



In vitro* assessment of the antibacterial activity of combinations of methanolic extracts of *Mangifera indica* L. bark and *Psidium guajava* L. leaf on multidrug-resistant *Pseudomonas aeruginosa

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Abstract

Pseudomonas aeruginosa causes various infections in humans both in hospital and community settings. *Mangifera indica* Linnaeus bark and dry *Psidium guajava* Linnaeus leaves have individually demonstrated activity against *P. aeruginosa*. This study aimed to assess the combined antibacterial activity of methanolic extracts of dry *M. indica* bark and dry *P. guajava* leaves on Multidrug-Resistant *P. aeruginosa*. Different proportion combinations of *P. guajava* and *M. indica* were assessed for antipseudomonal activity using Agar well diffusion method. Colistin was the positive control. The Fractional Inhibitory Concentration Index (FICI) was also determined. The combination of methanolic extracts of *P. guajava* leaves (35 mg/mL) and *M. indica* bark (6.25 mg/mL) had a superior antibacterial effect on Multidrug-Resistant *P. aeruginosa* when compared with the individual extracts used alone ($p < 0.05$), save for *P. guajava* (100 mg/mL) ($p = 0.1373$). Colistin was significantly more active on MDR *P. aeruginosa* than all the test extract concentrations used. This combination of *M. indica* bark and *P. guajava* leaves methanolic extracts had a FICI of 0.2434. This study demonstrates that the combination of *P. guajava* leaves (35 mg/mL) and *M. indica* bark (6.25 mg/mL) has synergistically enhanced activity against MDR *P. aeruginosa*.

Keywords: Antipseudomonal, MDR *Pseudomonas aeruginosa*, *Mangifera indica*, and *Psidium guajava*

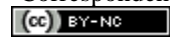
INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and a common cause of various infections especially in hospitalized patients [1, 2]. Incidences of nosocomial infections in South-East Asia and sub-Saharan Africa are over 75% with *P. aeruginosa* being the most common causative agent [3]. *P.*

aeruginosa causes various life-threatening diseases such as nosocomial pneumonia, urinary tract infections, endocarditis, meningitis, and septicaemia [4, 5]. Hospital-Acquired Infections (HAIs) are a major global safety concern for both patients and health care professionals. These infections cause prolonged hospital stay, potential disability,

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excessive costs, and sometimes death. The burden is substantial in developed countries affecting 5% to 15% of regular ward patients and as many as 50% or more of Intensive Care Unit (ICU) patients, while in developing countries, the magnitude remains underestimated and largely unknown [6].

Resistance to antibiotics, like antipseudomonal penicillins, carbapenems, fluoroquinolones, aminoglycosides, and cephalosporins commonly used to treat pseudomonal infections, is on the rise hence rendering them ineffective [7]. In a study carried out in selected animals, *P. aeruginosa* was isolated in 41.8% and resistance studies showed that 40.9% were susceptible to gentamicin, 77.3% to ciprofloxacin, 77.3% to imipenem, and 72.7% to ceftazidime [8]. In a study carried out in Uganda at Mulago National Referral Hospital, most HAIs were bloodstream Enterobacteriaceae infections, of which 22.4% were found to produce carbapenemase which confers resistance to carbapenems; the drugs of choice for the management of Pseudomonas infections [9]. This means that there is a very limited option of drugs when it comes to treating infections caused by *P. aeruginosa*. Therefore, there is an urgent need to find new antimicrobial agents to be used on *P. aeruginosa* [10].

Synthetic drugs currently suffer the problems of reduced efficacy and increasing toxicity and as such, the search for more suitable alternatives is paramount [11]. Medicinal plants, including *Mangifera indica* L. and *Psidium guajava* L., have demonstrated activity against a wide range of both sensitive and drug-resistant microbes [12]. For instance, *M. indica* methanolic leaf extracts have been reported to possess antipseudomonal activity with MICs of 6.25-250 mg/mL [13, 14]. Antipseudomonal activity has also been demonstrated in *P. guajava* leaves using methanolic extracts with MIC of 50 mg/mL and MBC of 100 mg/mL [15], and ethanolic extracts with MICs of 10 – 100 mg/mL [16].

M. indica and *P. guajava* have also demonstrated moderate antimicrobial activity against MDR *P. aeruginosa* with MICs of 0.512 mg/mL and 1.024 mg/mL respectively and have been found to improve the antimicrobial activity of several synthetic antibacterial drugs against various drug-resistant genotypes [12]. Since both *M. indica* and *P. guajava* are widely used for their antimicrobial effects, our study was aimed at establishing the potential improved activity against MDR *P. aeruginosa* when they are used in combination.

EXPERIMENTAL METHODS

Collection, identification, and preparation of plant extracts. The fresh bark pieces of the *Mangifera indica* Linnaeus tree and leaves of *Psidium guajava* Linnaeus were collected from the Mbarara district (Uganda), transported to and identified, and verified by a Botanist at Makerere University Herbarium, and then transported to the pharmaceutical analysis laboratory at the Department of Pharmacy, Makerere University. The fresh bark pieces of *M. indica* were washed thoroughly with distilled water and then openly dried on shelves at room temperature for 10 days in line with the Mada *et al.*, 2012 [14] protocol. After drying, the pieces were pounded, ground into a fine powder using a mortar and pestle, then sieved and the powder obtained was then weighed. The collected guava leaves were also thoroughly washed with distilled water, dried in the shade for 30 days, and then ground into a coarse powder with the help of mortar and pestle. *M. indica* bark powder (20 g) was macerated in methanol (200 mL) and allowed to soak at room temperature for 48 hours with occasional shaking. This mixture was thereafter filtered through a Whatman filter paper. Filtering was repeated three times with the same plant material until the solution became clear. The filtrate was then evaporated in a weighed flask, with a water bath set at 60°C. The weight of the extract obtained was

used to calculate the percentage yield. The shade dried coarse powder (100 g) of *P. guajava* leaves were macerated in methanol (200 mL) placed in a sterile conical flask (500 mL). The flask was then covered with cotton wool and intermittent shaking was done for one week. This mixture was then filtered through Whatman No. 1 filter paper, and the residue was discarded. The filtrate was evaporated at 60°C in an oven to obtain a dried extract which was weighed and the weight was used to calculate the percentage yield [17].

Phytochemical analysis. The powdered bark extracts of *M. indica* and the powdered leaf extracts of *P. guajava* were evaluated for the presence of phytochemical compounds using standard methods [18].

Preparation of test extracts and controls. *M. indica* extract (1 g) was dissolved in 10% DMSO (50 mL) in a volumetric flask to make a stock solution of 20 mg/mL. The solution was then refrigerated. *P. guajava* extract (10 g) was dissolved in 10% DMSO (100 mL) in a volumetric flask to make a stock solution of 100 mg/mL and then stored in the refrigerator. *Mangifera indica* (12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL dilutions) and *Psidium guajava* (100 mg/mL, 50 mg/mL and 25 mg/mL dilutions) were prepared from their respective stock solutions. The combination of the two extracts was prepared in ratios of 30:70, 50:50, 70:30 using their validated MICs [14, 15]. The negative control was prepared by adding DMSO (10 mL) to distilled water (90 mL). The positive control was made of Colistin at a concentration of 1 µg/mL.

Preparation of the Mueller Hinton agar. This was prepared by dissolving the agar in distilled water with heating and agitation followed by sterilization by autoclaving at 12°C for 15 min. Cooling to room temperature was then done and then the agar was poured into sterile Petri dishes on a level workstation to ensure uniform depth. The agar plates were then stored in a fridge between 2-8°C until

required for use following the manufacturer's guidelines.

Preparation of the test organisms. The test organism, CC 235 MDR *P. aeruginosa*, was obtained from the Microbiology Department laboratory, College of Health Sciences, Makerere University (Uganda). The organisms were resuscitated using Mueller Hinton agar and incubated for 24 h at 37°C followed by refrigeration at 2-8°C.

Evaluation of susceptibility of MDR *P. aeruginosa* to *M. indica* and *P. guajava*. Three 6 mm diameter holes were bored in each of the plates with solidified agar using a sterile borer. The MDR *P. aeruginosa* was inoculated using a cotton swab by streaking after dissolving in MacFarland's standard. This ensured the uniform distribution of the microorganisms. Methanolic extracts of *M. indica* stem bark (1.5 mL) of the concentration of 6.25 mg/mL and methanolic extract of *P. guajava* (1.5 mL) of concentrations of 50 mg/mL were put in the holes to validate the MICs. The plates were incubated at 37°C for 24 h and zones of inhibition were then measured to the nearest millimetre using a calibrated vernier callipers and results were recorded. The combinations of the two extracts in ratios of 30:70, 50:50, 70:30 using these validated MICs were then also put in holes of the assigned inoculated plates as described above.

Determination of Minimum Inhibitory Concentration (MIC) of the most active combination of the extracts. From the results obtained above, a graph of squared inhibitory zone diameter (IZD²; mm²) against log concentration was plotted and the equation of the graph was used to get X-intercept, the antilog of which is the MIC. Then using the MICs calculated above, the Fractional Inhibitory Concentration Index (FICI) for each combination ratio was then calculated using the formula below, and the effects of the combinations were then be classified as;

synergistic, additive, indifferent and antagonistic, if the FICI is <1 , $=1$, $>1 \leq 2$ and >2 respectively [19].

$$\text{FIC (M. indica extract)} = \frac{\text{MIC (M. indica extract in combination)}}{\text{MIC (M. indica extract alone)}}$$

$$\text{FIC (P. guajava extract)} = \frac{\text{MIC (P. guajava extraction in combination)}}{\text{MIC (P. guajava extract alone)}}$$

$$\text{FICI} = \text{FIC (M. indica extract)} + \text{FIC (P. guajava extract)}$$

Data management and analysis. The data was collected in raw form and recorded. The concentrations of the extracts and mean zones of inhibition diameter were entered into an excel sheet and plots of squared inhibitory zone diameter against log concentration were made to determine MICs. Graphpad prism ver 7.03 was used to generate the mean zone diameter and standard deviation (SD). Then one-way ANOVA with Dunnett's multiple comparison test was run to generate p values to determine significance. A p-value of < 0.05 was considered significant.

Research ethical approval. Approval to carry out this research was obtained from the Makerere University School of Health Sciences- Institutional Review Board (MakSHS-IRB). Permission was also sought from garden owners before samples were collected.

RESULTS AND DISCUSSION

Extract yield and phytochemical analysis.

The methanolic extraction yields of the dry *M. indica* bark and *P. guajava* leaves were 12.6% and 9.3% respectively (Table 1). These were relatively lower than those reported in related studies [14, 15] and this can be attributed to variations in experimental procedure parameters such as room temperature, reagent quality, and maceration duration among others [20, 21]. The methanolic extracts of dry *M. indica* L. bark and *P. guajava* L. leaves were both found to have significant amounts of

tannins, glycosides, and flavonoids (Table 1). These phytochemicals are known to be responsible for antibacterial activity [22, 23]. Tannins are polyphenolic compounds that exhibit antibacterial effects through interfering with protein synthesis by binding to proline-rich protein [24]. Flavonoids are hydroxylated polyphenolic compounds and these exhibit antibacterial effects through forming complexes with both extracellular and soluble proteins and also bacterial cell walls [25].

Antibacterial activity against MDR *P. aeruginosa*.

The results of the study indicated that the methanolic extracts of *M. indica* bark and *P. guajava* leaves showed inhibitory activity against MDR *P. aeruginosa* (Table 2, Figure 1) at the concentrations used, save for *M. indica* at the concentration of 3.125 mg/mL. However, the antibacterial activity of these extracts on MDR *P. aeruginosa* was significantly lower ($p > 0.01$) than that of the positive control (Colistin). Among the crude extracts, *P. guajava* at a high concentration of 100 mg/mL had the greatest activity against MDR *P. aeruginosa*. The antipseudomonal activity of combination 3(CB3) comprising *P. guajava* (PG) and *M. indica* (MI) at concentrations of 35 mg/mL and 1.875 mg/mL respectively was not significantly different with its serial dilution, S1 (17.5 mg/mL PG: 0.9375 mg/mL MI) ($P=0.0764$) and with *P. guajava* (100 mg/mL) ($P=0.1373$). The individual antibacterial activity of methanolic extracts of *M. indica* bark and *P. guajava* leaves against MDR *P. aeruginosa* has been previously reported. Methanolic extracts of *M. indica* bark and *P. guajava* leaves have been reported to possess moderate activity against MDR test organisms including *Pseudomonas aeruginosa*, *Escherichia coli*, *klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Providencia stuartii* [12]. Amongst the combinations, the *P. guajava* (35 mg/mL) and *M. indica* (1.875 mg/mL) combination had the best activity against MDR *P. aeruginosa*, and this

combination was hence used to determine the MICs of the extracts and to determine the FICI.

MIC and FICI. In our study, the MIC of methanolic extracts of dry *M. indica* bark and dry *P. guajava* leaves on MDR *P. aeruginosa* were found to be 3.641 mg/mL and 21.513 mg/mL respectively. A study in Cameroon on MDR *P. aeruginosa* reported MICs for methanolic *M. indica* bark and dry *P. guajava* leaves extracts of 0.512 mg/mL and 1.024 mg/mL respectively. The *M. indica* and *P. guajava* extracts used in our study and in that done by Dzotam & Kuete on MDR *P. aeruginosa* were more potent than those used in studies done previously in Kenya and Nigeria of non-MDR *P. aeruginosa* [13-15]. This superior activity could be attributed to the higher composition of the phytochemicals (glycosides and tannins from the above phytochemical screening) in the extracts due to regional differences in climate, soil composition, and month of plant harvesting [26]. The phytochemical composition of mango bark has been shown to increase gradually from February peaking in April and it declines in May and with the lowest being in June [27]. The other cause for such differences in the reported potency of the extracts could be the varying sensitivity of the method used to determine the MICs. For instance, the study by Dzotam & Kuete used the more sensitive Rapid INT colorimetric assay; we used the Agar well diffusion method while Mada *et al* used nutrient broth turbidity. Agar and broth dilution methods are more susceptible to omission errors and misinterpretation [28, 29].

Fractional Inhibitory Concentration Index (FICI) of the combination of methanolic extracts of dry *M. indica* bark and *P. guajava* leaves using serial dilutions of Combination 3 (CB3- 70% PG: 30% MI) was determined to be 0.2434. The MICs of *M. indica* bark extract

alone and *P. guajava* leaves extracts alone in the combinations were 0.21 mg/mL and 3.97 mg/mL respectively. The FICI value obtained was <1 indicating synergism [19]. Methanolic extracts of *M. indica* bark or *P. guajava* leaves when combined with drugs such as ciprofloxacin, erythromycin, streptomycin, kanamycin, chloramphenicol, and tetracycline have been reported to improve their antimicrobial activity through synergism [12]. The synergistic action observed in our study could be attributed to the fact that both *M. indica* bark and *P. guajava* leaves contain tannins, glycosides, and flavonoids, all of which are known to have antibacterial activity [22, 23]. Tannins exhibit antibacterial effects through interfering with protein synthesis by binding to proline-rich protein [24] while Flavonoids exhibit antibacterial effects through forming complexes with both extracellular and soluble proteins and also bacterial cell walls [25].

Combinations of herbal extracts are done to combat resistance or reduce possible side effects [26, 30]. Toxicity studies in animal models using methanolic extracts of dry mango leaves have reported no acute toxicity at doses of up to 5000 mg/kg [31] and ethanolic extracts of *P. guajava* leaves at varying concentrations of up to 5000 mg/mL over fourteen days [32]. More so, no hepatotoxicity was observed with aqueous extracts of *P. guajava* leaves, and as such were considered safe [33]. However, a long term toxicity study with mild toxicities indicated mild toxicities that include: slight body weight gain, slight triglycerides, and cholesterol increase, a slight reduction in serum potassium, and a slight increase in weight of liver, kidneys, and adrenal glands at doses of 100 mg/kg, 300 mg/kg and 900 mg/kg of the long term toxicity study with mild toxicities extracts [34].

Table 1: Phytochemical yields and composition of the methanolic extracts

Extracts	<i>Mangifera indica</i>	<i>Psidium guajava</i>
Parts used	Bark	Leaves
Percentage yield*	12.6%	9.3%
Tannins	+++	++
Glycosides	++	++
Flavonoids	++	++

(++) moderate intensity reaction, (+++) strong intensity reaction

* Calculated as a ratio of the mass of methanolic extract obtained to the mass of plant powder

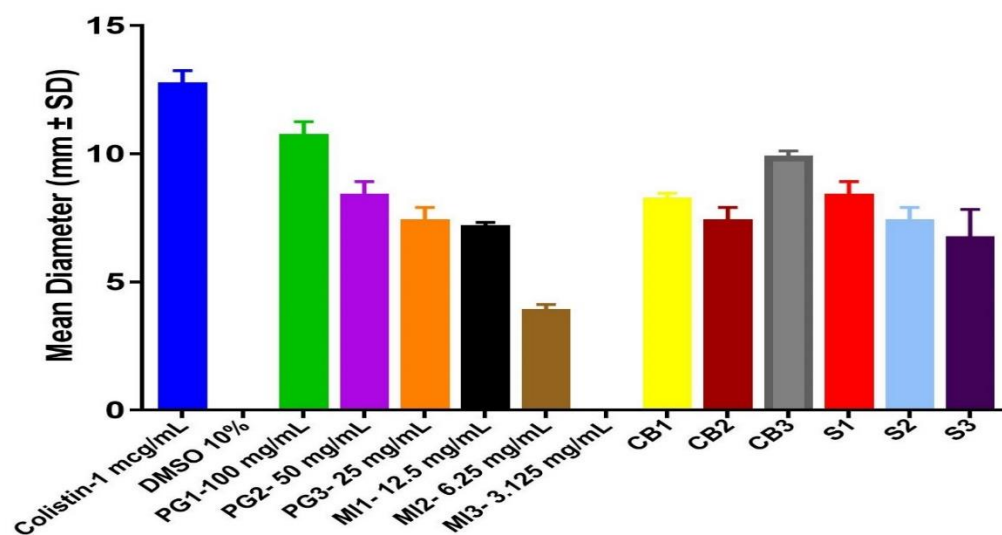
Table 2: Anti-pseudomonal activity of methanolic extracts of *Mangifera indica* bark and *Psidium guajava* leaves

Test material	Mean Diameter (mm) \pm SD
Positive control (colistin -1 μ g/ml)	12.67 \pm 0.58 [†]
Negative control (10% DMSO)	0.00 \pm 0
<i>Psidium guajava</i> (PG1)-100mg/ml	10.67 (\pm 0.58) ^{ns}
<i>Psidium guajava</i> (PG2)-50mg/mL	8.33 (\pm 0.58) ^{**}
<i>Psidium guajava</i> (PG3)-25mg/mL	7.33 (\pm 0.58) ^{****}
<i>Mangifera indica</i> (MI1)-12.5mg/mL	7.10 (\pm 0.22) ^{****}
<i>Mangifera indica</i> (MI2)- 6.25mg/mL	3.83 (\pm 0.29) ^{***}
<i>Mangifera indica</i> (MI3)-3.125mg/mL	0.00 (\pm 0) ^{****}
CB1-(25mg/mL PG: 3.125mg/mL MI)	8.17 (\pm 0.29) ^{**}
CB2-(15mg/mL PG: 4.375mg/mL MI)	7.33 (\pm 0.58) ^{****}
CB3-(35mg/mL PG: 1.875mg/mL MI)	9.83 (\pm 0.29)
S1 (17.5mg/mL PG: 0.9375mg/mL MI)	8.33 (\pm 0.58) ^{ns}
S2 (8.75mg/mL PG: 0.4688mg/mL MI)	7.33 (\pm 0.58) ^{***}
S3 (4.375mg/mL PG: 0.2344mg/mL MI)	6.67 (\pm 1.16) ^{***}

CB1- CB3 are combinations of *P. guajava* and *M. indica* in ratios of 50:50, 30:70, and 70:30 respectively calculated based on their reported individual MICs S1-S3 are serial dilutions of the most active combination (CB3)

[†]Colistin was significantly more activity on MDR *P. aeruginosa* compared to all the extracts

*or ^{ns} Indicates significance level of activity of the different extracts versus the most active combination (CB3).

**Figure 1:** Anti-pseudomonal activity of the different test samples against MDR *P. aeruginosa*

Potential toxicity of *M. indica* bark and *P. guajava* could as such be reduced by using them in combination with each at lower

concentrations of 6.25 mg/mL and 35 mg/mL respectively. However, in this study, the toxicity of this combination was not evaluated.

Conclusion. Our study results reveal that a combination of methanolic extracts of *P. guajava* leaves (35 mg/mL) and *M. indica* bark (6.25 mg/mL) has a superior anti-bacterial effect on Multidrug-Resistant *P. aeruginosa* as compared to when each extract is used alone. The combined anti-pseudomonal effect of the methanolic extracts of *P. guajava* leaves and *M. indica* bark is synergistic. These results thus justify the use of both plants' extracts in folk medicine for the management of various infections and provide grounds for further research on developing novel antibacterial agents that have activity against Multidrug-Resistant *P. aeruginosa*.

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