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Research Article

PUNICA GRANATUM RIND EXTRACT: ANTIBIOTIC POTENTIATOR AND EFFLUX PUMP INHIBITOR OF MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE* CLINICAL ISOLATES

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ABSTRACT

Objective: With a rise in multidrug resistant (MDR) bacterial isolates, search for antibiotics or compounds that could act synergistically with them is a significant area of research. Efflux-mediated resistance, in particular, is a great hurdle that needs to be overcome. In an effort to identify such synergistic compounds and potential efflux pump inhibitors (EPI), we analyzed the rind of *Punica granatum* (pomegranate) against MDR clinical *Klebsiella pneumoniae* isolates.

Methods: Sequential fractionation of *P. granatum* rind ethanol (PGR) extract was carried out to obtain hexane, butanol and water fractions. Antibacterial activity of the plant extracts was confirmed, and synergistic interaction with antibiotics was determined by the checkerboard assay. Gas chromatography-mass spectrometry (GC-MS) analysis was performed to identify the phytochemical constituents of the hexane extract. To study EPI activity of the extracts, norfloxacin accumulation assay was carried out.

Results: PGR ethanol extract was found to have synergistic activity with ciprofloxacin, levofloxacin, ceftazidime, cefoxitin, meropenem, and gentamicin resulting in fold decrease of minimum inhibitory concentration (MIC) ranging from 2 to 32 fold. The hexane fraction was found to have maximum synergistic activity resulting in a 32-fold reduction of ciprofloxacin MIC followed by butanol and water fractions. The PGR ethanol extract was also found to have efflux inhibition activity by the norfloxacin accumulation assay. Of the sequential fractions, the butanol fraction had maximum efflux inhibition activity.

Conclusion: Therefore, our study shows that PGR extract can potentiate the effect of antibiotics on MDR bacteria, and the mode of action is likely to be due to EPI.

Keywords: *Punica granatum* rind, Pomegranate, Synergy with antibiotics, Multidrug resistant, *Klebsiella pneumoniae*, Efflux pump inhibition.

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INTRODUCTION

Multidrug resistance (MDR) is increasing worldwide at an alarming rate [1]. To combat this, stronger antibiotics in higher dosages are prescribed, thereby resulting in a vicious cycle of further resistance. *Klebsiella pneumoniae*, an opportunistic pathogen, causes numerous diseases like *Pneumonia*, bloodstream infections, wound or surgical site infections, urinary tract infections and meningitis; with nosocomial infections, they possess a threat to patients with low immunity [2]. Their multidrug resistant nature poses serious healthcare issues. One of the reasons for high resistance is attributed to high efflux pump activity [3]. Efflux pumps are transport proteins that extrude toxic substances including antibiotics from the bacterial cells thereby resulting in resistance [4]. Efflux pump inhibitors (EPI) have a potential use in making antibiotics more effective against MDR strains [5]. However, the well-known EPIs-carbonyl cyanide m-chlorophenylhydrazone (CCCP), PAβN, reserpine, berberine, and verapamil [6] are toxic in nature and cannot be used commercially. Therefore, we focused on identifying EPI from natural products.

Punica granatum (pomegranate) has historically been known to have medicinal properties and has been used in herbal medicine to treat cancer, diarrhea, diabetes, blood pressure, leprosy, dysentery, hemorrhages, bronchitis, dyspepsia, and inflammation. It is also known to have antibacterial, antifungal, and anticancer activity [7]. While the antimicrobial activity of *P. granatum* pericarp (peel and rind) has been demonstrated [8,9] and its mode of action still remains unknown.

In this study, we analyzed the synergistic activity of *P. granatum* rind (PGR) extract with various classes of antibiotics against clinical MDR isolates of *K. pneumoniae* using the checkerboard assay [10]. Further, we confirmed the activity by sequential fractionation of the extract. The hexane extract which showed the best synergistic interaction was studied by gas chromatography-mass spectrometry (GC-MS) and potential compounds were identified. The ethanol extract was also shown to increase the intracellular norfloxacin accumulation, suggesting that it could be an EPI. When the sequential fraction was tested, it was found that the butanol fraction had maximum efflux inhibition activity. The identification of such EPI from natural products can lead to antibiotic combinations which are more effective against MDR isolates.

METHODS

General

The following chemicals were obtained from Sigma-Aldrich: Ciprofloxacin, norfloxacin, CCCP, magnesium sulfate, glycine hydrochloride, potassium l-lactate, and trizma hydrochloride. Resazurin, sodium hydroxide, Mueller-Hinton broth and agar, Luria Bertani broth and agar, Nutrient broth and agar and dimethyl sulfoxide were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Solvents-Ethanol, n-hexane, and n-butanol were obtained from Qualigens.

Bacterial isolates

About 22 MDR isolates were collected from tertiary care hospitals in Chennai. MDR nature of these isolates was clarified by

antibiotic susceptibility testing using the antibiotics norfloxacin (NX-10 µg), nalidixic acid (NA-30 µg), ciprofloxacin (CF-5 µg), amoxyclav (AMC-30 µg), cefotaxime (CTX-30 µg) cefepime (CPM-30 µg), cefoxitin (Cn-30 µg), and imipenem (IMP-10 µg) from (Himedia Laboratories Pvt. Ltd., Mumbai, India). The results were interpreted as per the EUCAST guidelines. *K. pneumoniae* MTCC 432 was used as a control strain.

Plant material and extraction

The rind of *P. granatum* variety Bhagwa was obtained from M/S "Sam Agritech, Hyderabad." The product was aseptically separated and shade dried. The dried material (1.5 kg) was ground, sieved and macerated with ethanol in the ratio 1:3 and kept for 5 days with intermittent shaking. The extract was filtered with Whatman number 1 filter paper (125 mm) and collected. It was then placed in a rotary vacuum evaporator and concentrated under pressure to give 100 g of a viscous material. 90 g of this crude extract was then dissolved in water, and sequential extraction was carried out resulting in hexane, butanol and water fractions which were concentrated in rotary vacuum evaporator [11].

Antibacterial activity

The antibacterial activity was tested by determining the minimum inhibitory concentration (MIC) of the plant extract using a stock solution of the plant extract (50 mg/ml of 50% dimethyl sulfoxide [DMSO]) by the resazurin assay [12]. The experiment was done in triplicates and positive control (isolate +LB broth), negative control (LB broth), and solvent control (50%DMSO + isolate +LB broth) were maintained. To determine the MIC of ciprofloxacin, the same experimental procedure was followed with stock solution of ciprofloxacin (100 mg/ml) added to the first well instead of the plant extract.

Synergistic studies

Synergistic interaction with ciprofloxacin was determined by checkerboard assay [13], in which 36 different combinations of plant extract and ciprofloxacin are tested to determine the best combination. The interaction between the antibiotics and plant extract was determined and quantified by calculating the fractional inhibitory concentration index (FICI) using the following formula:

$$\text{FIC index} = \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}} + \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

Interpretation of the FICI [13]: FICI = 0.5 synergy - joint effect is greater than sum of individual activity. FICI >0.5 to 4 indifference - joint effect is equal to sum of individual activity. FICI >4 antagonism - joint effect is less than sum of individual activity or effect of individual activity.

GC-MS analysis

GC-MS analysis of the PGR hexane extract was carried out using an AGILENT (Hewlett Packard) GC 5890, MS 5973 system. The following conditions were maintained for GC analysis. A HP-5 ms Agilent column was used (30 × 0.25 µm ID × 0.25 µmdf). Helium gas 99.9995% purity was used as a carrier with an injection volume of 1 µl (split ratio 20) and a flow rate of 1 ml/minute. The injector temperature was maintained at 250°C, and the oven temperature was programed at 35°C (for 2 minutes), with an increase to rate 15, 280°C for 5 minutes, lasting for a total of 35 minutes. Mass spectra were taken at 70 eV with a scan range 40-700 m/z. the solvent cut time was 1 minute, scan speed was 2000 and the total GC/MS running time was 36 minutes. Peaks were identified according to the MS library- NIST 11

Norfloxacin accumulation assay

Efflux inhibition activity was determined by the norfloxacin accumulation assay [14]. In this assay, the norfloxacin accumulation is determined by the fluorescence of supernatants measured as EX (excitation) at 281 nm and EM (emission) at 440 nm with a Shimadzu RF-5301pc fluorescence spectrophotometer. The experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Synergistic interaction between PGR ethanol extract and ciprofloxacin

The antimicrobial activity of PGR extract is known but the mode of action is unknown. To identify the mode of action, we analyzed the synergistic activity of the ethanol extract with antibiotics. As fluoroquinolone resistance is normally associated with nosocomial *K. pneumoniae* infections [15] and efflux-mediated resistance [16], ciprofloxacin-resistant strains were chosen for our study.

Our first step was to determine the ciprofloxacin resistance of the 22 clinical *K. pneumoniae* strains by determining MIC. As per the EUCAST guidelines, the ciprofloxacin breakpoint MIC is 1 µg/ml. 20 of the *K. pneumoniae* strains varied from high resistance (780 µg/ml) to less resistance (12 µg/ml) while two of them were found to be ciprofloxacin sensitive (Table 1). The antibacterial activity of PGR ethanol extract was determined by the MIC and was found to range from 25000 to 6250 µg/ml. From this, we infer that on its own, a high amount of plant extract is required to inhibit bacteria. It was also noted that, even for the two strains which were ciprofloxacin sensitive, a high amount of PGR extract was required to inhibit the bacteria (Table 1). The control strain *K. pneumoniae* MTCC 432 was ciprofloxacin sensitive, and the MIC of PGR ethanol extract was 195 µg/ml.

We then tested for synergy between PGR ethanol extract and ciprofloxacin by the checkerboard assay [10,13]. 36 different combinations were tested and the optimum combination of ciprofloxacin and plant extract concentration was determined. Based on an FICI, the combinations were classified as synergistic, antagonistic, and indifferent.

In 13 of the 20 strains, synergistic activity of PGR and ciprofloxacin was observed. In four of these strains, a 32-34 fold decrease in ciprofloxacin MIC was observed when in the presence of the PGR ethanol extract. Seven of the strains showed an 8-24 fold decrease and only 2 strains showed a 2-4 fold reduction of MIC. One of the strains K35 showed a 24-fold reduction of ciprofloxacin MIC in the presence of PGR making it a sensitive strain (MIC=0.5 µg/ml after combining with ethanol extract). These data show that the addition of PGR ethanol extract can reduce the ciprofloxacin MIC in *K. pneumoniae* isolates.

Further, despite the fact that seven strains out of 20 were classified as indifferent; a fold reduction was found in the MIC of ciprofloxacin in the presence of PGR. Of these, three strains showed an 8-fold reduction, three showed a 4-fold reduction and one showed no change.

As the ethanol extract of PGR had very good synergistic activity with ciprofloxacin (up to 34-fold reduction of MIC and re-sensitization of a resistant strain), we decided to determine whether the synergism of PGR extract was specific to ciprofloxacin or if it could interact with other antibiotics as well.

Synergistic interaction with other classes of antibiotics

Once it was determined that the PGR ethanol extract worked synergistically with ciprofloxacin and resulted in a reduction of the antibiotic MIC, we decided to study its interaction with other antibiotics.

The synergistic interaction with other classes of antibiotics such as cephalosporins, β-lactams, and aminoglycosides was determined for a representative *K. pneumoniae* strain U25 which showed a 16-fold reduction in MIC of ciprofloxacin and is known to have high efflux activity [17].

Out of the 12 antibiotics tested, 6 of them - ciprofloxacin, levofloxacin, ceftazidime, cefoxitin, meropenem and gentamicin, exhibited synergism with the ethanol extract for the strain U25 (Table 2). Ciprofloxacin showed maximum synergism with the ethanol extract showing a

16 fold reduction, followed by cefoxitin and meropenem showing a 12 and 8 fold reduction, respectively. Levofloxacin, gentamicin, and ceftazidime were found to decrease 4-fold in combination with ethanol extract.

In the 6 antibiotics in which indifference was noted, a 2-4 fold reduction of antibiotic was noted except for oxacillin where a 32-fold reduction was noted. In the case of oxacillin, despite the fold reduction being so high, it is classified as indifferent as the highest concentration of plant extract is used.

This shows that while the PGR ethanol extract shows synergy over a broad spectrum of antibiotics, the highest reduction of MIC was noted in combination with ciprofloxacin.

This supports the fact that the PGR ethanol extract could have potential EPI activity.

As our objective is to identify compounds with efflux inhibition activity, we sequentially fractionated the ethanol extract with low polar hexane and highly polar butanol in a hope to extract single compounds.

Synergistic extraction with the sequential plant fractions

Once the synergistic activity of the PGR ethanol extract with ciprofloxacin was determined, sequential hexane, butanol and water fractions were obtained and were found to have antibacterial activity (data not shown). The checkerboard assay was carried out to determine if the sequential fractions as well exhibited synergism with ciprofloxacin. Four representative strains were chosen for this assay, two in which, ethanol extract-antibiotic synergism activity was found (U25, K15) and two in which indifference was noted (K21, K37).

It was found that all of the sequential fractions-hexane, butanol and water, exhibited synergistic interaction with ciprofloxacin (Table 3). Even the strains K21 and K37 in which indifference was seen with the ethanol extract, synergism was seen with the sequential fractions and ciprofloxacin.

The hexane fraction showed maximum synergistic activity with ciprofloxacin. The strains U25 and K15 which showed a 16 fold MIC reduction with ethanol extract, exhibited a 32-fold reduction with hexane fraction. An 8-32 fold reduction was seen with the water fraction while the butanol fraction showed a 2-4 fold reduction.

Table 1: Synergistic interaction between *Punica granatum* rind ethanol extract and ciprofloxacin

Strain	MIC µg/ml			FIC	Interpretation
	Ciprofloxacin	PGR ethanol	Ciprofloxacin/PGR ethanol concentration		
K38	780	6250	390/390	0.56	Synergy
K14	625	25000	39/12500	0.56	Synergy
K13	625	25000	78/6250	0.37	Synergy
U25	390	12500	24/6250	0.56	Synergy
K21	390	25000	97.5/12500	0.75	Indifference
K3	390	25000	24/12500	0.56	Synergy
K39	390	6250	12/3125	0.53	Synergy
K31	312.5	12500	9.75/6250	0.53	Synergy
K32	312.5	12500	9.75/6250	0.53	Synergy
K15	312.5	25000	19.5/12500	0.56	Synergy
K12	312	12500	78/3125	0.50	Synergy
K9	312	12500	39/12500	1.13	Indifference
K36	156	6250	156/6250	2.00	Indifference
K29	156	12500	4.5/6250	0.53	Synergy
K11	78	25000	20/12500	0.76	Indifference
K34	78	12500	9.75/6250	0.63	Indifference
K40	45	6250	5.5/1560	0.37	Synergy
K28	39	12500	4.5/12500	1.12	Indifference
K37	12	6250	3.5/6250	1.29	Indifference
K35	12	25000	0.5/12500	0.54	Synergy
K10	S	25000	ND	ND	ND
K33	S	12500	ND	ND	ND
MTCC 432	S	195	ND	ND	ND

MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration where FIC of<0.5 is considered and>0.5 to 4 as indifference, PGR: *Punica granatum* rind, ND: Not determined, *P. granatum*: *Punica granatum*

Table 2: Synergistic interaction between *Punica granatum* rind ethanol extract and other antibiotics for the strain U25

Antibiotic	MIC µg/ml			FIC	Interpretation
	AB MIC for U25	PGR ethanol	Antibiotic/PGR ethanol concentration		
Ciprofloxacin	390	12500	24/6250	0.56	Synergy
Nalidixic acid	6250	12500	3125/6250	1.00	Indifference
Levofloxacin	390	12500	97.5/1560	0.37	Synergy
Norfloxacin	6250	12500	3125/3125	0.75	Indifference
Chloramphenicol	780	12500	195/6250	0.75	Indifference
Ceftazidime	25000	12500	6250/3125	0.50	Synergy
Cefotaxime	25000	12500	12500/3000	0.74	Indifference
Cefoxitin	625	12500	50/6250	0.58	Synergy
Meropenem	62.5	12500	7.8/1560	0.25	Synergy
Oxacillin	50000	12500	1560/12500	1.03	Indifference
Cloxacillin	25000	12500	12500/3125	0.75	Indifference
Gentamicin	12500	12500	3125/1560	0.37	Synergy

P. granatum: *Punica granatum*, MIC: Minimum inhibitory concentration, AB: Antibiotic, FIC: Fractional inhibitory concentration where FIC of≤0.5 is considered and>0.5 to 4 as indifference, PGR: *Punica granatum* rind

The strains K21 and K37 in which indifference was seen with the ethanol extract and showed synergy with all three solvent fraction resulting in a 2-4 fold reduction of ciprofloxacin MIC. The strain K37 was even made sensitive with the butanol fraction - antibiotic combination. From this, we can infer that each of the fractions has synergistic activity, but the highest fold reduction of MIC is seen in the hexane fraction. To determine the active compounds that could play a role in the synergism, GC-MS of this fraction was carried out.

GC-MS analysis

GC-MS analysis plays a major role in identifying active compounds in plant extracts [18]. The phytochemical constituents of the hexane fraction which exhibited very good synergistic activity with ciprofloxacin were analyzed by GC-MS. Eight peaks of interest indicating phytochemical constituents were observed (Fig. 1). On comparison of the spectra with the NIST database, the compounds were identified (Table 4). The results showed that the major components were linoleic acid ethyl ester and hexadecanoic acid ethyl ester with an area % of 36.21 and 24.83, respectively. This is followed by ethyl 9-hexadecenoate (13.45%), n-hexadecanoic acid (7.79%), octadecanoic acid, ethyl ester (6.02%), eicosanoic acid, ethyl ester (1.31%), octadecanoic acid (1.11%), and nonadecanoic acid ethyl ester 0.94%.

These are fatty acids and their esters whose antimicrobial activity has been observed [19]. These fatty acids and their esters might be interacting with the bacterial membrane to enhance the susceptibility to antibiotics. To specifically find out if the PGR extracts had an effect of the efflux pumps, we tested the fractions by the norfloxacin accumulation assay [14].

EPI activity

The efflux inhibition activity of PGR ethanol extract and the hexane, butanol and water fraction was analyzed by the norfloxacin accumulation assay [14] using a representative *K. pneumoniae* isolate U25 which is known to have high efflux activity [17]. A well-known EPI CCCP that dissipates the proton gradient was used as a control [20]. In this assay, the amount of norfloxacin accumulated within the cell is estimated after the addition of an EPI. It was found that on addition of PGR ethanol extract a high intracellular accumulation of norfloxacin was seen. When compared to the standard CCCP, the PGR ethanol extract was found to lead to higher intracellular norfloxacin accumulation (Fig. 2).

When the sequential fractions were tested, it was found that addition of the butanol fraction resulted in higher norfloxacin accumulation. Despite the fact that the hexane fraction showed maximum synergistic activity, it was found that the butanol fraction had a much higher EPI activity. These data suggest that the components of PGR butanol fraction can inhibit the efflux pumps, and therefore, one or more components could be a potential EPI.

Studies have been carried out with the PGR extract by disc diffusion method and MIC [9 21] against MTCC strains (using 1 *K. pneumoniae* isolate) and methicillin resistant *Staphylococcus aureus*, respectively. Our report analyses the synergistic activity of PGR extract with antibiotics using the checkerboard method against 22 clinical isolates of MDR *K. pneumoniae.*

Studies have also found that *P. granatum* has the ability to inhibit NorA efflux pumps in *Staphylococcus aureus* [22]. Taken together with our data, it is likely that PGR butanol fraction inhibits the efflux pumps in *K. pneumoniae*. Further analysis of the compounds in the butanol fraction is in progress.

P. granatum tannins punicalagin, punicalin, and ellagic acid have been found to specifically block the HCV NS3/4A protease activity *in vitro* and have been found to be bioavailable [23].

Table 3: Synergistic interaction between ethanol extract, sequential fractions in hexane, butanol and water with ciprofloxacin

Culture	CIP	PGR extract	CIP+PGR butanol fraction	Activity	CIP+PGR hexane fraction	Activity MIC in µg/ml	CIP+PGR water fraction	Activity	CIP+PGR ethanol extract	Activity
U25	390	25000	97.5/1560	S	12/12500	S	48.5/780	S	24/6250	S
K15	312.5	25000	156/780	S	10/12500	S	10/12500	S	19.5/12500	S
K21	390	25000	97.5/6250	S	120/6250	S	156/3120	S	97.5/12500	I
K37	12	25000	S	S	6/1560	S	6/1560	S	3.5/6250	I

MIC: Minimum inhibitory concentration, CIP: Ciprofloxacin, PGR: *Punica granatum* rind, S: Synergy, I: Indifference, *P. granatum: Punica granatum*

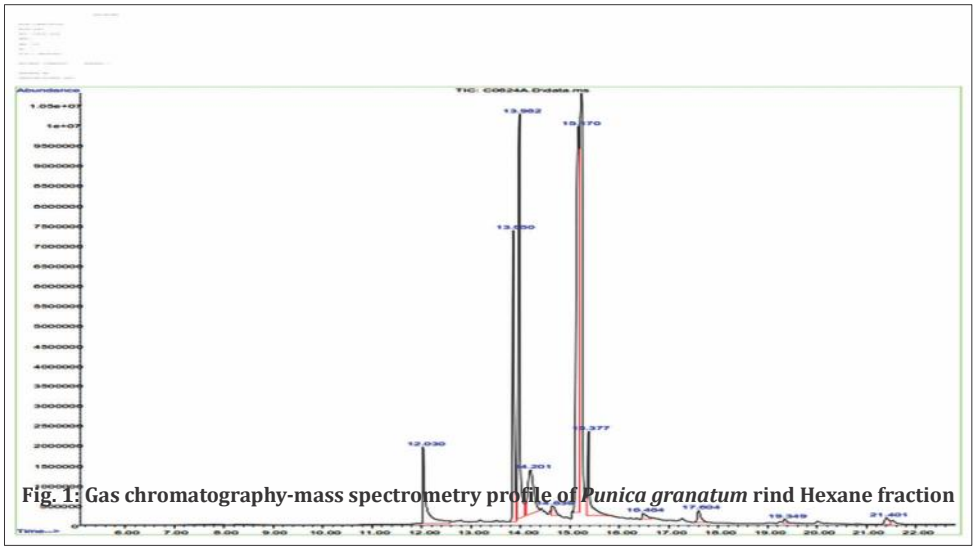

Fig. 1: Gas chromatography-mass spectrometry profile of *Punica granatum* rind Hexane fraction

Table 4: Compounds from PGR hexane fraction identified by GC-MS analysis

Retention time	Name of compound	Area %
13.85	Ethyl 9-hexadecenoate	13.45
13.982	Hexadecanoic acid, ethyl ester	24.83
14.201	n-Hexadecanoic acid	7.79
14.639	Octadecanoic acid	1.11
15.17	Linoleic acid ethyl ester	36.21
15.377	Octadecanoic acid, ethyl ester	6.02
17.604	Eicosanoic acid, ethyl ester	1.31
21.401	Nonadecanoic acid, ethyl ester	0.94

PGR: *Punica granatum* rind, GC-MS: Gas chromatography-mass spectrometry

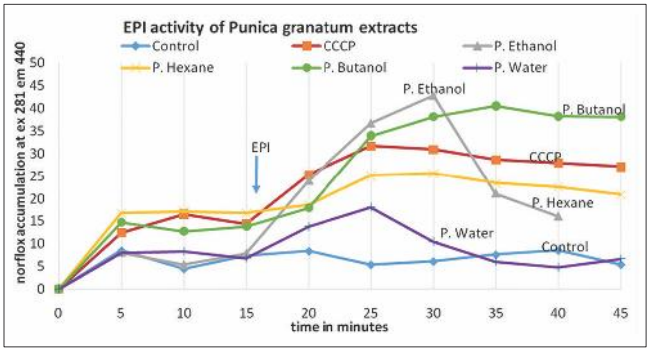

Fig. 2: Efflux inhibition activity of *Punica granatum* rind ethanol extract and sequential fractions

Our study shows that PGR extracts have synergistic activity with ciprofloxacin and the butanol soluble fraction from PGR specifically inhibits efflux pump activity. Analyzing leads from traditional knowledge to identify compounds that can be used as adjuvant for antimicrobial therapy is an important field of research. The use of such synergistic compounds can re-sensitize resistant isolates and make antimicrobial therapy more efficient.

CONCLUSION

In this report, we show that PGR ethanol extract exhibits a consistent reduction of antibiotic MIC. Even in the strains that the plant-antibiotic interaction was classified as indifferent, a fold reduction of the antibiotic is still seen. This synergistic interaction was seen with a broad spectrum of antibiotics such as ciprofloxacin, levofloxacin, ceftazidime, cefoxitin, meropenem, and gentamicin. To identify the individual compounds with EPI activity, sequential extraction of the PGR ethanol extract was done. Of the sequential fractions, the hexane fraction was found to have the most synergistic activity with ciprofloxacin, resulting in a 32-fold reduction of MIC. The mode of action of PGR ethanol and sequential fractions were also studied and it was found that addition of PGR extract results in increased intracellular norfloxacin accumulation. In this case, it was found that the PGR butanol fraction had the best EPI activity. This indicates one or more components of the PGR butanol fraction could be a potential EPI.

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