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**Original Article**

# In vitro combinations of antibiotics and phytochemicals against *Pseudomonas aeruginosa*

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**Background and purpose:** Antibiotic combinations are used to enhance antibacterial efficacy and to prevent the development of resistance. In this study, the in vitro activities of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa* were tested by the fractional inhibitory concentration method, derived from the minimal inhibitory concentrations (MICs) of the agents in combination.
**Methods:** The antimicrobial activity of phytochemicals, alone and in combination with antibiotics, was evaluated using the checkerboard assay and time-kill curve methods.
**Results:** There was synergism between gentamicin and caffeic acid, and sulfadiazine and the 3 phytochemicals under investigation (protocatechuic acid, quercetin, caffeic acid). The MIC of sulfadiazine was 256 μg/mL, and of gentamicin was 2 μg/mL. When gentamicin was combined with one-quarter the MIC of caffeic acid, the MIC of gentamicin was reduced 4-fold. When sulfadiazine was tested with one-quarter the MIC of protocatechuic acid, quercetin, and caffeic acid, the MIC was reduced 4-fold in combination with each of the drugs.
**Conclusions:** These results indicate the potential efficacy of phytochemicals in combination with antibiotics for enhancing total biological activity.

**Key words:** Caffeic acid; Combined modality therapy; Drug resistance; Protocatechuic acid; Quercetin

## Introduction

*Pseudomonas aeruginosa* is a highly drug-resistant and opportunistic pathogen. Due to the permeability barrier in the outer membrane it is naturally resistant to many antibiotics. Antimicrobial resistance in *P. aeruginosa* can be acquired by enzymatic inactivation, target alterations, or efflux pump inhibition [1]. The incidence of infections caused by *P. aeruginosa* is increasing, both in hospitals and in the community, and it has been reported as one of the principal causes of nosocomial infection, particularly among immunocompromised patients [2]. At the same time, the extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the emergence of multidrug-resistant bacteria [3,4]. Hence, the efficacy of many antibiotics for treatment of infections has become limited [5,6]. As the development of resistance to monotherapy is a common problem, dual antimicrobial coverage is often a necessity for *Pseudomonas* spp. infections [7], and attempts have been made to use combination therapy [8].

Several studies have investigated the interactions of antimicrobial combinations with multiresistant planktonic strains of *P. aeruginosa* [9-11]. Recently, Cernohorska and Votava demonstrated the in vitro effect of 8 antibiotic combinations on *P. aeruginosa* biofilms using biofilm susceptibility testing [12]. Earlier, Neu reviewed the data on combinations of fluoroquinolones with other antimicrobial agents against several bacteria, including *P. aeruginosa* [13]. Vancomycin in combination with cephalosporins and penicillins has been shown to synergistically inhibit a number of Gram-negative bacilli [14]. However, the threat from antimicrobial-resistant organisms is

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accumulating and accelerating [15]. With the dearth of new antibiotics becoming available and the advance of multidrug-resistant bacteria, it is not difficult to predict untreatable life-threatening bacterial infection becoming common [16]. Moreover, it is difficult to identify strategies to prevent or delay the emergence of resistance. Recently, Amyes et al discussed the issue of a good principle for antibiotic usage to limit resistance development [17]. To this end, there is a need to find new ways to control *P. aeruginosa* and continue the search for new antimicrobial compounds.

Plants have traditionally provided a source for novel drug compounds, as plant and herbal mixtures have made a contribution to human health and wellbeing [18]. Owing to the popular use of plants as remedies for many infectious diseases, the search for substances in plants with antimicrobial activity is common. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties [18,19]. The minimal inhibitory concentrations (MICs) of these plant antimicrobials are often reported to be between 100 and 1000 mg/L. Cai et al reviewed the antibacterial activity of allicin alone and in combination with β-lactam antibiotics against *Staphylococcus* spp. and *P. aeruginosa* [20]. Allicin is one of the most effective antibacterial compounds isolated from garlic. The diterpenes isolated by Batista et al have been reported to work synergistically against *Staphylococcus aureus, Vibrio cholerae*, and *P. aeruginosa* [21]. *P. aeruginosa*, which is considered to be multidrug resistant, has also been reported to have had its growth inhibited by extracts from clove, jambolan, pomegranate, and thyme [22].

Novel combinations of antibiotics and phytochemicals may provide a new therapeutic option for *P. aeruginosa* infections. Furthermore, the strategy of combining antibiotics with phytochemicals may have ramifications for the treatment of other multidrug-resistant organisms. The aim of this study was to evaluate the synergistic effects of the antibiotics gentamicin, levofloxacin, and sulfadiazine in combination with the phytochemicals protocatechuic acid, quercetin, and caffeic acid against *P. aeruginosa*.

## Methods

### Bacterial strain

*P. aeruginosa* American Type Culture Collection (ATCC) 15692 was used in this study. This strain showed no resistance and was susceptible to the control antibiotic chloramphenicol. Inoculum preparation was done by selecting 3 to 5 well-isolated individual colonies from an agar plate and the growth was transferred to a tube containing 4 to 5 mL of Iso-sensitest broth. The broth culture was incubated at 35°C for 2 to 6 h.

### Antibiotics and phytochemicals

Antibiotics and phytochemicals were obtained from Sigma-Aldrich, Inc. (Lenexa, KS, USA). Gentamicin 600 μg/mL, levofloxacin 98%, and sulfadiazine ≥99% were chosen for this analysis, as they have been used as antipseudomonal agents, either alone or in combination with other drugs. Protocatechuic acid 98%, quercetin ≥98%, and caffeic acid ≥98% were the phytochemicals used. Antibiotic stock solutions were prepared and dilutions made according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and the manufacturer's recommendations [23].

### Determination of minimal inhibitory concentrations

The MICs for the antibiotics and phytochemicals under study were determined in duplicate by the microbroth dilution method in Iso-sensitest broth, according to the CLSI methods [24]. The antibiotic concentrations ranged from 0.0125 to 128 μg/mL for gentamicin and levofloxacin and 8 to 8152 μg/mL for sulfadiazine, protocatechuic acid, quercetin, and caffeic acid. The final bacterial inoculum in each well was approximately $7.5 \times 10^5$ colony-forming units (CFUs)/mL. The microtiter plate was incubated at 35°C and read at 18 to 19 h for the optical density using a spectrophotometer at 600 nm.

### Checkerboard assay

The range of concentrations tested for each antimicrobial agent was 4- to 5-fold lower than the MIC and at least 2-fold higher than the MIC, if antagonism was suspected. Testing was performed using 96-well microtiter trays. MICs were determined for each drug by broth microdilution according to the National Committee for Clinical Laboratory Standards method. Synergism by the checkerboard method was defined as fractional inhibitory concentration (FIC) index of ≤0.5, indifference was defined as an FIC index of >0.5 to ≤4, and antagonism was defined as an FIC index of >4. Concentrations within the FIC panel were such that the MIC of each antibiotic was in the middle of the range of concentrations tested.

The FIC indices for all combinations were calculated using the formulae below. The FIC for a drug in a given well was derived by dividing the drug concentration in the given well by the control MIC of the test organism to that drug.

$$FIC_A = \frac{MIC_A \text{ combination}}{MIC_A \text{ alone}}$$

$$FIC_B = \frac{MIC_B \text{ combination}}{MIC_B \text{ alone}}$$

The FIC index for a well is the sum of the FICs for each of the drugs present in the well:

$$FIC_{index} = FIC_A + FIC_B$$

### Time-kill curves

The bactericidal activity was determined according to the CLSI protocol [24]. Viable cells were counted by performing serial dilutions and removing an aliquot at different time intervals. Antibiotics were tested at one-quarter the MIC for each isolate and the concentrations were incrementally increased until a maximal concentration of 4 x MIC was reached. At zero time the final inoculum was determined and samples were taken at 0, 4, 8, and 24 h of incubation at 35°C. All experiments were performed in triplicate.

Time-kill curves were plotted as $\log_{10}$ CFU/mL over 24 h. Synergism was defined as a decrease in colony count of ≥100 CFU/mL at 24 h for the combination compared with the count obtained for the most active single agent. Antagonism was defined as an increase in colony count of ≥100 CFU/mL at 24 h.

**Table 1.** Minimal inhibitory concentrations (MICs) of antimicrobials used against *Pseudomonas aeruginosa*.

| Antimicrobial | MIC (μg/mL) |
|---|---|
| Gentamicin | 2 |
| Levofloxacin | 1 |
| Sulfadiazine | 256 |
| Protocatechuic acid | 2000 |
| Quercetin | ≤500 |
| Caffeic acid | ≥250 |

## Results

### Minimal inhibitory concentrations

For the susceptibility range of *P. aeruginosa* evaluated for the 3 antibiotics and 3 phytochemicals, the MIC values were higher for the phytochemicals than for the antibiotics. The results are shown in Table 1 and the dose response of the antimicrobials is represented in Fig. 1.

### Combined drug effects

Protocatechuic acid, quercetin, and caffeic acid alone had limited inhibitory effects against *P. aeruginosa*. However, synergism was observed between gentamicin and caffeic acid, and sulfadiazine and the 3 phytochemicals. Indifference was observed for combinations of gentamicin and protocatechuic acid, gentamicin and quercetin, and levofloxacin and the phytochemicals. The combination effects of the antibiotics and phytochemicals are shown in Fig. 2.

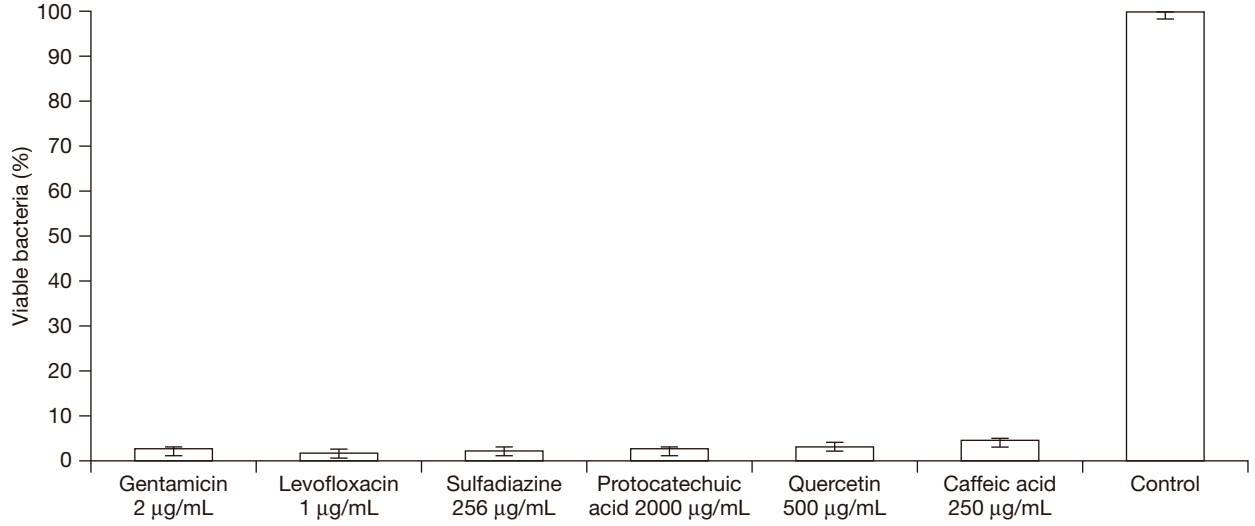


**Fig. 1.** Effect of select antimicrobials (dose, 1 × minimal inhibitory concentration) on *Pseudomonas aeruginosa*.

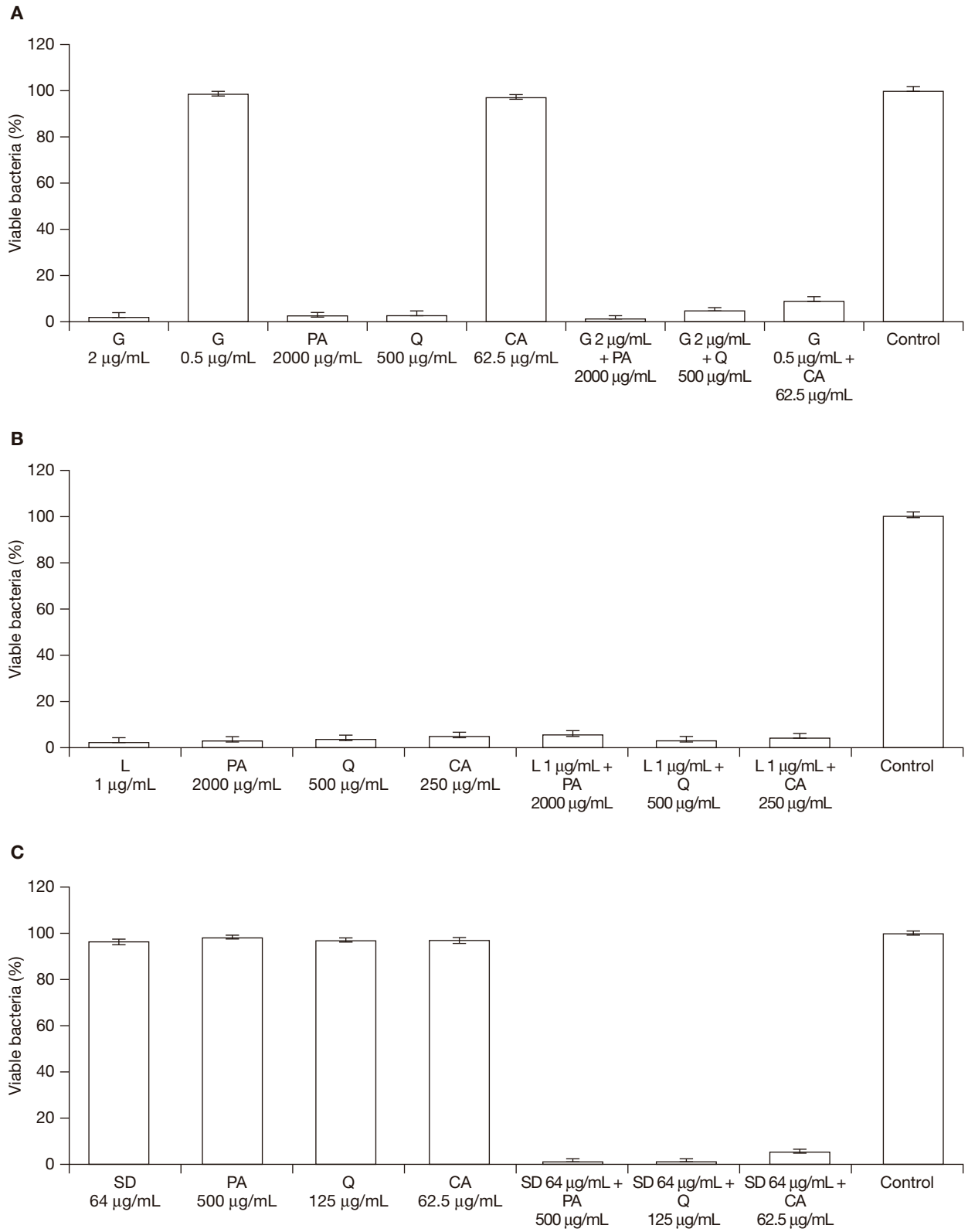


**Fig. 2.** Combination effects of antimicrobials against *Pseudomonas aeruginosa*. (A) Gentamicin and phytochemicals; (B) levofloxacin and phytochemicals; and (C) sulfadiazine and phytochemicals.
Abbreviations: G = gentamicin; PA = protocatechuic acid; Q = quercetin; CA = caffeic acid; L = levofloxacin; SD = sulfadiazine.

## Time-kill curves

The combination of sulfadiazine plus protocatechuic acid, and quercetin, caffeic acid, and gentamicin plus caffeic acid at one-quarter the MIC for the ATCC strain resulted in synergism, with a higher rate of killing for the first 2 to 4 h (Fig. 3). The kill rates for the combinations for the first 4 h were higher than those for any monotherapy against the chosen ATCC strain

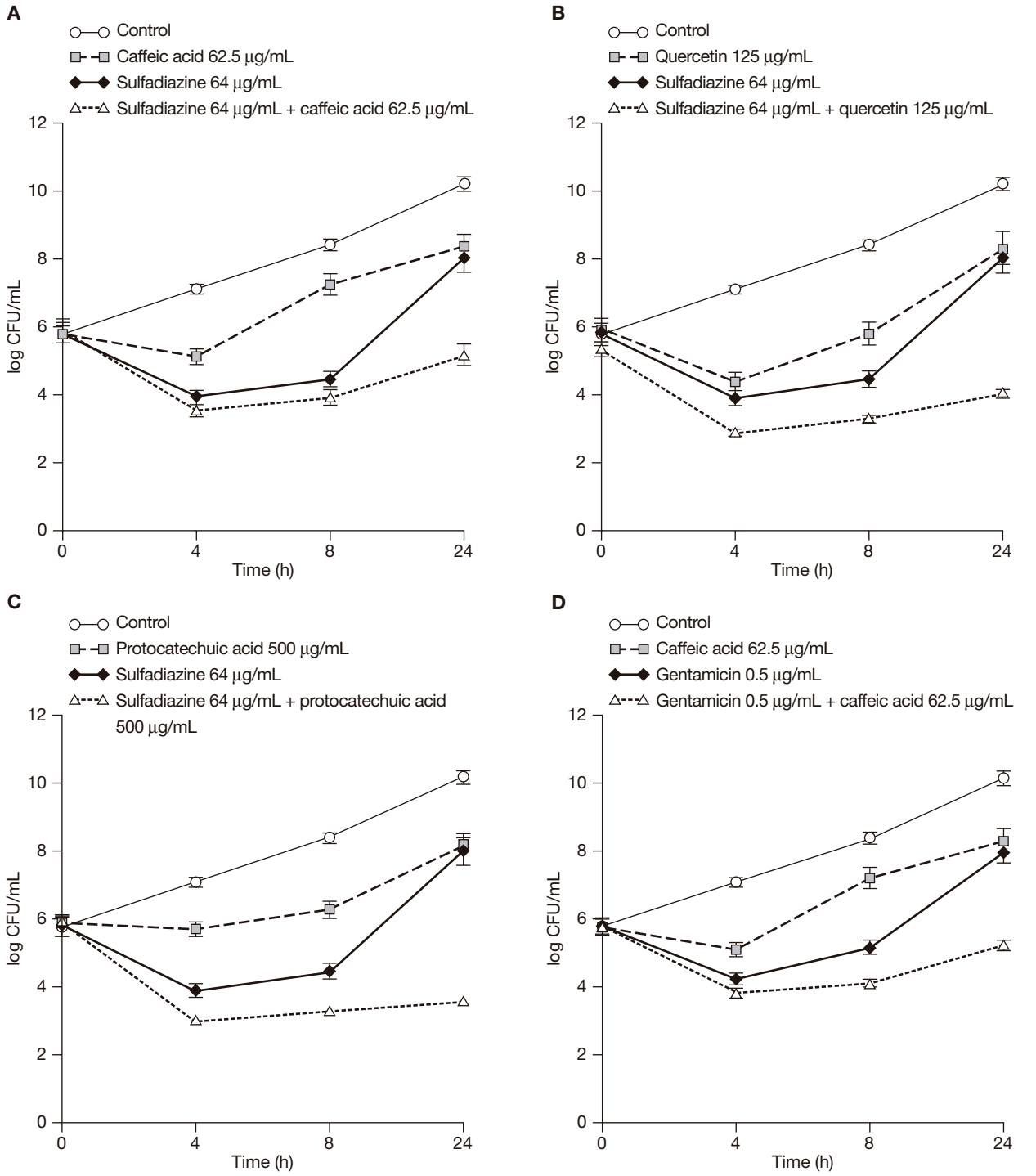


**Fig. 3.** Time-kill curves of antimicrobials at one-quarter the minimal inhibitory concentrations for synergistic combinations. (A) Sulfadiazine and caffeic acid; (B) sulfadiazine and quercetin; (C) sulfadiazine and protocatechuic acid; and (D) gentamicin and caffeic acid.
Abbreviation: CFU = colony-forming unit.

of *P. aeruginosa*. All the above listed combinations at one-quarter the MIC of each agent resulted in synergism at 8 and 24 h. There were no significant differences in activity between combinations of levofloxacin and phytochemicals and gentamicin and protocatechuic acid and quercetin. Antagonism was not observed in any of the combinations being investigated.

## Discussion

Phytochemicals have great potential as antimicrobial compounds, and have been proven to have great therapeutic potential as phytochemicals [25]. Phytochemicals also have the ability to increase the susceptibility of the organism to various drugs [25]. This study has shown that a combination of gentamicin and caffeic acid is synergistic. Sulfadiazine in combination with protocatechuic acid, quercetin, or caffeic acid is synergistic against the ATCC strain of *P. aeruginosa*. Both the static information on drug-herb interactions provided by the checkerboard and the dynamic approach measuring the bactericidal activity of the combinations — the time-kill assays — show this effect (Fig. 3). The most successful drug combinations against *P. aeruginosa* were sulfadiazine plus protocatechuic acid and sulfadiazine plus quercetin, with the killing curve showing high bactericidal rates at 8 and 24 h. For all the combinations, synergy was observed from 4 to 8 h, showing the higher rate of killing with the phytomedicine and antibiotic combination therapy than with monotherapy, with a ≥2 $\log_{10}$ decrease in CFU/mL at 24 h. These results suggest the possibility of drug-herb combinations for lowering the dose of antibiotics needed to treat infections caused by *P. aeruginosa*. However, it is important to know the reported toxicity levels before pursing future experiments in this direction. The acute toxicity levels for protocatechuic acid (50% lethal dose [$LD_{50}$], >800 mg/kg), quercetin ($LD_{50}$, 159 mg/kg), and caffeic acid ($LD_{50}$, >721 mg/kg) were obtained from the manufacturer. Thus, the MIC dose levels of the 3 phytochemicals at synergistic concentrations are lower than the reported toxicity levels, suggesting that they have potential as therapeutic agents in combination with antibiotics.

The mechanism of action of protocatechuic acid is unknown. Despite the lack of knowledge for the underlying mechanism of the synergistic effect of these combinations or of the phytochemicals alone, there is potential for their clinical use. They could make some untreatable resistant infections treatable at the currently recommended doses that are often only marginally effective against resistant strains when used alone. For example, the combinations of sulfadiazine and gentamicin with phytochemicals may help to reduce the amount of antibiotic used and deliver a medicine with a similar or greater potency to an antimicrobial. Combination therapy has earlier been reported to increase activity and prevent the development of resistance [26]. More importantly, since phytochemicals are structurally different from antibiotics and often have different modes of action, they may provide novel means of studying the mechanisms of bacterial control at a molecular level. With the increased prevalence of multidrug-resistant strains (specifically *P. aeruginosa*), synergism testing using various combinations of phytochemicals with antibiotics could be a powerful tool to aid selection of appropriate antibiotic therapy. These data encourage further studies of these agents plus other antimicrobial classes and in vivo animal experiments to validate this finding. These authors are now testing other combinations and preparing animal work for validation of the synergistic effect.

One of the major concerns is the difference in the reported MIC values for the phytochemicals under investigation. These differences may arise due to differences in climate and ecological conditions of the plant or plant parts from which the phytochemicals are extracted. The use of laboratory grade phytochemicals may help to overcome this limitation to some extent.

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