Antimicrobial pomegranate rind extracts: enhancement by Cu(II) and vitamin C combinations against clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Recently, natural products have been further evaluated as sources of antimicrobial agents with efficacies against a variety of microorganisms. This study reports the antimicrobial activities of pomegranate rind extract (PRE) in combination with Fe(II) and Cu(II) salts against extended-spectrum multidrug-resistant *Pseudomonas aeruginosa*. Antimicrobial suspension assays were carried out using aqueous extract of pomegranate alone or in combination with metals salts against *P. aeruginosa*. The extract:metal salt combination was also enhanced with the addition of vitamin C. Marked activities were observed for the aqueous PRE/Cu(II) preparations, which were greatly enhanced by the addition of the reductant vitamin C. In contrast, the aqueous PRE/Fe(II) preparations were inactive, regardless of addition of vitamin C. The combination of PRE and Cu(II) salts and vitamin C showed the greatest activity against clinical isolates of *P. aeruginosa*. These results warrant further investigation of PRE as a potential source of new antimicrobial agents.

KEY WORDS: Antibacterial agents.
Ascorbic acid.
Copper.
Pomegranate.
Punicaceae.

Introduction

With the growth in incidence of reported cases of hospital-based drug-resistant microbial infections, an immediate demand exists for the development of new antimicrobial agents.[1–6] Within the clinical setting, alarming increases in rates of infection by drug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* are commonly reported.[7,8] However, infections with other drug-resistant organisms such as the pseudomonads continue. The total reported bacteraemia cases for all *Pseudomonas* spp. reported in England, Wales and Northern Ireland was 3272 in 2005.[9]

Recently, considerable research activity has focused on the identification of new antibacterial agents from botanicals.[10–13] A wide variety of species have been tested against microbial panels in both isolation and combination where synergistic effects are sought. A recent report of the activities of extracts of 15 medicinal plants against extended-spectrum β-lactamase (ESBL)-producing multidrug-resistant bacteria highlights the potential for this approach.[1]

Natural products such as pomegranates (*Punica granatum*) have also been used in combination with known antibiotics to achieve synergistic activities.[14] Furthermore, natural products have been shown to inhibit the efflux pumps in multidrug-resistant bacteria.[15]

Previous work carried out by the authors has demonstrated that PRE in combination with different metals, extensions of the incubation period and the addition of a stabiliser have shown an antibacterial effect against a range of laboratory strains of Gram-positive and -negative bacteria.[16] Pomegranate rind extract (PRE) with the addition of $CuSO_4$ reduced the level of three Gram-negative laboratory bacterial strains (*Pseudomonas aeruginosa, Proteus mirabilis* and *Escherichia coli*) from 10^8 to no detectable growth within 30 minutes.[16]

The aim of this study is to explore the known antimicrobial effects of combinations containing metal ions and PREs against clinical isolates of *Pseudomonas aeruginosa*.

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Materials and methods

Preparation of pomegranate rind extracts

Pomegranate rind extracts were prepared by cutting the rind into small cubes (approximately 5 mm^3) which were then dried at 55°C for 24 h. Dried rind was stored in air-tight containers in the dark until further use. Stock solutions were prepared by adding 10 g dry rind to 150-mL distilled water and shaking (at 80 rpm) at room temperature for 24 h.[17] The crude extract was passed through muslin and a Whatman filter No.1 to remove the particulate matter, and then filter-sterilised by passing through a 0.2 µm filter (Millipore) into a sterile bottle. The PRE stock solutions were stored at –20°C.

Bacterial cultures

Clinical isolates of *P. aeruginosa* were collected at the Royal Marsden Hospital (Sutton, UK) and were kindly provided by Miss Jackie Kenny. Upon arrival at Kingston University, the isolates were grown overnight on nutrient agar (Oxoid, UK) and then frozen in cryovials (Pro-Lab, UK) for future use.

Evaluation of effect of pomegranate rind extract and metal salts

All reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) and distilled water was used throughout. *P. aeruginosa* isolates were removed from the freezer and passaged on nutrient agar twice before use. Overnight cultures on nutrient agar were then suspended in Ringer's solution (Oxoid, UK) to a turbidity equivalent to 0.5 McFarland (1.5×10^8 colony-forming units [cfu]/mL). A sample of the PRE extract (330 µL) was added to 700 µL freshly prepared solutions (4.8 mmol/L) of metal salts ($FeSO_4$, $CuSO_4.5H_2O$). The final solution was protected from light.[18]

The appropriate bacterial dilution was prepared and 50 µL placed in a sterile Eppendorf microcentrifuge tube with 100 µL extract/metal salt solution. After exposure of the bacteria for 30 min at room temperature, the activity of the bactericidal agent was neutralised by adding an equal volume of 2% (v/v) Tween-80 (Sigma, UK) in Lambda buffer.[18] Serial dilutions were prepared in Ringer's solution, and 10-µL of each dilution was spotted on a nutrient agar plate and incubated for 24 h at 37°C. Each assay was conducted in triplicate.

Evaluation of pomegranate rind extract and metal salts, plus increasing concentration of vitamin C

The assay was carried out as described above but with the addition of vitamin C (Sigma, UK) to the metal ion solution immediately prior to mixing with the PRE. Aliquots of vitamin C were prepared to give final metal ion:vitamin C ratios (and vitamin C concentrations) of 1:1 (4.8 mmol/L), 1:5 (24 mmol/L) and 1:20 (96 mmol/L). A 700 µL aliquot of this solution was then added to PRE.[16]

Results

The antimicrobial effects of PRE and metal salts alone and in combination, plus additions of increasing concentrations of vitamin C, are shown in Figures 1a–1c. Nine multidrug-resistant clinical isolates of *P. aeruginosa* were subjected to challenge by PRE alone or in combination with metal ions and were assessed using the suspension test.

In the controls, PRE alone showed no significant effect and the Fe(II) and Cu(II) treatments resulted in only modest (approximately 10^1 $\log_{10}$) reduction in growth (mean values) (Fig. 1a). In contrast to the control Lambda buffer and PRE tests, the modest mean $\log_{10}$ reductions in growth observed for both metal ions were characterised by a wide range covering four and two log units for Fe(II) and Cu(II), respectively. The median and mean values were similar for Fe(II) and Cu(II) treatments.

In addition to the wide range, the variation seen between the mean (▲) and median (middle bar in box) values indicated a degree of heterogeneous response. In contrast, the relatively compact box height, indicating lower and upper quartiles, suggests some uniformity of effect on the majority of organisms tested.

A minor reduction in growth (approximately 10^1 $\log_{10}$) was observed following treatment with the PRE/Fe(II) combination compared to the Lambda and PRE controls (Figs. 1a and 1b). However, the effect of the PRE/Fe(II) combination was not significantly different to the Fe(II) control when expressed as the mean reduction in growth.

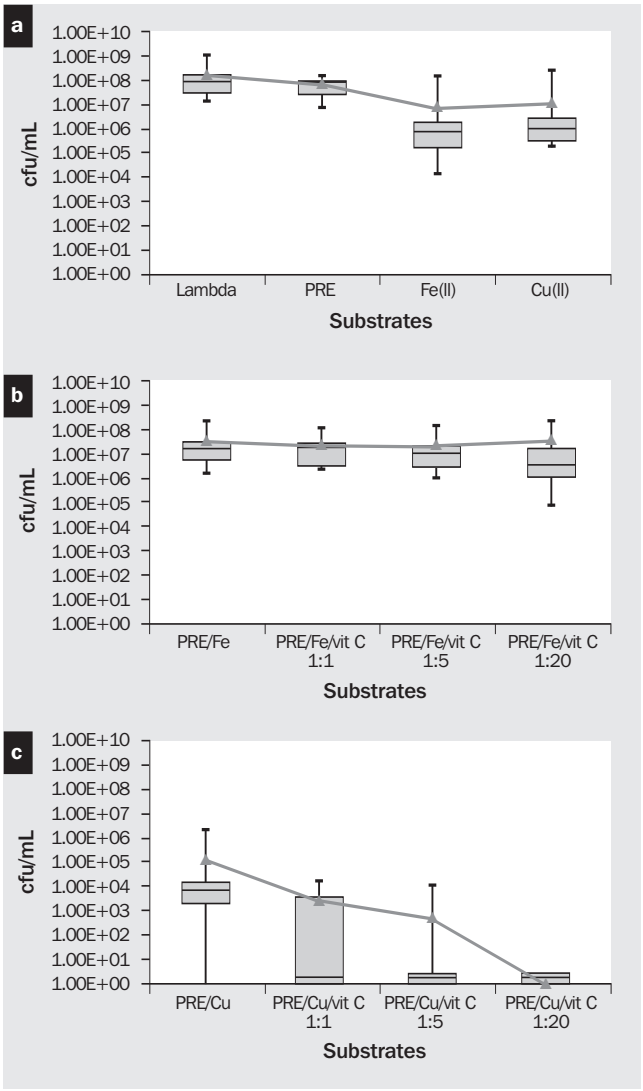

Fig. 1. Antimicrobial activities of a) PRE, Fe(II) and Cu(II) alone and in combination with b) PRE/Fe(II) or c) PRE/Cu(II) ions and vitamin C. Vitamin C was added to Cu(II) at a ratio of 1:1, 1:5 or 1:20 after a 30 minute incubation against nine clinical isolates of multidrug-resistant *Pseudomonas aeruginosa* using Lambda buffer as a control. Box represents 25% and 75% quartiles, bar represents median and error bars represent range. Mean cfu/mL value shown by ▲.

Addition of vitamin C had no effect on the mean, median or percentile values for activity with the PRE:Fe(II) combination.

In contrast, a moderate mean reduction in growth (approximately 10^2 $\log_{10}$) was observed following treatment with the PRE/Cu(II) combination compared to the Lambda and PRE controls (Figs. 1a and 1c). However, a large range is apparent between 10^6 $\log_{10}$ and no detectable growth and the median reduction is four $\log_{10}$ units.

Addition of vitamin C greatly enhanced the activity of the PRE/Cu(II) combination (Fig. 1c). Addition of one equivalent of vitamin C enhanced the mean antimicrobial activity of PRE/Cu(II) to below 10^4 $\log_{10}$ cfu/mL reductions, with the median and lower quartile demonstrating considerable antimicrobial activity.

Five equivalents of vitamin C afforded a mean reduction in growth of 10^5 $\log_{10}$ cfu/mL, with the upper quartile being

considerably reduced in comparison to the PRE/Cu/vitC 1:1 combination. No detectable growth was observed following the addition of 20 equivalents of vitamin C to the PRE/Cu combination.

Discussion

The antimicrobial effects of pomegranate extract have been well publicised,[17,19–21] and in recent years significant advances have been made which include reports of synergistic activity with antibiotics[14] and inhibition of enterotoxin release by *S. aureus*.[22] Enhancement of virucidal activity by addition of ferrous salts has yet to be fully explained in mechanistic terms.[18] As for antibiotics such as bleomycin, where ferrous ions generate oxidants, the requirement for enhancing pomegranate activity with ferrous ions may be through redox cycling at the metal centre. However, this is only one of several putative mechanisms currently under investigation.

This proposed oxidative damage mechanism, coupled to the instability of ferrous solutions, previously led the current authors to extend their studies to investigate other common redox-active metals found in biological systems.[16] This work investigated the combination of PRE with three other metal salts ($CuSO_4$, $MnSO_4$, ZnO) as well as $FeSO_4$ against a range of laboratory strains of both Gram-positive and Gram-negative bacteria. The findings of that work demonstrated that no detectable growth could be detected with any of the Gram-negative bacteria tested (*P. aeruginosa, Proteus mirabilis* and *E. coli*) after 30 minutes' incubation with the PRE/$CuSO_4$ combination. The combination of PRE with the other three metals salts demonstrated less antibacterial effect than was achieved with the metal salts alone.[16]

As shown in Figure 1b, and in line with the results obtained previously,[16,18] the PRE/Fe(II) system had negligible effect on the bacterial growth, with only modest retardation of growth occurring with Fe(II) treatment alone. In contrast, however, the PRE/Cu(II) combination exhibited an approximate 10^2 $\log_{10}$ mean reduction compared to Cu(II) treatment alone.

Following this result and in line with previous work,[16] further investigation involved addition of the reductant vitamin C to explore if the mechanism was attributable to redox cycling, as observed for bleomycin. The profound effects produced following addition of vitamin C indicated that reducing the metal ion may be paramount. In the aqueous preparations it is notable that this enhanced effect was only seen with the PRE/Cu(II) combination.

High levels of vitamin C did not activate the PRE/Fe(II) system, whereas complete retardation of growth was observed for the PRE/Cu(II) system following addition of 20 equivalents of vitamin C. These results suggest that the combination of PRE/Cu(II) and vitamin C may be a possible antimicrobial agent for treating the pseudomonads that are becoming increasingly resistant to currently available antibiotics.

In conclusion, the combination of aqueous solutions of PRE and Cu(II) salts show antimicrobial activity against pseudomonads, and the effect is further enhanced by the addition of vitamin C. This is the first report of the activation of a natural product by addition of a redox-active metal ion and the reductant vitamin C. Further investigations are underway to determine the mechanism of action of these novel antimicrobial compositions.

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