant sample before extraction

Sterility test of the extracts

The sterility test method was carried out using both Nutrient agar (NA) and Potato Dextrose Agar (PDA) for bacterial and fungal growth respectively. Incubation was done at 24 hrs at 37°C and 7 days at 25°C for NA and PDA respectively.

Phytochemical Screening of *M. indica* and *A. muricata*.

Qualitative phytochemical screening of *M. indica* and *A. muricata* was done individually for tannins, flavonoids, steroids and phenols according to methods by Thoden et al., 2012, alkaloids, phlobatannins, saponins according to methods by Odutayo et al., 2017, terpenoids, glycosides according to methods by Okiki et al., 2015. Quantitative determination of the identified phytochemicals: flavonoids, saponins, tannins, glycosides, terpenoid, phenols, steroids, and alkaloids. Procedure described by Singleton et al. (1999) was used to quantify flavonoids and phenols, while procedure described by Sofowora (1995) was used to quantify saponins, tannins, cardiac glycosides, terpenoid and steroids.

M. indica and *A. muricata* Synergistic Testing

The broth microdilution checkerboard method was performed with six serial dilutions, two-fold dilutions each for *M. indica* and *A. muricata* at concentration of 200- 6.25 mg/mL was prepared. 20 µL aliquots of *M. indica* was added to the tubes in a vertical orientation, and 20 µL aliquots of *A. muricata* was added in a horizontal orientation so that the tubes contained various concentration combinations of the two extracts. Solvent and microbial controls were also added to the assay tube. Positive and Negative controls was used in tubes without antimicrobial samples and tubes with antimicrobial samples respectively. Following this, each tube was inoculated with a 50 µL (5×10^3 CFU/well) microorganism suspension (0.5 MacFarland Standard), and was incubated at 37°C for 24 hours.

Fractional inhibitory concentration index (Σ FIC) defined as the sum of the MIC of each sample, when used in combination divided by the MIC of the sample when used alone was used to determine synergy using the equation below:

$$\Sigma \text{FIC} = \text{FIC X} + \text{FIC Y}$$

FIC X = (MIC value of combined sample and antibiotic)/ (MIC value of antibiotic alone)

FIC Y = (MIC value of combined sample and antibiotic)/ (MIC value of sample alone)

Consequently, the antibacterial activity was defined as follows:

$\Sigma \text{FIC} \leq 0.5$ = synergism;

$\Sigma \text{FIC} 0.5 \leq 1$ = additive effect;

$\Sigma \text{FIC} > 1-4$ = indifferent effect; and as

$\Sigma \text{FIC} \geq 4$ = antagonism (Van Vuuren et al., 2009)

Statistical Analysis

Antimicrobial susceptibility of the isolates to different antibiotics data was presented as mean and standard error in duplicates was used to present the zone of inhibition for the bacteria. The new Duncan's Multiple Range test was used to establish significant relationship ($P < 0.05$). The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were presented in values according to the CLSI criteria unit for antibacterial study. The mode of deviation of interaction of the extracts in combination using fractional inhibitory concentration (FIC) were interpreted as: synergistic, additive, indifferent and antagonist antimicrobial effect.

RESULTS

A total of forty (40) wound samples were examined. The highest percentage of bacteria isolated from the wound swabs: *S. aureus* (47.5%), followed by *P. aeruginosa* (30%), *E.coli* (17.50%) and the least isolate was *K. pneumoniae* (5.00%). Isolates with purplish and pinkish colouration after gram staining were interpreted as gram positive (*S. aureus*) and gram negative (*E.coli*, *K. pneumoniae*, and *P. aeruginosa*) bacteria respectively. *Staphylococcus aureus* isolates were catalase-positive and coagulase- positive. For the gram negative bacteria: *E.coli* was citrate-negative and indole-positive, *K. pneumoniae* was citrate-positive and indole-negative, while *P. aeruginosa* was citrate-positive and oxidase-

positive. The gram negative bacteria and gram positive bacteria were further confirmed on differential and selective media using a standard microbiological methods as described by Giuliano et al. (2019).

Table 1 shows the antibiotic susceptibility patterns of randomly selected wound isolates against 13 different antibiotics with varied susceptibility but high resistance was observed among *P. aeruginosa*, *E. coli* and *K. pneumoniae*. *Escherichia coli* was the only gram negative isolate with sensitivity to one antibiotics (Augmentin). While *S. aureus* showed intermediate pattern to 46% of the antibiotics tested.

The percentage recovery of the ethanol leaf extract for *A. muricata* showed the highest yields recovery with 22.16% compared to *M. indica* with recovery of 8.48% (Table 2). The qualitative and quantitative analysis of *M. indica* and *A. muricata* was done in triplicates and indicated the most abundant phytochemical as Tannin (107.21 mg/100g) and Alkaloids (101.96 mg/100g) for *A. muricata* and *M. indica* respectively while Phlobatannin was absent in both plant extracts as shown in table 3 and 4 respectively.

The antibacterial evaluation of *M. indica* and *A. muricata* alone and in combination revealed synergism for the antibiotic-resistant wound isolates with Σ FIC $\leq$ 0.5. For the antibacterial activity of *M. indica* alone, the MIC and MBC for the isolates ranged from 12.5 -50 mg/mL and 25 - 100 mg/mL respectively. For *A. muricata*, the MIC and MBC ranged from 6.25 – 12.5 mg/mL and 12.5 - 50 mg/mL respectively. On the other hand, the MIC and MBC of the combined extracts was the same with 2 mg/mL for all isolates.

DISCUSSION

The phytochemical constituents of various plants including *M. indica* and *A. muricata* has been previously reported (Nwachukwu et al., 2016). According to a previous work of Baumann et al. (2020) saponins were found in *M. indica* and it has anti-bacterial activities (Killeen and Konishi et al., 2018). In another study by Ouf et al. (2020), alkaloids, saponins, tannins and glycosides were reported as the main phytochemical constituents of *M. indica* obtained from different areas in Egypt. In other study, flavonoid (11.25 mg/100 g), saponins (3.23 mg/100 g), phenolic (0.08 mg/100 g), and tannins (0.46 mg/100 g) were found as main phytochemical constituents in *M. indica* leaves (Okwu et al., 2008). For *A. muricata*, Nwaehujor et al., (2019) reported alkaloids (294.70 mg/g), glycosides (86.57 mg/g) and tannins (3.41 mg/g) from Kwara State, Nigeria. In these studies, alkaloids and tannins were not the most abundant phytochemical in *M. indica* and *A. muricata* respectively which differs from our study which could be due to difference in geographical location and methods. However, the qualitative analysis of *A. muricata* by Agu and Okolie (2017) from Edo State, Nigeria showed similar report with our study. No significant inhibitory activity against *S. aureus* was reported whereas the MIC for *E. coli* was 6.25 mg/ml and 12.5 mg/mL for *P. aeruginosa* pathogen by *M. indica* (Kumar et al., 2021). Furthermore, Hannan et al.,

2013 reported MIC of *M. indica* as 11-52 mg/mL with 36.3 mg/mL for *E. coli* and Ouf et al. (2020) reported 125mg/mL and 500 mg/mL for *E.coli* and *P. aeruginosa*. In contrary, to study, *M. indica* showed no activity against five different gram-negative bacteria (Islam et al., 2010). Our results reported the MIC for *M. indica* alone against *S. aureus*, *E.coli*, *P. aeruginosa* and *K. pneumoniae* as 12.5 – 50 mg/mL, with high concentrations of alkaloids (101.96 mg/100g).

Silva et al. (2022) also reported the inhibition results of *A. muricata* against *S. aureus* with a value of MIC=156 mg/mL; for *E. coli*, MIC=256 mg/mL. Additionally, Ozor and Okwute (2023) reported MIC values for *E. coli*, MIC= 25 mg/mL; for *S. aureus*, MIC=12.5 mg/mL. Other MIC values of *A. muricata* have been reported for *S. aureus* and *E.coli* (Da Silva et al., 2014; Cagnini et al., 2021). The mode of action for *A. muricata* due to the presence of tannins was reported as disruption of enzymatic activities, disintegration of lipid bilayer, and promotion of metal ion chelation by Konishi et al. (2013). *A. muricata* was reported as inhibitory against *S. aureus* (MIC=12.5 mg/mL), *E. coli* (MIC=6.25 mg/mL), *K. pneumoniae* (MIC=6.25 mg/mL) and *P. aeruginosa* (MIC= 25 mg/mL) with tannins (107.21 mg/100g) as the most abundant phytochemical. Furthermore, *M. indica* and *A. muricata* showed enhanced antibacterial action compared with other plant extracts using broth dilution method from previous studies.

Pathogens such as *S. aureus*, *E. coli*, *P. aeruginosa* (both gram positive and gram negative) are commonly found in wound infections are major causes of wound infection resulting in delayed healing (Han, 2017). Interestingly, in our study we isolated *K. pneumoniae* although, it was the least pathogen with 5% prevalence. *Staphylococcus* spp. was the most predominant bacteria species observed in this study with 47.5%. Similar to studies conducted by Lambe and Oladunmoye (2023). In particular, wound pathogens are challenging to eliminate in the treatment of multi drug resistance. Antibiotic susceptibility patterns of bacterial isolates obtained in this study showed that *Staphylococcus* spp. was resistant to amoxicillin (30 µg) used in this study. This is consistent with the report by Maddocks (2017) and Muhammad et al. (2018) reported that *Staphylococcus* spp. isolated from wounds were resistant to macrolides, β -lactam antibiotics, and aminoglycosides. This may be due to the presence of multiple resistance genes in most *Staphylococcus* spp. such as the *mec* and *ccr* genes reported by Chambers and Deleo (2010).

When plant extracts are used in combination, synergistic activity can increase efficiency (Akharaiyi et al., 2021; Ezeanya-Bakpa et al., 2023). Previous published work on the synergistic activity by checkerboard of two plant extracts; non available for *A. muricata* and *M. indica* resulted in a synergistic effect against tested antibiotic-resistant bacterial strains (Mtani et al., 2024). The available study by Silva et al. (2019) where *A. muricata* was combined with different classes of antibiotics such as Ceftriaxone resulted in an increase of the antibacterial action against antibiotic-resistant bacterial strains.

This study was performed to evaluate the antibacterial action of *M. indica* and *A. muricata* in combination

against antibiotic-resistant wound pathogens. To the best of our knowledge, the combination of the two plant extracts: *M. indica* and *A. muricata* against common wound pathogens performed in this present study is novel as there are no previous reports. Moreover, *M. indica* and *A. muricata* showed synergistic antibacterial activities when combined on the specific antibiotic – resistant bacterial strains understudied. Our inhibitory and bactericidal results were also in concordance with current literature.

In conclusion, these present results showed that the combinations of *M. indica* and *A. muricata* could be an alternative treatment approach for drug (antibiotic) resistance in microbial infections of public health significance in particular originating from wound sources, which requires further in-depth in-vivo studies.

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Table 1: Antibiotic susceptibility pattern of the wound isolates

Antibiotics	SA	EC	PA	KP
LEV	17.5±0.5 ^b (I)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)
E	18.0±1.0 ^b (I)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)
PEF	16.5±0.5 ^b (I)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)
CN	16.5±0.5 ^b (I)	11.00±0.00 ^b (R)	0.00±0.00 ^a (R)	0.00±0.00 ^a (R)
APX	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)
Z	0.0±0.0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)
AM	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	0.0±0.0 ^a (R)	10.00±0.00 ^a (R)
R	22. 0.0±1.0 ^d (S)	19.50±0.5 ^c (I)	17.0±0.0(I)	20.50±1.5 ^c (I)
CPX	17.5±0.5 ^c (I)	11.00±0.00 ^b (R)	0.00±0.00 ^a (R)	0.00±0.00 ^a (R)
AZ	20.0±1.0 ^c (I)	18.50±0.0 ^b (I)	14.50±1.5(I)	16.00 ±1.0 ^b (I)
S	0.00±0.00 ^a (R)	0.00±0.00 ^a (R)	11.00±0.00 ^b (R)	0.00±0.00 ^a (R)
CHL	12.00±0.00 ^a (R)	0.00±0.00 ^a (R)	0.00±0.00 ^a (R)	11.00±0.00 ^a (R)
AU	0.00±0.00 ^a (R)	18.00±2.12 ^b (S)	0.00±0.00 ^a (R)	12.00±0.00 ^a (R)

Keys: PA: *Pseudomonas aeruginosa*; EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; SA: *Staphylococcus aureus*

LEV: Levofloxacin (30 µg), PEF: Pefloxacin (30 µg), CN: Gentamycin (30 µg), APX: Amplicox (30 µg), Z: Zinnacef (20 µg), AM: Amoxicillin (30 µg), R: Rocephin (25 µg), CPX: Ciprofloxacin (30µg), E:

Erythromycin (10 µg), S: Streptomycin (30 µg), CH: Chloramphenicol (30 µg), AU: Augmentin (10 µg).

(S): Sensitive, (R): Resistant, (I): Intermediate. Values are presented as mean±SE of duplicates, values in the same column carrying same superscript are significantly different (p<0.05) according to new Duncan's Multiple Range test.

Table 2: Percentage Recovery of Ethanol Leaf Extract of *M. indica* and *A. muricata*

Extract	<i>M. indica</i>	<i>A. muricata</i>
Original Weight (g)	105	73
Extract Weight (g)	8.9	16.18
Percentage recovery (%)	8.48	22.16

Table 3: Qualitative phytochemical screening of *M. indica* and *A. muricata*

Characteristics	<i>M. indica</i>	<i>A. muricata</i>
Tannin	+	+
Saponin	-	+
Steroid	+	+
Terpenoids	-	+
Alkaloids	+	+
Phlobatannin	-	-
Phenol	-	+
Flavonoid	+	+
Glycoside	+	+
Reducing sugar	+	+

Keys: +: Present, -: Absent

Table 4: Quantitative Phytochemical screening (mg/100g) of *M. indica* and *A. muricata*

Phytochemicals	<i>A. muricata</i>	<i>M. indica</i>
Tannin	107.21	90.18
Terpanoid	24.26	-
Steroid	18.13	22.61
Flavonoid	30.14	27.10

Phenol	22.34	-
Saponin	44.62	-
Cyanogenic glycoside	22.91	30.17
Alkaloids	82.17	101.96
Reducing Sugar	69.29	73.82

Table 5: *M. indica* and *A. muricata* combination testing for antibiotic-resistant wound isolates

	<i>M. indica</i> (MIC/MBC) mg/mL	<i>A. muricata</i> (MIC/MBC) mg/mL	Combined MIC/MBC mg/mL	ΣFIC Value (Interpretation)
<i>S. aureus</i>	25/50	12.5/25	2*	0.24 (Synergistic)
<i>E. coli</i>	12.5/25	6.25/12.5	2*	0.40 (Synergistic)
<i>K. pneumoniae</i>	12.5/25	6.25/12.5	2*	0.06 (Synergistic)
<i>P. aeruginosa</i>	50/100	25/50	2*	0.12 (Synergistic)

Keys: ΣFIC value: Fractional Inhibitory Concentration Index; MIC: Minimum inhibitory Concentration; MBC: Minimum Bactericidal Concentration (MBC), *MIC=MBC