The Thai Journal of Pharmaceutical Sciences

Volume 38
Issue 0 *supplement issue*

Article 33

1-1-2013

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KEYWORDS: *Streptococcus pyogenes*, FT-IR microspectroscopy, Flavonoids

INTRODUCTION

Streptococcus pyogenes is one of the most frequent pathogens of humans. *S. pyogenes* can infect when defenses are compromised or when the organisms are able to penetrate the constitutive defenses. It is the cause of many important human diseases, ranging from mild superficial skin infections to life-threatening invasive illness. One of the most severe invasive manifestations is streptococcal toxic shock syndrome (STSS)[1)]. Beta-lactam antibiotics such as penicillin and amoxicillin are uniformly effective against most strains of *S. pyogenes*. However, increasing antimicrobial resistance of *S. pyogenes* has been observed during the last decade in Europe and worldwide[2)]. Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compound[3)]. Phenolic compounds and flavonoids exhibit a wide range of physiological properties such as anti-allergenic, anti-inflammatory and antimicrobial. The antibacterial activity of flavonoids is being increasing documented and some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria. Although the antibacterial activity of flavonoids against various pathogens has been reported, the little is known about its activity on *S. pyogenes*. FT-IR microspectroscopy technique can be employed to perform in depth molecular level and also use to study spectral change resulting from bacterial injuries. The spectra of bacterial cells reflect the biochemical structure and composition of the cellular constituent that include fatty acids, proteins, polysaccharides and nucleic acids. This technique has been applied to differentiate intact microbial cells base on the unique surface biochemistry of bacterial cell[4)]. Therefore, this study was aimed to investigate antibacterial effect and synergistic effect with antibiotic of flavonoids against *S. pyogenes*. Furthermore, the FT-IR microspectroscopy technique was used to characterized biochemical compound on whole bacterial cell after treatment with antibacterial compound.

MATERIALS AND METHODS

Strain and growth conditions *S. pyogenes* ATCC 19615 was used in this study and cultivated with aeration at 37 °C in Brain Heart Infusion (BHI) broth. The cell culture was centrifuged at 4,000 rpm for 10 min. The cell pellets were washed with saline (0.85% NaCl), recentrifuged and resuspended in saline. The cell was adjusted to appropriate concentration for antibiotic susceptibility testing.

Drug and chemical preparations Stock solution of ceftazidime was dissolved in distilled water and isolated flavonoids contain luteolin, baicalein and quercetin were prepared in 5% DMSO.

Minimum Inhibitory Concentrations (MICs) determination Overnight culture (18 hr.) was adjusted to give 5 x 10^5 CFU/ml in BHI broth, which plus 10% serial dilutions of the ceftazidime or isolated flavonoid, Tubes of broth without antibacterial agent was used as the control. The lowest concentration of each antibacterial which inhibited growth was taken to be the MIC[5-6)].

Checkerboard determination The culture from 18 hr was adjusted to give final concentration of 1x10^5 CFU/ml in BHI broth, which plus 10% serial dilutions of the ceftazidime alone and in combination with isolated flavonoids, tubes of broth without antibacterial agent was used as the control. MICs were determined for antibacterial combination and the isobologram was plotted. The Fractional Inhibitory Concentration (FIC) indexes were calculated[5-6)].

Viability curve determination Bacterial concentration of 5 x 10^5 CFU/ml in BHI broth was exposed to the ceftazidime alone or in combination with isolated flavonoids at selected concentrations an incubation temperature at 37 °C. Viable count was determined after contact time of 0, 0.5, 1, 2, 4 and 6 hr on BHI agar plates in triplicate. An incubation at 37 °C for 18-24 hr was allowed counting of growing colonies and viability curve was plotted[7)].

Effect of flavonoids on cytoplasmic membrane of S. pyogenes *S. pyogenes* ATCC 19615 was cultured into 100 ml BHI broth and incubated at 37 °C for 18–24 h. After incubation, the bacterial cells were separated by centrifugation at 10,000g for 10 min and resuspended in PBS (pH7.4). Suspensions were adjusted to OD_{600} of 2.0. Different conditions of treatment were added to the cell suspension. The

experiment was done in triplicates. Cells without ceftazidime and flavonoids were used as control. All the samples were incubated at 37 °C for 60 min. After treatment, the cell suspension was centrifuged at 13,400g for 15 min and OD_{260} value of the supernatant was taken as a percentage of the extracellular UV-absorbing materials released by cells[8].

Bacterial whole cells preparation for FT-IR technique After treatment with ceftazidime and isolated flavonoids. Bacterial cells were centrifuged and discard supernatant, cells were washed in saline for 2 times to remove culture media. Cell suspensions in saline were washed in sterile distilled water for 2 times and then deposited into Mirr IR low e-microscope slides. Dried cell in desiccator was resuspended in pure water before used[9].

FT-IR microspectroscopy analysis Cell suspensions was dissolved in sterile distilled water and then deposited into Mirr IR low e-microscope slides (Kevey slide) used as a substrate for FT-IR microscope analysis. The samples were then desiccated under vacuum for several hours and stored in desiccators to form films suitable before analysis. Spectra were collected on a Bruker IR spectrometer (tensor 27) coupled to an IR microscope (Hyperion 2000) with 36x magnification. Second derivative and vector normalize were manipulated to account for difference in sample thickness, minimize baseline variation and allowed visual comparison. All data analysis were carried out in the spectral range from 3000-2800 $cm^{-1}$and 1800-900 cm^{-1}, which covers the mixed region of lipid, protein and polysaccharide. The original spectra of bacterial whole cells were generated second derivatives, and subsequently vector normalized for multivariate statistical analysis, using the Unscrambler 9.7 software (Camo, Norway) and Cluster analysis, using the OPUS 6.5 software (German)[9].

RESULTS

Minimum Inhibitory Concentrations (MICs) MIC is defined as the lowest concentration of the antimicrobial agent in the BHI broth resulting in the complete inhibition visible growth. The MICs for the ceftazidime, luteolin, baicalein and quercetin against *S. pyogenes* ATCC 19615 are shown in Table 1. This strain was susceptible to ceftazidime, luteolin, baicalein and quercetin at MIC of 0.25 µg/ml, 128 µg/ml, >256 µg/ml and 128 µg/ml respectively.

Checkerboard assay The fraction inhibitory concentration of ceftazidime plus isolated luteolin, baicalein and quercetin were 0.625, <0.625 and 0.531 respectively (Table 1). The results showed that ceftazidime at concentration of 0.25 µg/ml was significantly reduced when combined with isolated flavonoids against this strain.

Table 1 Minimum Inhibitory Concentrations (MICs), fractional inhibitory concentrations (FICs) and FIC indexes determined by checkerboard assays of ceftazidime, luteolin, baicalein, quercetin alone and ceftazidime in combination with flavonoids against *S. pyogenes* ATCC 19615

S. pyogenes ATCC 19615	MIC (µg/ml)	FIC (CF + FV) (µg/ml)	FIC index
Ceftazidime	0.25	-	-
Luteiolin	128	0.125 + 16	0.625
Baicalein	>256	0.125 + <32	<0.625
Quercetin	128	0.125 + 4	0.531

CF = Ceftazidime; FV = Flavonoids

Viability curve The synergistic activity of ceftazidime in combination with luteolin, baicalein and quercetin were confirmed in viability curve. The combination effect of ceftazidime and these selected flavonoids were more effective than ceftazidime alone. It can be seen in figure 1 that the viable counts of *S. pyogenes* ATCC 19615 were significantly reduced in the presence of the combination of ceftazidime and these selected flavonoids compared to ceftazidime, these selected flavonoids alone and control group. The cells in the combination groups of either ceftazidime plus quercetin or baicalein were decreased to 10^4 CFU/ml at 6 hr.

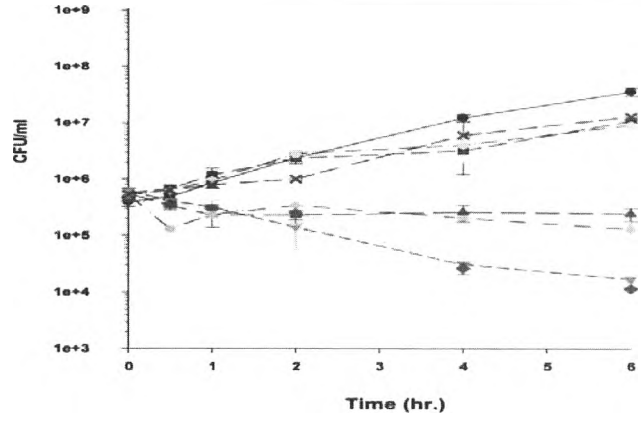

Figure 1 Viability of *S. pyogenes* ATCC 19615 after treatment with ceftazidime alone and in combined with flavonoids. —●—, control; , ceftazidime 0.125 μg/ml; – – –, baicalein 84 μg/ml; – ■ –, quercetin 43 μg/ml; —✱– , luteolin 43 μg/ml; – –▼– –, ceftazidime 0.125 μg/ml + quercetin 4 μg/ml; · ◆ ·, ceftazidime 0.125 μg/ml + baicalein 32 μg/ml; · – – –, ceftazidime 0.0625 μg/ml + luteolin 4 μg/ml. The bars represent the standard deviations of three replicates.

Effect of flavonoids on cytoplasmic membrane of S. pyogenes The leakage of cytoplasmic membrane was analysed by determining the release of cell materials. Figure 2 shows that the OD_{260} of bacterial cell material after *S. pyogenes* ATCC 19615 suspensions were treated with either ceftazidime alone and in combination with flaovnoids. After treatment with combination of ceftazidime and flavonoids, the OD was increased from 0.01 in control group to 0.02 in ceftazidime alone treated group. Especially, the ceftazidime plus either luteolin or baicalein were clearly risen up to 0.12 and 0.14 respectively. The results suggest that combination of ceftazidime and selected flavonoids damages cytoplasmic membrane and causes subsequent leakage of intracellular constituents.

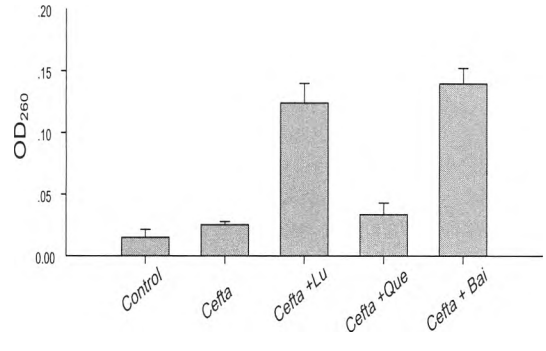

Figure 2 Measuring absorbance of the cell materials contents at 260 nm releasing from *S. pyogenes* ATCC 19615 after treated with ceftazidime alone and in combination with flavonoids. (Cefta = Ceftazidime, Lu = Luteolin, Que = Quercetin, Bai = Baicalein)

FT-IR characterization studies The spectra of *S. pyogenes* ATCC 19615 cultivated in BHI broth are shown in Figure 3a-b. The effect of treated group of ceftazidime and isolated flavonoids were harvested and analyzed using FT-IR microspectroscopy. Each spectrum contains information about functional groups arising from predominantly carbohydrate, protein and nucleic acids. In Figure 3a the band at ~2921, ~2852 cm^{-1} of treated groups that correspond to the C–H stretching of CH_2 vibration, were significantly increased compared to control group. No difference was observed in band content at ~2963, ~2875 cm^{-1} that assigns to C–H stretching of CH_3 vibration on control and treated groups. This band centered corresponding to stretching mode of asymmetric CH_3, CH_2 vibration mainly due to membrane lipids. The conformational change of protein amide I noted between 1700-1600 cm^{-1} can give information of protein secondary structure such as α-helix (centered at ~1650 cm^{-1}), β-sheet (centered at ~1635 cm^{-1}), β-turn (centered at ~1685 cm^{-1}). FT-IR spectral was changed over the growth phase of *S. pyogenes* ATCC 19615 treatment with ceftazidime plus baicalein. The spectra show in figure 3b indicated that the α-helix, β-sheet of amide I and amide II band were higher than all treated and control groups. In addition, the α-helix of amide I and amide II band of ceftazidime plus baicalein was shifted after bacterial growth phase

was treated. However, the P=O symmetric stretch of nucleic acid ribose or deoxyribose moieties (~1080 cm^{-1}) of ceftazidime plus baicalein was clearly decreased. While, ceftazidime plus quercetin treated group revealed high content of P=O symmetric stretch of nucleic acid ribose or deoxyribose.

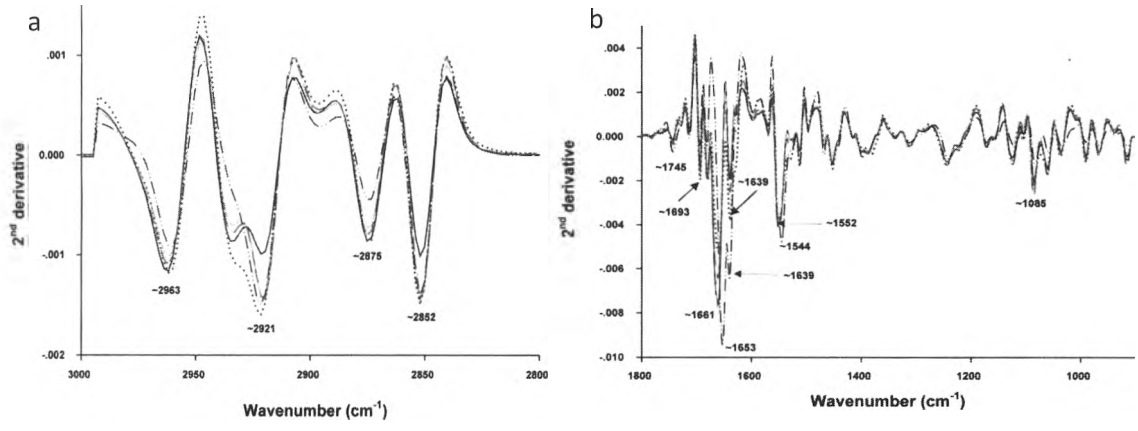

Figure 3a Representative 2nd derivative transformation spectra (~3000-2800 cm^{-1}) and **3b** Representative 2nd derivative transformation spectra (~1800-900 cm^{-1}) of *S. pyogenes* ATCC 19615 after treatment with ceftazidime alone and in combination with flavonoids. The spectra were collected and averaged from 627 spectra.

DISCUSSION

The results from MICs determination indicated that the isolated flavonoids inhibited *S. pyogenes* ATCC 19615. These results are in substantial agreement those of Banso and Mann, they found that the antibacterial test of the flavonoid fraction from *Antiaris africana* showed activity against *B. subtilis*, *S. pyogenes* and *E. coli* [10]. The checkerboard assay revealed that FIC indices of ceftazidime plus flavonoids were lower than 1.0 against this strain. It has been proposed that synergy can be announced when the FIC index < 1.0 [11]. Therefore, the ceftazidime plus these flavonoids exhibited synergistic effect against this train. These synergistic effects were confirmed by killing curve effect. The measurement of UV-absorbing materials releasing is an index of cell lysis and non selective pore formation[12]. After treatment with ceftazidime and isolated flavonoids, the leakage of cytoplasmic membrane was analysed by determining the release of cell materials such as nucleic acid which was absorbed at 260 nm. The results indicated that the combination of ceftazidime plus flavnoids led to higher cell leakage that appeared to lose nucleic acid, critical molecule and ions. The obtained spectral changes of the bacteria treated with ceftazidime plus flavonoids provide evidence that the chemical composition of these cells were varied. Our results showed significant increase in band area at ~2920 to ~2850, ~1653, ~1639 and ~1544 cm^{-1} which seem to represent the amounts of CH_2 of fatty acid, alpha-helix, beta-sheet of amide I and amide II, respectively when treated with the combination of ceftazidime and flavonoids. In addition, a major reduction in the band area of amide I and amide II was found in ceftazidime alone treated group. Moreover, there were undoubtedly decreased in spectrum at ~1085 cm^{-1} of ceftazidime plus baicalein treated group compared to others, this spectral variation suggests that phosphodiester functional group is lower.

CONCLUSION

Our findings provide evidence that all selected flavonoids have synergistic effect with ceftazidime aginst *S. pyogenes* ATCC 19615. The primary mechanism of action of ceftazidime plus these flavonoids is 1. Disruption of the cytoplasmic membrane function of *S. pyogenes* ATCC 19615 which resulted in a loss of cytoplasmic constituents and ions. 2. Alter protein synthesis and disrupt nucleic acid synthesis whereas do not effect fat synthesis. Of the three compounds, baicalein plus ceftazidime showed good inhibited viability curve. In conclusion, FT-IR microspectroscopy may use to determine the presence of injured antibacterial agent that could be underestimated by conventional microbial technique.

ACKNOWLEDGMENT

The authors wish to thank the School of Pharmacology, Faculty of Science, Suranaree University of Technology for research funds. And grateful acknowledge Synchrotron Light Research Institute (Public Organization) Thailand to support the FT-IR microspectroscopy technique.

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