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Original Research Article

Mode of Action and Synergy of Ceftazidime and Baicalein against *Streptococcus pyogenes*

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Abstract

Purpose: *To investigate the antibacterial activity of baicalein used alone, or in combination with ceftazidime, against Streptococcus pyogenes.*
Methods: *Minimum inhibitory concentration (MIC), checkerboard assay parameters, and viability curves were determined for S. pyogenes DMST 30653, 30654, and 30655. Cytoplasmic membrane (CM) permeability technique, enzyme assays, transmission electron microscopy and Fourier transform-infrared microspectroscopy were used to investigate the changes in the bacterial biomolecules.*
Results: *The MIC of ceftazidime and baicalein against all the S. pyogenes strains were 0.50 and > 256.0 µg/ml, respectively. A synergistic effect against these strains was exhibited by the ceftazidime/baicalein combination (fractional inhibitory concentration index, < 0.37). The results for the viable counts indicate that this synergistic activity was present. Baicalein exerted inhibitory activity against β-lactamase. Compared with the controls, combining baicalein with ceftazidime caused peptidoglycan and morphological damage, significantly increased CM permeability and protein concentrations, and decreased cellular fatty acid and nucleic acid concentrations.*
Conclusion: *Baicalein is a potential synergistic adjunct to ceftazidime for the treatment of S. pyogenes infections.*

Keywords: *Streptococcus pyogenes, Cytoplasmic membrane permeability, Baicalein, Ceftazidime, Synergistic activity, Fourier Transform-infrared microspectroscopy, Transmission electron microscopy*

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INTRODUCTION

Streptococcus pyogenes (*S. pyogenes*), group A streptococcus, is the most common cause of bacterial pharyngitis, scarlet fever, and impetigo. *S. pyogenes* is also the group mostly associated with streptococcal toxic shock syndrome [1]. In the past two decades, increases in severe *S. pyogenes* diseases have been reported worldwide [2]. Recommended therapies for *S. pyogenes* infections include the use of penicillins and cephalosporin. However, the failure of penicillin to eradicate streptococci from the throat occurs in up to 35 % of patients with pharyngeal-tonsillitis [3].

Previous research results indicate that 1.9% of *S. pyogenes* infections exhibit intermediate resistance to ampicillin [4]. Khan and co-worker found *S. pyogenes* resistant to cloxacillin, oxacillin, and cefoperazone [5]. The only two described *S. pyogenes* strains are β-lactamase producers, and bla-TEM has been detected and amplified on genomic DNA of 28 β-lactam

resistant isolates from 42 *S. pyogenes* infections from Mansoura university hospitals [6].

Co-existing oropharyngeal beta-lactamase-producing bacteria (BLPB) may not only have survived penicillin therapy, but also could have protected other penicillin-susceptible bacteria from penicillin. Increased failure rates for penicillin used for treatment of otitis, sinusitis, and pharyngeal-tonsillitis infections associated with these bacteria have been reported [7]. Effective antibiotics available for the treatment of *S. pyogenes* and coexisting BLPB infections are frequently associated with β-lactam failure and unwanted side effects. Discovery of new combination agents to treat these bacteria, overcome β-lactam failures during *S. pyogenes* treatment and the resurgence of BLPB infections, and reduce adverse drug effects, is urgently needed. Many flavonoids isolated from plants have shown synergistic antibacterial activity [8].

The aim of the present study was to investigate the antibacterial and synergistic activities of baicalein, alone and in combination with ceftazidime, against *S. pyogenes*. The basic mechanism of action was also examined.

EXPERIMENTAL

Materials and bacterial strains

Streptococcus pyogenes DMST 30653, 30654, and 30655 were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. *Staphylococcus aureus* ATCC 29213 (*S. aureus*) was purchased from the American Type Culture Collection (ATCC; Virginia, USA), and was used as a positive control. Baicalein (purity 98 %) was purchased from the Indofine Chemical Company (New Jersey, USA). Ceftazidime, amoxicillin, penicillin, β-lactamase type IV, dimethylsulfoxide, glutaraldehyde (grade I, 25 %; for electron microscopy (EM)), osmium tetroxide (4 %; for EM), a Spurr Low-Viscosity Embedding Kit, and nisin (from *Lactococcus lactis*, 2.5 %, balanced sodium chloride and denatured milk solids) were obtained from Sigma (Sigma-Aldrich, Dorset, UK). Mueller-Hinton agar (MHA), Mueller–Hinton broth with sheep blood (5 % v/v) agar (MHBSB), cation-adjusted Mueller-Hinton broth (CAMBH), and cation-adjusted Mueller-Hinton broth with lysed horse blood (2.5 % v/v) (CAMHBHB) were obtained from Oxoid (Basingstoke, UK).

Bacterial suspension standard curve

The bacterial suspensions standard curve method was used to determine known viable counts, following the method of Richards and Xing with few modifications [9]. Viable counts for each absorbance reading were determined in triplicate using an agar plate counting method [8,10].

Minimum inhibitory concentration (MIC)

MIC determinations of ceftazidime, amoxicillin, penicillin, nisin, and baicalein against the *S. pyogenes* and *S. aureus* strains were performed using previously reported methods [8,10,11]. Briefly, in addition to an antibacterial or baicalein, an inoculum of 5 x 10^6 cfu/ml bacterial suspension was added to each tube containing CAMHBHB (for *S. pyogenes*) or CAMBH (for *S. aureus*), to result in approximately 5 x 10^5 cfu/ml per tube. The MIC determination was accomplished by observing turbidity after 20 h of incubation at 35 °C.

Checkerboard assay

Checkerboard assays to determine the synergistic activity of baicalein in combination with ceftazidime against all tested *S. pyogenes* strains were performed following Eumkeb *et al* [8]. The interaction between the two agents was calculated using the fractional inhibitory concentration index (FICI) [12]. The FICI categories were: synergistic (FICI, ≤ 0.5), partially synergistic (FICI, > 0.5 and < 1.0), additive (FICI, 1.0), indifferent (FICI, > 1 and ≤ 4.0), and antagonistic (FICI, > 4.0). *Staphylococcus aureus* was used as positive control.

Determination of viability curves

Killing curve determination was performed to confirm the synergistic activity of the combination following the methods of Richards and Xing, Eumkeb *et al*, and the Clinical and Laboratory Standards Institute, with slight modifications [8,9,11]. After the FIC index was obtained, the MIC of each compound that was associated with the synergism FIC index for the combination was chosen for investigation. The half-MICs of ceftazidime and baicalein separately, and that of their combination, yielded the synergistic FIC index against *S. pyogenes*.

Cytoplasmic membrane (CM) permeability test

The CM permeabilization experiment was performed, with some modifications, to confirm results as previously described by Shen *et al* and Zhou *et al* [13,14]. Shortly after the FIC index was determined from the checkerboard assay,

the half-MIC values for ceftazidime and baicalein alone, and the 3/4 MIC values for these combinations that resulted in synergistic FIC indices, were selected against *S. pyogenes* to measure CM permeability. This method was performed by measuring the release of UV-absorbing material (Varian's Cary 100 UV-Vis spectrophotometer, Varian, Inc, California, USA) [15].

Enzyme assay

The β-lactamase type IV of Enterobacter cloacae inhibition activity was previously described by Reading and Farmer [16]. The analyses of the remaining substrates were performed using reverse-phase HPLC with an acetronitrile/ammonium acetate mobile phase [16].

Transmission electron microscopy (TEM)

Cellular damage of the bacteria was examined using TEM. Briefly, after the FIC index was obtained from checkerboard assay, the half-MIC values for ceftazidime and baicalein alone, and the 3/4 MICs for the combinations that gave synergistic FIC indices, were chosen against *S. pyogenes* to investigate the morphological appearance. Subcultures were prepared for examination using TEM, based the method of Eumkeb *et al* [8].

Fourier Transform-Infrared (FT-IR) microspectroscopy

FT-IR measurement was performed following the methods of Eboigbodin and Biggs, and Toubas *et al* [17,18]. Briefly, after the FIC index was illustrated from the checkerboard assay, the half-MIC for ceftazidime alone and the 0.75 MIC for the combination that gave a synergistic FIC index against *S. pyogenes* were used in FT-IR studies [17-19]. The cells were incubated at 37 °C in a shaking water bath for 4 h. The cell pellets were centrifuged at 3000 x g for 10 min and washed twice with saline. The cells were then washed twice with Milli-Q water. A small portion of pellet was then deposited into a Mirr IR low e-microscope slide (Kevey slide) to use as a substrate for FT-IR microscope analysis. The cells were then desiccated under a vacuum for approximately 20 min and stored in desiccators to form acceptable films before analysis. To achieve high S/N ratios, 64 scans were coded for each measurement in the wavenumber between 4000 and 400 cm^{-1} resolution of 6 cm^{-1}. Spectra were recorded in reflection mode on a Bruker IR spectrometer (Tensor 27, Massachusetts, USA) coupled to an IR microscope (Hyperion 2000, Bruker) with 36x magnification. The data for the effects of variation of the composition and distribution of the biochemical components in the bacterial cells during cell culture were analyzed using principal components analysis (PCA). All data analysis was performed in the 3000 – 2800 cm^{-1} and 1800 – 850 cm^{-1} spectral ranges, which include the fingerprint region.

Statistical analysis

All experiments were carried out in triplicate; results were expressed as mean ± standard error of the mean (SEM). Significant differences in CM permeability, the enzyme assay data between each treated group at the same interval times, and the peak area in each group of FT-IR range were analyzed using one-way ANOVA. A *0*-value < 0.01, based on Scheffe's post-hoc test, was considered to indicate a statistically significant result.

RESULTS

MIC and FIC

The MIC results for ceftazidime, nisin, and baicalein against *S. pyogenes* tested strains are presented in Table 1. The results revealed that the MICs for ceftazidime, nisin, and baicalein against all *S. pyogenes* strains were 0.50, 1.0, and > 256 µg/ml, respectively. These results indicated that these strains were sensitive to ceftazidime [11]. *Staphylococcus aureus* ATCC 29213, a positive control, was susceptible to amoxicillin and penicillin [11]. Baicalein exhibited little inhibitory effect against this strain. The FIC indices for ceftazidime plus baicalein against all *S. pyogenes* strains were < 0.37. These results indicated that these combinations showed synergistic activity against this strain.

Killing curves

The results for the separate and combined effects of ceftazidime and baicalein on viable counts of *S. pyogenes* 30653 are presented in Figure 1. The viable counts for the cells treated with ceftazidime were slightly lower than that of baicalein (between 2 and 24 h). The combination of ceftazidime and baicalein dramatically decreased the cell count to 6 x 10^3 cfu/ml after 6 h and up to 24 h.

Table 1: Minimum inhibitory concentrations (MICs), fractional inhibitory concentrations (FICs), and FIC indices for ceftazidime, amoxicillin, penicillin, nisin, and baicalein, alone or in combination, against *S. pyogenes* strains

Strain	MIC (μg/ml)					FIC (μg/ml)	FIC index
	cef	amo	pen	nis	bai	cef + bai	cef + bai
S. pyogenes DMST 30653	0.50[s]	N/D	N/D	1.0	>256.0	0.12 +32.0	<0.37
S. pyogenes DMST 30654	0.50[s]	N/D	N/D	1.0	>256.0	0.12 +32.0	<0.37
S. pyogenes DMST 30655	0.50[s]	N/D	N/D	1.0	>256.0	0.12 +32.0	<0.37
S. aureus ATCC 29213*	N/D	<0.2[s]	<0.1[s]	N/D	N/D	N/D	N/D

**S. aureus ATCC 29213, amoxicillin and penicillin were used as positive control; cef = ceftazidime, amo = amoxicillin, pen = penicillin, nis = nisin, bai = baicalein; [s] = susceptible; N/D = No data; n = 3*

These results were confirmed by the checkerboard assay results, which indicated that the combination produced a decrease of ≥ 2 log10 cfu/ml, compared with ceftazidime treatment alone [20].

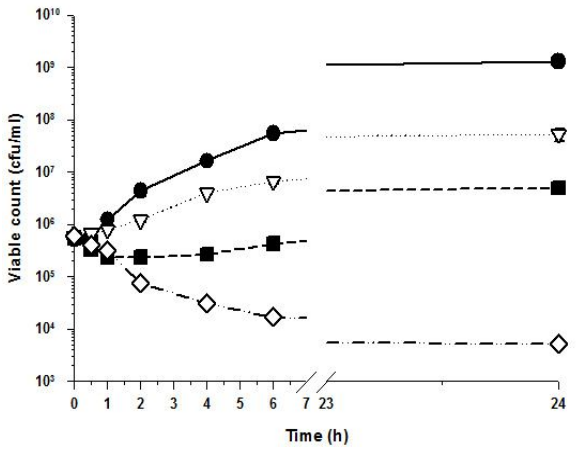

Figure 1: The effects of ceftazidime and baicalein, alone and in combination, on the viable counts of *S. pyogenes* DMST 30653; (●) = control (drug-free); (▽) = baicalein, 128 μg/ml; (■) = ceftazidime, 0.25 μg/ml; (◇) = ceftazidime, 0.12 μg/ml, plus baicalein, 32 μg/ml; n = 4; error bars indicate the standard error of the mean (SEM)

CM permeability

The CM permeability was measured by examining the release of UV-absorbing materials (Figure 2). After treatment, *S. pyogenes* 30653 cells with nisin, ceftazidime, and the ceftazidime plus baicalein combination could induce the release of 260 nm absorbing materials at significantly higher levels compared with the control or baicalein alone ($p < 0.01$). The CM permeability strength was nisin > ceftazidime plus baicalein > ceftazidime > baicalein > control ($p < 0.01$). These results suggested that the synergistic activity of ceftazidime plus baicalein mostly resulted in increased cytoplasmic membrane permeability of DNA, RNA, and cellular metabolites [13,14].

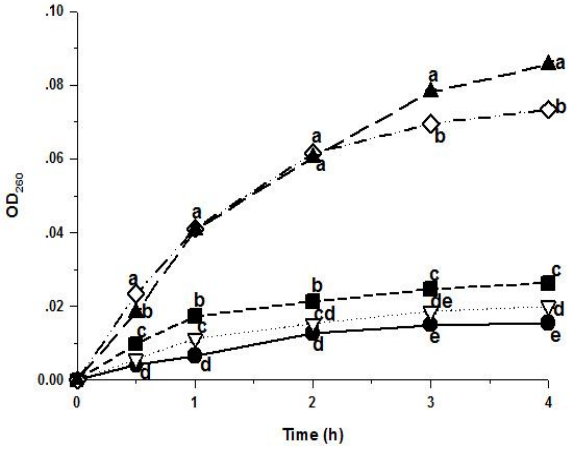

Figure 2: Effect of 260 nm absorbing material (DNA, RNA, and metabolites) in the *S. pyogenes* DMST 30653 supernatants treated with baicalein and ceftazidime either alone or in combination. (●) = control (drug-free); (▽) = baicalein, 128 μg/ml; (■) = ceftazidime, 0.25 μg/ml; (◇) = ceftazidime, 0.09 μg/ml, plus baicalein. 24 μg/ml; (▲) = nisin, 0.50 μg/ml. Nisin (0.50 μg/ml) was used as a positive control and untreated cells were used as a negative control; n = 3; means sharing the same superscript are not significantly different (Scheffe's test, $p < 0.01$)

Enzyme assay

The enzyme assay results for the baicalein treatment revealed that the levels of benzylpenicillin were significantly higher compared with the controls ($p < 0.01$). The levels of benzylpenicillin depended on the baicalein concentration in a decreasing, dose-dependent manner ($p < 0.01$).

TEM

The transmission electron micrographs of cells from the log phase of growth of *S. pyogenes* 30653 in the presence of ceftazidime, baicalein, and ceftazidime + baicalein are presented in Figure 3. The peptidoglycan and cytoplasmic membranes could be distinguished, and the cells had the normal appearance of the control group (Figure 3a). The micrographs of the *S. pyogenes* 30653 cells treated with ceftazidime or baicalein alone revealed that cell division may have been interrupted, which resulted in cell shape distortions (Figure 3b and 3c). The results for the combination-treated cells also indicated that cell division in many of these cells may have been interrupted, leading to twisted and irregular cell shapes, and peptidoglycan and cytoplasmic membrane damage (Figure 3d). The average areas of these cells were significantly larger compared with the control cells ($p < 0.01$).

FT-IR spectra

The 3-dimensional PCA clustering results from the FT-IR spectral data for *S. pyogenes* 30653 after treatment with ceftazidime alone and in combination with baicalein are presented in Figure 4a. The biomolecule fingerprint clusters for the control and the ceftazidime alone or in combination with baicalein groups were clearly differentiated [23,27].

The *S. pyogenes* 30653 strain was grown in CAMHBHB medium in the presence of ceftazidime or the ceftazidime plus baicalein combination, and examined using FT-IR microspectroscopy. The results for the loading plots are presented in Figure 4b. The signal intensities and peak areas of these treated cells at ~1655 and ~1637 cm^{-1} were ceftazidime plus baicalein > ceftazidime > control, which corresponded to an absorption peak for the secondary structure of protein amide I (α-helix and β-sheet). The peak intensity pattern for the areas at ~1085 cm^{-1} was ceftazidime > control > ceftazidime plus baicalein, which correlated with an absorption peak for a nucleic acid (DNA and RNA) phosphodiester backbone (Figure 4c) [23,24].

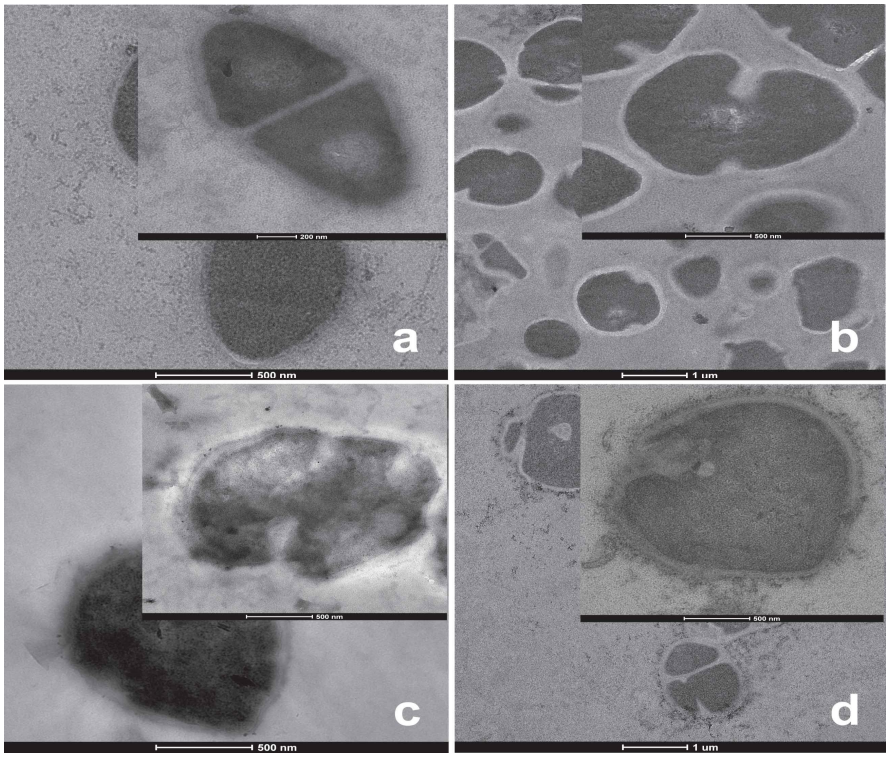

Figure 3: Ultrathin sections of log phase *S. pyogenes* DMST 30653 grown in cation-adjusted Mueller-Hinton broth with lysed horse blood (2.5 %v/v) containing: **a**, control (drug-free); **b**, ceftazidime, 0.25 µg/ml; **c**, baicalein, 128 µg/ml; **d**, ceftazidime, 0.09 µg/ml, plus baicalein, 24 µg/ml. (**a**, 195,000x, bar 500 nm; **b**, 7,000x, bar 1 µm; **c**, 19,500x, bar 500 nm; **d**, 7,000x, bar 1 µm; *Inset*: **a**, 26,000x, bar, 200 nm; **b**, 19,500x, bar, 500 nm; **c**, 19,500x, bar, 500 nm; **d**, 19,500x, bar, 500 nm)

The second loading of the treated and control groups indicated that the obvious regions at 3000–2800 cm^{-1} (~2963, ~2921, ~2875, ~2852 cm^{-1}) could be attributed to the stretching modes of CH_2 and CH_3 in the fatty acids located on the various membrane amphiphiles and ester bands, respectively (Figure 4d) [25,26]. The treated cells exhibited a peak area and intensity at ~2921 and ~2852 cm^{-1}, respectively, of ceftazidime > control > ceftazidime plus baicalein (Figure 4d) [23, 27].

DISCUSSION

The presence of BLPB after treatment indicated that the bacteria may not only have survived penicillin therapy, but also could have protected other penicillin-susceptible bacteria from the drug and led to the failure of penicillin to eradicate this strain from the patients with pharyngeal-tonsillitis [28]. A novel antibacterial combination that can conquer β-lactam failures for treatment of *S. pyogenes* and the coexistence of the BLPB infections is urgently required. The MIC results revealed that all of the *S. pyogenes* strains were still sensitive to ceftazidime alone. The standard values for the sensitivities of cefotaxime (also a third-generation cephalosporin) against these strains are ≤ 0.50 µg/ml [11].

The checkerboard assay revealed that there were synergistic effects against all *S. pyogenes*

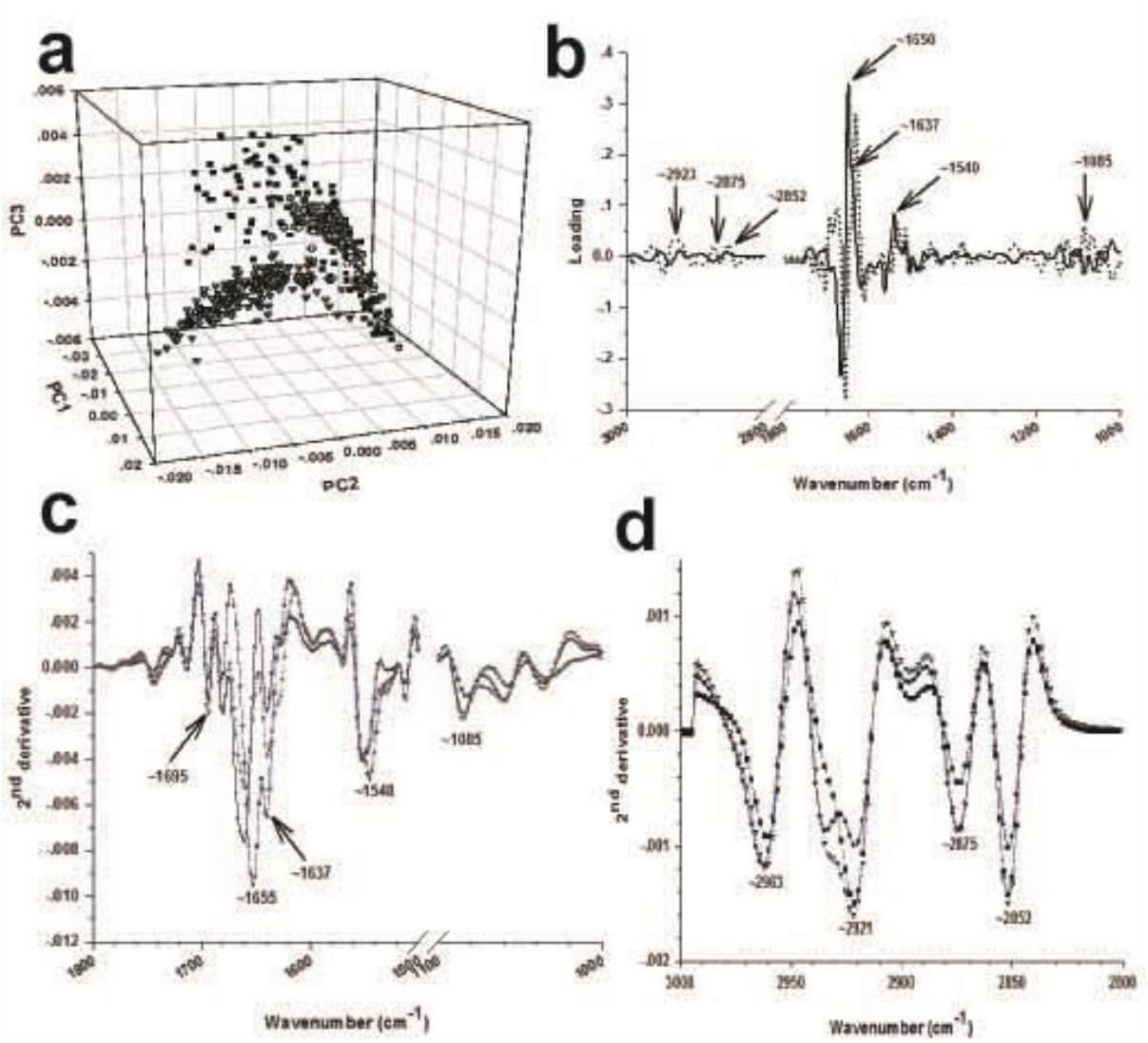

Figure 4: Principal components analysis (PCA) results **(a);** the loading plot for the first (PC1) and the second (PC2) principal components obtained from the PCA of *S. pyogenes* DMST 30653 **(b);** the representative second derivative transformation spectra (1800–1000 cm^{-1}) **(c);** and the representative second derivative transformation spectra (3000–2800 cm^{-1}) **(d).** Symbols represent the FT-IR spectra of, **a, c, d,** (●) = control (drug-free), ▽(▽) = ceftazidime, 0.25 µg/ml (■) = ceftazidime, 0.09 µg/ml, plus baicalein, 24 µg/ml; **b,** solid line = PC1, dotted line = PC2

strains when ceftazidime was combined with baicalein (FIC index < 0.37) [12]. The killing curve result (≥ 2 log10 cfu/ml reduction) also confirmed the synergistic effect of ceftazidime plus baicalein, compared with ceftazidime alone [20].

The CM permeability results indicated that the ceftazidime plus baicalein combination clearly increased CM permeability of *S. pyogenes*. To our knowledge, this study is the first to reveal this effect. An increase in CM permeability may be one of the synergistic actions of this combination against *S. pyogenes*. These findings provide evidence that the phospholipid bilayer in the cytoplasmic membrane might have been destroyed, with a resultant leak in the plasma membrane [14].

The enzyme assay revealed that baicalein had an inhibitory activity against β-lactamase. Whether *S. pyogenes* produces beta-lactamase or not, a previous study found that the beta-lactamase produced by other bacteria in the pharynx can inactivate penicillin, which can result in increased treatment failures or infection relapses [29]. These findings provide evidence that baicalein in combination with beta-lactam antibiotics may useful for the inhibition of the coexistence of the BLPB and *S. pyogenes* in oropharyngeal infections.

The TEM results for the changes in the *S. pyogenes* cells after exposure to ceftazidime plus baicalein indicated that the division of many of the bacterial cells may have been interrupted. This effect likely led to the twisted and irregular cell shapes, the peptidoglycan and cytoplasmic membrane damage, and the significant increase in average cell area ($p < 0.01$). These results can be explained by assuming that baicalein may interact synergistically with ceftazidime to inhibit peptidoglycan synthesis. This synergistic effect can result in marked morphological damage and delayed cell division.

Our FT-IR results indicated that compared to the controls, *S. pyogenes* cells treated with baicalein in combination with ceftazidime had increased the α-helical and β-pleated sheet structures of amide I, but had decreased nucleic acids and fatty acid content in the bacterial cells [24]. These results suggested that this combination may damage the composition of the fatty acid chains on the phospholipid bilayer of the plasma membrane and interrupt peptidoglycan synthesis. The amide I transformation results in CM damage and increased permeability, DNA and RNA leakage, and amide I protein accumulation within the cells.

CONCLUSION

This study provided evidence that baicalein and ceftazidime have a synergistic effect against *S. pyogenes*. To our knowledge, this is the first report on the mechanisms of synergistic action of baicalein and ceftazidime against this strain using FT-IR. The modes of actions of this combination included inhibition of peptidoglycan synthesis, increased CM permeability and enhanced amino acid accumulation, but reduced fatty acid and nucleic acid quantities, in the bacterial cells. Because baicalein has limited toxicity, it can potentially be used as an adjunct to ceftazidime for the treatment of coexisting BLPB and *S. pyogenes* infections. Future studies are required to confirm that this synergism occurs in humans, and in animal species.

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