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Psidium guajava (Myrtaceae) re-sensitizes multidrug-resistant Pseudomonas aeruginosa over-expressing MexAB-OprM efflux pumps to commonly prescribed antibiotics

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Abstract

Background: *Pseudomonas aeruginosa* is a critical-class priority pathogen showing high resistance to almost all classes of conventional antibiotics. As a result, discovering new drugs capable of combating *Pseudomonas* infections becomes critical. The current study looked at the antibacterial and antibiotic-resistance reversal properties of the leaf (PGL) and bark (PGB) methanol extracts of *Psidium guajava* (Myrtaceae), a popular food plant, towards multidrug-resistant (MDR) *P. aeruginosa* over-expressing active efflux.

Methods: The activities were assessed using a 96-well plate microdilution technique, with iodonitrotetrazolium chloride (INT) to detect living bacteria. The action of herbals was further carried out on *Pseudomonas* kinetic growth, cell membrane, and H^+-ATPase mediated proton pumps, using standards.

Results: PGL and PGB produced inhibitory effects on all the fourteen MDR strains and clinical isolates of *P. aeruginosa*, with the minimum inhibitory concentrations (MIC) recorded ranging from 64 to 2048 µg/mL. PGB was shown to be more effective, having MICs ≤ 512 µg/mL on 100% of the MDR pathogens tested. It also exhibited the lowest MICs (best activity) of 64 µg/mL against three MDR clinical isolates P124, P57, and P29, with activity higher than that of the reference medication (chloramphenicol). PGL and PGB were shown to have significant antibiotic-resistance reversal action when combined with conventional antibiotics, with PGB enhancing the efficacy of all standard drugs employed. PGB was shown to lengthen the latent phase of kinetic growth, also, it significantly inhibited the H^+-ATPase-mediated proton pump and altered cell membrane integrity, at MIC and 2×MIC.

Conclusion: The current investigation provides justification for considering *P. guajava* extracts, alone or in combination with antibiotics, as potential treatments for MDR *P. aeruginosa* infections.

Keywords: Antibiotics; efflux pumps; food plants; multidrug-resistant bacteria; *Pseudomonas aeruginosa*; *Psidium guajava*.

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Background

Antimicrobial resistance (AMR) is a serious hazard to public health, driving the ongoing search for novel and effective medicines. The World Health Organization (WHO) has identified *Pseudomonas aeruginosa* as a critical priority pathogen to guide and support research and development of novel antibacterial drugs [1, 2]. *P. aeruginosa* infections are most common in hospitalized patients or those with compromised immune systems. It is especially harmful to those suffering from chronic pulmonary disorders. Some types of multidrug-resistant (MDR) *P. aeruginosa* are resistant to nearly all antibiotics (pan-drug resistant), including carbapenems [3, 4]. *P. aeruginosa* has evolved many resistance tactics, including active efflux, which appears to be one of the key processes contributing to MDR [5, 6]. Several measures are being made to combat MDR in bacteria. Recent decades have seen a surge in interest in medicinal plants and their derived phytoconstituents, including food plants, where research has revealed immense potential in the battle against resistant pathogens, either alone or in combination with conventional treatments [7-18]. The scholarly literature on the utilization of food plants for their many health advantages and possible therapeutic uses has lately bloomed in pharmaceutical and nutritional sciences. Health practitioners increasingly acknowledge that combining drugs and diet may yield the best results in the battle against infectious diseases. Because of the existence of pharmacologically active chemicals, the preventive advantages of food plants are being researched for possible application as innovative therapeutic therapies [19-22]. Furthermore, various studies have shown that food plants have a remarkable ability to combat MDR in bacteria [23-31]. Although various food plant extracts have been investigated for their antibacterial effects against MDR microbes, few explicitly indicate antipseudomonal activity.

The plant of interest in this study is *Psidium guajava* (Myrtaceae), a common food plant. The plant is a well-known tropic tree that is often planted for its fruit. This herb is used to treat a variety of disorders including dysentery, gastroenteritis, diarrhea, malaria, diabetes, hypertension, cavities, rheumatism, and pain. *P. guajava* leaf extract is used to treat coughs, diarrhea, oral ulcers, and wounds with swollen gums [32, 33]. *P. guajava* has a high concentration of organic and inorganic substances, such as secondary metabolites, especially polyphenols and terpenes [32]. The bioactive constituents extracted from *P. guajava* include phytochemicals and essential oils. The main phytochemicals are gallic acid, casuariin, catechin, chlorogenic acid, rutin, vanillic acid, quercetin, syringic acid, kaempferol, apigenin, cinnamic acid, luteolin, quercetin-3-O-α-L-arabinopyranoside, morin, ellagic acid, guaijaverin, pedunculoside, asiastic acid, ursolic acid, oleanolic acid, methyl gallate and epicatechin, whereas the major essential oils include limonene, trans-caryophyllene, α-humulene, γ-muurolene, selinene, caryophyllene oxide, bisabolol, isocaryophyllene, δ-cadinene, α-copaene, α-cedrene, β-eudesmol, α-pinene, β-pinene, β-myrcene, linalool, α-terpineol and eucalyptol [33]. These chemicals are responsible for *P. guajava*'s diverse bioactivity. The most active antioxidant discovered in *P. guajava* is quercetin. Furthermore, various in vitro and in vivo investigations have shown that *P. guajava* has pharmacological activity such as antidiarrheal, hepatoprotective, anticancer, anti-inflammatory, antiestrogenic, and antibacterial properties, which support its traditional usage [32]. According to the findings, *P. guajava* is a strong herbal product with remarkable pharmacological qualities that should be used in the development of pharmaceutical agents and functional foods. To further demonstrate *P. guajava*'s biopharmaceutical potential, we tested the antibacterial and antibiotic-resistance reversal activities of its leaf and bark extracts against a critical class pathogen, *P. aeruginosa*, overexpressing active efflux pumps. The potential modes of action of extracts, tested alone, have also been investigated.

Methods

Plant material and extraction procedure

The leaves (PGL) and barks (PGB) of *Psidium guajava* were harvested in Dschang (West Region, Cameroon, 5.4459° N, 10.0472° E) in October 2020. The plant was identified and authenticated at the Cameroon National Herbarium (HNC) by comparing it to a sample registered under the reference number 2884/SRFCam. The plant parts harvested were cleaned and dried in the shade before being ground. The resulting powders were macerated in methanol 95° (1:3 w/v) for 48 hours. The mixture was then agitated 3 to 4 times each day to increase the yield, followed by filtration through Whatman paper N°1. The filtrate was evaporated under a vacuum using a rotary evaporator (BÜCHI R-200) at 40 °C. The crude extracts were then recovered in dry and sterile vials and dried in an oven at 40 °C for complete solvent evaporation. The extraction yield of each extract was determined relative to the mass of dry plant powder, which was 13.51% and 14.66% for PGL and PGB, respectively. The extracts were then placed in a freezer at 4°C for future experiments.

Chemicals

Dimethylsulfoxide (DMSO) was used to solubilize plant extracts and antibiotics, and para-iodonitrotetrazolium chloride (INT) was employed to detect living bacteria during susceptibility testing. Phenylalanine arginine beta naphthylamide was used as an efflux pump inhibitor. Antibiotics belonging to different classes were included, comprising beta-lactams (ampicillin, penicillin), cyclins (tetracycline and doxycycline), phenicols (chloramphenicol), cephalosporins (ceftriaxone, cefixime), fluoroquinolones (levofloxacin, ciprofloxacin), carbapenem (imipenem), aminopenicillin (augmentin), and polypeptide (polymixin B). All chemicals were provided by Sigma-Aldrich (Germany).

Microorganisms and culture media

A multidrug-resistant (MDR) clinical strain PA124 overexpressing MexAB-OprM and a reference strain PA01, as well as 12 clinical isolates of *P. aeruginosa* expressing MDR characteristics, were employed. In a recent study, Tiotsop et al. [34] provided the bacterial characteristics of the examined *P. aeruginosa* strains and laboratory collection. Cetrimide agar (Titan Biotech Ltd, Rajasthan, India) was used to first confirm the *P. aeruginosa* strains and isolates. Microorganisms were then grown in Mueller Hinton Agar (MHA, Liofilchem, Italy) before any susceptibility testing. The Mueller Hinton broth (MHB, Titan Biotech LTD, India) served in the microdilution test for the determination of minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), antibiotic-resistance modulating factor (AMF), and the antibacterial mechanisms of test samples. All culture media were prepared according to the manufacturer's instructions.

Preparation of working solutions

The stock solution for PGL and PGB was prepared at 8192 µg/mL, whereas the antibiotics solution was made based on their respective potency. Therefore, chloramphenicol was prepared at 1024 µg/mL, ampicillin and penicillin at 512 µg/mL, ceftriaxone, cefixime, imipenem, augmentin at 256 µg/mL, levofloxacin and ciprofloxacin at 64 µg/mL, and the remaining antibiotics at 32 µg/mL. Plant extracts and antibiotics were dissolved in DMSO (For innocuity, the final DMSO content was less than 2%), then the mixture was homogenized, and the final volume was obtained by adding MHB.

Inoculum preparation

Bacterial suspensions were prepared from 18-hour-old bacterial culture colonies. A bacterial colony was dissolved in 10 mL sterile distilled water, comparing the turbidity of the bacterial suspension obtained after homogenization with that of the corresponding McFarland 0.5 ($1.5x10^8$ CFU/mL). Then, a volume of prepared bacterial suspension was collected and introduced into the MHB to obtain a corresponding solution ($2x10^6$ CFU/mL).

Rapid colorimetric INT assay for MIC and MBC determination

The minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) of PGL and PGB were determined by microdilution using a rapid colorimetric INT test as reported by Eloff [35] and thoroughly described in our previous studies [34, 36-38]. The negative control consisted of MHB and inoculum, whereas the positive control consisted of chloramphenicol, a reference antibiotic, with a concentration range of 2 to 256 µg/mL. Bacterial growth was revealed using INT (0.2%) and the MICs were defined as the minimum concentrations for which no bacterial growth (absence of pink coloration in the wells) was observed. For the MBCs, a volume of 50 µL of the content of the wells corresponding to concentrations greater than or equal to the MIC was taken and introduced into the wells of microplates containing 150 µL of MHB. Afterward, the plates were incubated for 48 h at 37°C followed by the addition of INT. The concentrations of the wells in which no pink staining was present were taken as bactericidal and the lowest of these was noted as MBC [39]. The work was done in triplicate and repeated three times.

Antibiotic-resistance reversal potential of P. guajava extracts in association with standard antibiotics

P. guajava extracts at sub-inhibitory concentrations were combined with antibiotics. The assay was performed in the same manner as previously described for the MIC test, with the exception that successive dilutions were performed in the presence of the antibiotics, followed by the addition of 50 µL of extract at sub-inhibitory concentrations in each well. Afterward, the volume was completed to 200 µL by adding 50 µL of bacterial inoculum (4×10^6 CFU/mL). The activity of PGL and PGB at sub-inhibitory concentrations (MIC/2, MIC/4, MIC/8, MIC/16) was first evaluated by a preliminary test performed on the most resistant strain (PA124) which allowed to select MIC/2 and MIC/4 for further testing on extended MDR *P. aeruginosa* strains. The antibiotic-resistance modulating factor (AMF) was calculated from the following formula:

$$AMF = MIC_{antibiotic\ alone} / MIC_{antibiotic\ in\ combination}$$

The biological significance of antibiotic-resistance modifying effects was set at AMF ≥ 2 [40].

Antibacterial activity of P. guajava extracts in the presence of PAßN

The procedure was carried out in the presence of an efflux pump inhibitor (PAßN), as reported by Kuete et al. [41]. The MIC was determined in the same way as described above except that 50 µL of the inhibitor solution was then introduced, followed by a 50 µL of bacterial inoculum (4×10^6 CFU/mL) for a final volume of 200 µL/well. A total of four controls were provided: two negative controls, one formed by wells consisting of inoculum, MHB, and the inhibitor, the other consisting of inoculum, MHB, and 10% DMSO, the neutral control consisting of wells containing only MHB and the positive control consisting of a reference antibiotic (chloramphenicol). The work was done in triplicate and repeated three times.

Action of P. guajava bark extract on kinetic of bacterial growth

The effect of PGB on the kinetic of bacterial growth was examined on *P. aeruginosa* PA124 using optical densities (OD) at 600 nm [42]. Briefly, the bacterial suspensions were produced at a concentration of 10^8 CFU/mL in the corresponding flasks. They were subsequently treated with PGB at 0.5×MIC, MIC, and 2×MIC. The vials were incubated at 37 °C under an orbital shaker (REMI) at 200 rpm. The positive and negative controls consisted of the vials containing ciprofloxacin at MIC as well as the vial containing MHB and the bacterial suspension, respectively. A volume of 500 µL was then collected at different time intervals (0 h, 1 h, 2 h, 4 h, 6 h, 10 h, 12 h, 14 h, 16 h, 18 h, and 20 h) and the OD was read using a spectrophotometer (Thermo Scientific, Langenselbold, Germany) at 600 nm. Each test was repeated 3 times. OD = f(t) was plotted using the OD values in Microsoft Office Excel 2016.

Action of P. guajava bark extract on H^+-ATPase-mediated proton pump

The ability of PGB to inhibit the H^+-ATPase-mediated proton pump of *P. aeruginosa* PA124 was performed by controlling the acidification of the bacterial growth medium as described previously [43] with minimal modifications. Briefly, 100 mL of bacterial suspension was cultured in MHB for 18 hours at 37 °C. The resulting culture was centrifuged (3000 rpm) for 10 minutes at 4 °C. Afterward, the pellet was washed twice in distilled water, then in 50 mM KCl, and re-suspended in 50 mL of KCl (50 mM). After that, the cell solution was incubated at 4 °C for 18 hours to induce glucose deprivation. To 4 mL of the cell medium, 0.5 mL of the PGB at 0.5×MIC, MIC, and 2×MIC, as well as an antibiotic (ciprofloxacin) were added. The pH was adjusted to 6.4 with 1 M HCl or 0.1 M NaOH. After 10 min of pre-incubation at 37 °C, acidification of the medium was started by adding 0.5 mL of 20% glucose. The pH was measured every 10 min for 1 h using a pH meter (Thermo Scientific, USA). The extract was replaced with DMSO to create the negative control. pH=f(t) was plotted using the reported pH values in Microsoft Office Excel 2016.

Action of P. guajava bark extract on the bacterial cell membrane

The intracellular content of *P. aeruginosa* PA124 after exposure to different concentrations of PGB (0.5×MIC, MIC, and 2×MIC) and polymixin B was performed by measuring the absorbance of the supernatant at 260 nm as previously reported [44, 45], with few changes. Briefly, young colonies of bacteria were harvested from a fresh culture on MHA medium and a suspension of 1×10^6 CFU was prepared. Bacterial cells were then treated with PGB at 0.5×MIC, MIC, and 2×MIC, then incubated for 12 hours at 37°C.

The preparations were centrifuged, and the absorbance of the supernatant was measured at 260 nm for control and treated cells, using a spectrophotometer (Thermo Scientific, Langenselbold, Germany). As a negative control, the tube containing the inoculum and DMSO was employed. Each assay was performed in triplicate.

Results

Antibacterial activity

P. guajava extracts (PGL and PGB) were examined for their antibacterial activity on a panel of fourteen *P. aeruginosa* strains and clinical isolates expressing MDR phenotypes, through determination of MIC and MBC. The bacteriostatic or bactericidal effect of test extracts was ascertained following the determination of the MBC/MIC ratio. Table 1 summarizes the findings. PGL and PGB produced inhibitory effects against all tested bacteria, with MIC recorded ranging from 64 to 1024 µg/mL. PGB displayed better efficacy than PGL, with MIC ≤ 512 µg/mL against all the studied MDR *P. aeruginosa*. More so, PGB exhibited the lowest MICs (best activity) of 64 µg/mL towards the clinical isolates P124, P57, and P29, with activity higher than that of the reference drug (chloramphenicol). PGL showed MBC/MIC ≤ 4 in 13 of 14 studied microorganisms, compared to 11 of 14 with PGB. MBC of the reference antibiotic exceeded the initial test concentration of 256 µg/mL in all cases (Table 1).

Influence of PAβN on the efficacy of P. guajava leaf and bark extracts

As presented in Table 2, the MICs of PGL and PGB, as well as the reference antibiotic (chloramphenicol) significantly improved in the presence of the efflux pump inhibitor, PAβN, with enhancement factor ranging from 2 to >128.

Antibiotic-resistance modifying activity of P. guajava extracts

The ability of *P. guajava* extracts to re-sensitize MDR *P. aeruginosa* strain and clinical isolates to standard antibiotics was assessed. The results from the preliminary investigations showed a better potentiation of antibiotics at MIC/2 and MIC/4, which were then selected for testing on further MDR isolates (Table 3). The results depicted outstanding antibiotic-resistance breaker potential of PGB, which significantly improved the activity of all standard antibiotics against all studied MDR *Pseudomonas*. Similar effects were obtained with PGL in combination with ceftriaxone, imipenem, and augmentin at sub-inhibitory concentrations of MIC/2 and MIC/4.

Antibacterial mechanisms of P. guajava bark extract

Kinetic growth

A significant and concentration-dependent reduction of bacterial growth was recorded over 20 hours of exposure of *P. aeruginosa* PA124 to PGB (Figure 1). More so, a prolonged latent phase was observed, up to 2 hours for PGB and 4 hours for the reference drug ciprofloxacin.

Cell membrane

After treatment of *P. aeruginosa* PA124 with PGB at 0.5×MIC, MIC, and 2×MIC, a significant increase of OD at 260 nm was observed, as compared with the control (Figure 2). The highest OD was recorded at MIC and 2×MIC.

H^+-ATPase-mediated proton pumps

PGB induced a significant inhibition of PA124 H^+-ATPase-mediated proton pumps, from 20 min of exposure up to 60 min, as evidenced by the reduction of acidity of the solution (Figure 3). Better effects were obtained at MIC and 2×MIC.

Discussion

African medicinal plants have shown promising activity against various types of multidrug-resistance phenotypes of bacterial and parasitic infections as well as cancer [46-59]. Their role as source of antibacterial phytomedicines and potential pharmaceuticals have also been demonstrated [25, 60-64]. The antibacterial activity of the leaf (PGL) and bark (PGB) methanol extracts of *P. guajava* was investigated towards MDR strains and clinical isolates of *P. aeruginosa*, overexpressing efflux pumps. The testing included determining the activity of herbals alone, in the presence of an efflux pump inhibitor (EPI), and association with standard antibiotics. Also, the possible modes of action of the most active extract were examined. *P. aeruginosa* has been identified as a critical priority class pathogen for guiding antibiotic research and development [1]. This, therefore, emphasizes the relevance of the present study. *P. aeruginosa* is highly resistant to conventional treatments leading to high prevalence of hard-to-treat infections. Interestingly, PGL and PGB displayed remarkable antipseudomonal potential, acting on all tested *Pseudomonas* strains with MIC recorded ranging from 64 to 1024 µg/mL (Table 1). The MIC range suggests the best to moderate activity of test herbals [65]. PGB produced better inhibitory potential than PGL. Indeed, most of the MIC obtained with PGB were ≤ 512 µg/mL against all the studied MDR *P. aeruginosa*. Furthermore, PGB depicted the lowest MIC (best activity) of 64 µg/mL towards three clinical MDR isolates (P124, P57, and P29), which activity was found higher than that of the reference drug used (chloramphenicol). This suggests that PGB may contain potent antipseudomonal phytoconstituents that work synergistically in the herbal complex. Further research using bio-guided separation of these phytoconstituents will be beneficial in identifying the bioactive components responsible for the observed actions. The genetic difference which influences the synthesis of secondary metabolites in plant parts could explain the variation in the potency of PGL and PGB [66]. PGL and PGB showed MBC/MIC ratio ≤ 4 in most cases, suggesting bactericidal effects [67]. The high antimicrobial activity of various parts of *P. guajava* has been reported in the literature [32, 68]. However, a few research have been published on its antipseudomonal activity involving MDR strains. As a result, the current study adds essential data to the field on the antipseudomonal properties of *P. guajava* leaf and bark methanol extracts. Aqueous and ethanol extracts have been shown to have limited antimicrobial activity whereas methanol extracts show great efficiency [69], as shown in the current investigation. As a result, it is possible to infer that methanol should be considered the solvent of choice for *P. guajava* antimicrobial research. The antipseudomonal activity of PGL and PGB was enhanced in the presence of PAβN (Table 2). This validates efflux activity as the primary mechanism in studied MDR *P. aeruginosa*. It was reported that resistance to antibiotics in *P. aeruginosa* is mediated through

various efflux pumps [70]. The strains tested in the present study are characterized as over-expressing MexAB-OprM efflux pumps. Indeed, MexAB-OprM pumps are responsible for resistance to various classes of antibiotics such as β-lactams, β-lactam inhibitors, fluoroquinolones, tetracyclines, tigecycline, novobiocin, thiolactomycin, sulfonamides, macrolides, aminoglycosides, etc [70]. Thus, the addition of an EPI could be considered an interesting approach to improve the antibacterial efficacy of PGL and PGB in the process of drug development. The ability of PGL and PGB to re-sensitize MDR *P. aeruginosa* to standard antibiotics (penicillin, ceftriaxone, cefixime, doxycycline, tetracycline, chloramphenicol, levofloxacin, ampicillin, imipenem, and augmentin) was assessed; outstanding results were obtained. Both extracts demonstrated a strong capacity to increase the efficiency of chosen antibiotics against the most resistant *P. aeruginosa* strains or isolates tested (Table 3). PGB improved the activity of all standard antibiotics used against all studied MDR *Pseudomonas*, whereas PGL enhanced the activity of ceftriaxone, imipenem, and augmentin against all tested strains. The results are relevant and could be exploited in rejuvenating the old antibiotics which have lost their efficacy due to resistance. *P. guajava* methanol and aqueous extracts synergized the efficacy of various common antibiotics against drug-resistant Salmonella bacteria, according to recent research by Ngwanguong et al. [31]. This shows that the phytochemicals contained in P. guajava are potent antibiotic-resistance breakers that should be studied further. PGB produced a significant decrease in the bacterial count of *P. aeruginosa* PA124 over 20 hours of exposure at 0.5×MIC, MIC, and 2×MIC; an extended latent phase was also recorded (Figure 2). This demonstrates PGB's ability to suppress *Pseudomonas* growth. The constituents of PGB may act by preventing the bacteria from effectively using the nutrients of the milieu, probably by inhibiting some key digestive or metabolic enzymes produced by bacteria. A significant increase of OD at 260 nm was observed when PA124 was exposed to PGB, better effects were recorded at MIC and 2×MIC. This suggests the disturbance of cell membrane composition and alteration of cell membrane integrity induced by PGB, resulting in cell membrane leakage and release of intracellular constituents (DNA, RNA). Another noteworthy effect of PGB was registered on H^+-ATPase-mediated proton pumps of PA124, with a significant reduction of the pH as compared with the control; marked effects were obtained at MIC and 2×MIC (Figure 3). This shows that PGB may be an effective bacterial proton pump inhibitor. *P. guajava*'s antibacterial activity is attributed to its rich and diverse bioactive secondary metabolites. The reported chemical composition includes compounds such as tannins, phenols, flavonoids, saponins, carbohydrates, alkaloids, sterols, and terpenoids [71]. Some major *P. guajava* constituents comprising oleanolic acid, guaijavarin, quercetin, and flavonoids are well-established antibacterial phytochemicals [19, 32, 72]. The flavonoid compounds and their derivatives isolated from *P. guajava* have been reported to inhibit the growth of different bacteria in different dilutions [20]. Terpinene and pinene found in the aqueous extract of *P. guajava*'s leaves showed antimicrobial activity [32].

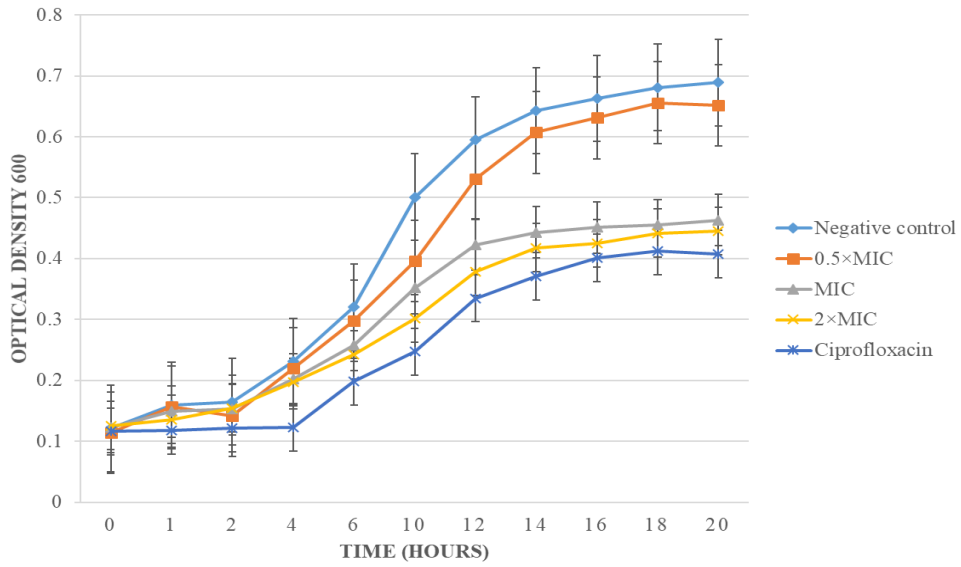

Figure 1. Effect of *Psidium guajava* (bark) extract on the kinetic growth of *P. aeruginosa* PA124

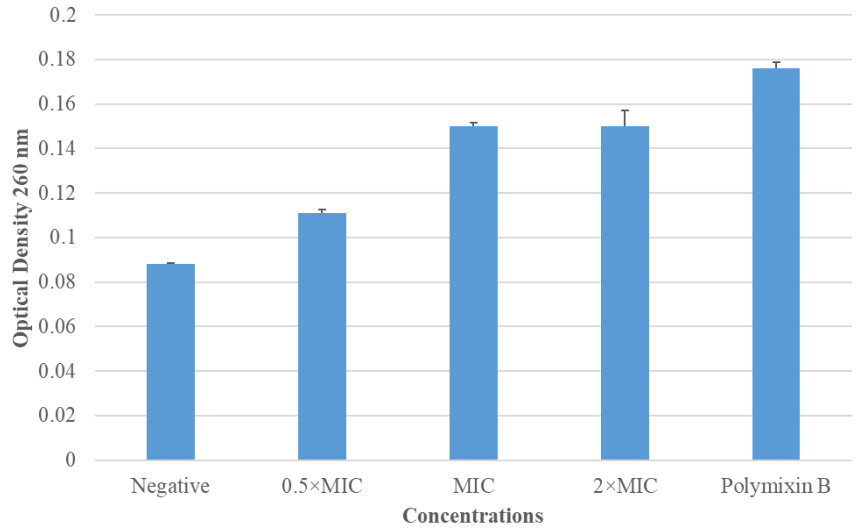

Figure 2. Effect of *Psidium guajava* (bark) extract on the cell membrane of *P. aeruginosa* PA124

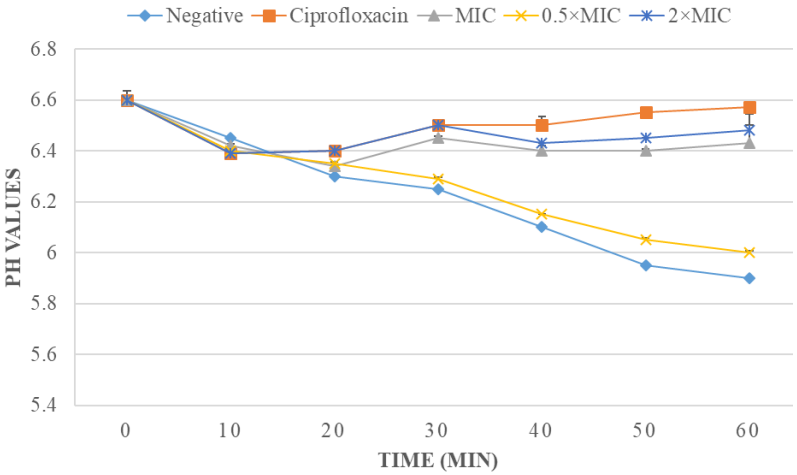

Figure 3. Effect of *Psidium guajava* (bark) extract on H^+-ATPase-mediated proton pumps of *P. aeruginosa* PA124

Table 1. Antipseudomonal activity (MIC and MBC) of *P. guajava* leaf and bark methanol extracts (in µg/mL).

Bacterial strains	*Psidium guajava*						Chloramphenicol		
	Leaves			Bark					
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
PA124	256	512	2	128	256	2	256	>256	nd
PA01	1024	1024	1	256	512	2	256	>256	nd
P081	512	2048	4	512	1024	2	128	>256	nd
P61	256	1024	4	256	512	2	256	>256	nd
P120	256	1024	4	256	512	2	128	>256	nd
P124	256	512	2	**64**	512	8	128	>256	nd
P57	128	256	2	**64**	512	8	128	>256	nd
P29	256	2048	4	**64**	512	8	128	>256	nd
P2	512	1024	2	256	512	2	128	>256	nd
P121	1024	1024	1	256	512	2	128	>256	nd
P97	128	2048	16	256	1024	4	128	>256	nd
P060	256	1024	4	256	1024	4	128	>256	nd
P21	512	1024	2	256	512	2	128	>256	nd
P1	256	1024	4	256	1024	4	128	>256	nd

MIC: minimal inhibitory concentration (in µg/mL); MBC: minimal bactericidal concentration (in µg/mL); R: MBC/MIC ratio; nd: not determined

Table 2. Influence of PAβN (efflux pump inhibitor) on the efficacy of *P. guajava* leaf and bark methanol extracts against selected MDR *P. aeruginosa.*

Bacterial strains	*P. guajava*						Chloramphenicol		
	Leaves			Bark					
	MIC alone	+PAβN	R	MIC alone	+PAβN	R	MIC alone	+PAβN	R
PA124	256	<8	**>32**	128	<8	**>16**	256	<8	**>32**
PA01	1024	<8	**>128**	256	<8	**>32**	256	128	**2**
P97	128	>8	**>16**	256	<8	**>32**	128	16	**8**
P21	512	<8	**>64**	256	<8	**>32**	128	<8	**>16**
P081	512	<8	**>64**	512	<8	**>64**	128	128	**1**
P121	1024	<8	**>128**	256	<8	**>32**	128	<8	**>16**
P124	256	128	**2**	64	<8	**>8**	128	64	**2**
P1	256	128	**2**	256	<8	**>32**	128	<8	**>16**

R: Ratio of MIC (+PAβN)/MIC (alone); MIC: minimal inhibitory concentration (in µg/mL); Values in bold: improved activity.

Table 3. Efficacy of antibiotics in combination with *Psidium guajava* extracts at MIC/2 and MIC/4 towards selected MDR *P. aeruginosa.*

Bacterial strains	MIC(µg/mL)	*Psidium guajava* extracts			
		Bark		Leaves	
	0	MIC/2	MIC/4	MIC/2	MIC/4
Penicillin					
PA01	16	4(**4**)	4(**4**)	16(1)	16(1)
P081	64	4(**16**)	4(**16**)	8(**8**)	8(**8**)
P124	32	4(**8**)	4(**8**)	16(**2**)	16(**2**)
P21	64	4(**16**)	4(**16**)	4(**16**)	4(**16**)
P1	64	2(**32**)	2(**32**)	8(**8**)	8(**8**)
P121	32	8(**4**)	8(**4**)	8(**4**)	8(**4**)
P97	32	4(**8**)	4(**8**)	4(**8**)	8(**4**)
Ceftriaxone					
PA01	32	2(**16**)	2(**16**)	16(**2**)	16(**2**)
P081	16	4(**4**)	4(**4**)	8(**2**)	8(**2**)
P124	64	16(**4**)	16(**4**)	32(**2**)	32(**2**)
P21	32	4(**8**)	4(**8**)	4(**8**)	4(**8**)
P1	32	4(**4**)	16(**2**)	16(**2**)	16(**2**)
P121	32	4(**4**)	4(**4**)	4(**8**)	4(**8**)
P97	64	8(**4**)	8(**4**)	32(**2**)	32(**2**)
Cefixime					
PA01	256	128(**2**)	128(**2**)	128(**2**)	256(1)
P081	128	4(**32**)	4(**32**)	4(**32**)	4(**32**)
P124	256	128(**2**)	128(**2**)	256(1)	256(1)
P21	64	8(**8**)	16(**4**)	8(**8**)	8(**8**)
P1	128	32(**4**)	32(**4**)	128(1)	128(1)
P121	128	32(**4**)	32(**4**)	64(**2**)	64(**2**)
P 97	128	32(**4**)	32(**4**)	64(**2**)	64(**2**)
Doxycycline					
PA01	8	4(**2**)	8(**2**)	16(0.5)	8(1)
P081	32	2(**16**)	2(**16**)	4(**8**)	2(**16**)
P124	16	4(**4**)	4(**4**)	16(1)	4(**4**)
P21	32	8(**4**)	8(**4**)	8(**4**)	8(**4**)
P1	8	0.5(**16**)	0.5(**16**)	1(**8**)	0.5(**16**)
P121	8	0.5(**16**)	0.5(**16**)	0.5(**16**)	0.5(**16**)
P97	32	4(**8**)	4(**8**)	8(**4**)	4(**8**)
Tetracycline					
PA01	32	8(**4**)	8(**4**)	32(1)	32(1)
P081	8	0.25(**32**)	0.25(**32**)	0.5(**16**)	0.5(**16**)
P124	32	4(**8**)	4(**8**)	16(**2**)	16(**2**)
P21	32	16(**2**)	16(**2**)	32(**2**)	32(1)
P1	8	2(**4**)	4(**8**)	8(**2**)	8(**2**)
P121	8	1(**8**)	1(**8**)	2(**4**)	2(**4**)
P97	16	4(**4**)	8(**2**)	8(**2**)	8(**2**)

Table 3. *continued and end.*

Bacterial strains	MIC(µg/mL)	*Psidium guajava*			
		Bark		Leaves	
	0	MIC/2	MIC/4	MIC/2	MIC/4
Chloramphenicol					
PA01	128	64(**4**)	64(**4**)	128(1)	128(1)
P081	256	32(**8**)	32(**8**)	32(**8**)	32(**8**)
P124	256	64(**4**)	64(**4**)	128(**2**)	128(**2**)
P21	256	128(**2**)	128(**2**)	256(1)	256(1)
P1	128	16(**8**)	16(**8**)	128(1)	128(1)
P121	128	16(**8**)	16(**8**)	16(**8**)	32(**8**)
P97	256	64(**4**)	64(**4**)	128(**2**)	128(**2**)
Levofloxacin					
PA01	8	2(**4**)	2(**4**)	8(1)	8(1)
P081	4	0.5(**8**)	0.5(**8**)	0.5(**8**)	0.5(**8**)
P124	4	1(**4**)	1(**4**)	4(1)	4(1)
P21	4	0.5(**8**)	0.5(**8**)	1(**4**)	1(**4**)
P1	8	1(**8**)	1(**8**)	2(**4**)	2(**4**)
P121	8	2(**4**)	2(**4**)	2(**4**)	2(**4**)
P97	4	1(**4**)	1(**4**)	1(**4**)	1(**4**)
Ampicillin					
PA01	32	8(**4**)	8(**4**)	16(**2**)	16(**2**)
P081	32	2(**16**)	2(**16**)	4(**8**)	16(**2**)
P124	128	8(**16**)	8(**16**)	32(**4**)	32(**4**)
P21	32	4(**8**)	4(**8**)	8(**4**)	8(**4**)
P1	64	32(**2**)	32(**2**)	64(1)	64(1)
P121	128	8(**32**)	8(**32**)	16(**8**)	16(**8**)
P97	64	8(**8**)	8(**8**)	16(**4**)	16(**4**)
Imipenem					
PA01	64	8(**4**)	8(**4**)	8(**8**)	16(**4**)
P081	32	2(**16**)	2(**16**)	2(**16**)	2(**16**)
P124	16	2(**8**)	2(**8**)	8(**2**)	8(**2**)
P21	16	4(**4**)	4(**4**)	8(**2**)	8(**2**)
P1	32	4(**8**)	4(**8**)	32(**4**)	32(**4**)
P121	32	2(**16**)	2(**16**)	4(**8**)	8(**4**)
P97	32	1(**32**)	1(**32**)	4(**8**)	8(**4**)
Augmentin					
PA01	32	2(**16**)	2(**16**)	2(**4**)	2(**16**)
P081	32	2(**16**)	2(**16**)	2(**16**)	2(**16**)
P124	16	1(**16**)	1(**16**)	4(**4**)	4(**4**)
P21	8	2(**4**)	2(**4**)	2(**4**)	2(**4**)
P1	32	2(**16**)	2(**16**)	8(**4**)	16(**2**)
P121	32	8(**4**)	8(**4**)	16(**2**)	16(**2**)
P97	32	0.5(**64**)	0.5(**64**)	1(**32**)	1(**32**)

MIC: minimal inhibitory concentration (in µg/mL); (): antibiotic-resistance modulating factors (AMF); Values in bold: improved efficacy of antibiotics.

Conclusion

The current study established the noteworthy antipseudomonal potential of *Psidium guajava* leaf (PGL) and back (PGB) methanolic extracts against multidrug-resistant phenotypes over-expressing efflux pumps. The bark extract (PGB) was determined to be the most active. Interestingly, both plant parts displayed outstanding antibiotic-resistance reversal potential towards MDR *P. aeruginosa* tested, as evidenced by improved efficacy of standard antibiotics once in combination. PGB is most likely acting by affecting bacterial cell membrane integrity and blocking proton pumps. More research is needed to uncover specific antipseudomonal phytochemicals from *P. guajava*, as well as the combination's mechanisms of action, in addition to in vivo testing and toxicity.

Abbreviations

AMF, antibiotic-resistance modulating factor; DMSO, dimethylsulfoxide, HNC, Cameroon national herbarium; INT, para-Iodonitrotetrazolium chloride; MDR, multidrug-resistant; PAβN, phenylalanine arginine beta naphthylamide; MBC, minimal bactericidal concentrations; MHA, Mueller Hinton Agar; MHB, Mueller Hinton Broth; MIC, minimum inhibitory concentrations.

Authors' Contribution

RST, MYV, PN, BENW, and MGF carried out the study; AJS analyzed data and wrote the manuscript; ATM and VK supervised the study; All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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