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Baicalin Synergy with β-Lactam Antibiotics Against Methicillin-resistant *Staphylococcus aureus* and Other β-Lactam-resistant Strains of *S. aureus*

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Abstract

Bacterial resistance to antibiotics is a serious global problem and includes strains of β-lactam-resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). Novel antimicrobials and/or new approaches to combat the problem are urgently needed. The Chinese herb Xi-nan Huangqin (*Scutellaria amoena* C.H. Wright) has been used in traditional Chinese medicine to treat a wide range of infectious diseases. In this study we have examined the antibacterial action of baicalin, a flavone isolated from the herb.

When combined with 16 $\mu g\,mL^{-1}$ baicalin, minimum inhibitory concentrations (MICs) of benzylpenicillin against MRSA and penicillin-resistant *S. aureus* were reduced from 125 and 250 $\mu g\,mL^{-1}$ to 4 and 16 $\mu g\,mL^{-1}$, respectively. This activity of baicalin was dose-dependent. Viable counts showed that the killing of MRSA and β-lactam-resistant *S. aureus* cells by 10 to 50 $\mu g\,mL^{-1}$ ampicillin, amoxycillin, benzylpenicillin, methicillin and cefotaxime was potentiated by 25 $\mu g\,mL^{-1}$ baicalin.

From the study it was concluded that baicalin has the potential to restore the effectiveness of β-lactam antibiotics against MRSA and other strains of β-lactam-resistant *S. aureus*. In view of its limited toxicity baicalin offers potential for the development of a valuable adjunct to β-lactam treatments against otherwise resistant strains of microorganisms.

Bacterial resistance to antibiotics is a global problem and nearly twenty years ago over 90% of *Staphylococcus aureus* strains were reported β-lactamase positive (O'Brien 1986). Strains of β-lactam-resistant *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), now pose a serious problem to hospitalized patients and their care providers (Mulligan et al 1993). Novel antimicrobials and/or new approaches to combat the problem are urgently needed. The Chinese herb Xi-nan Huangqin (*Scutellaria amoena* C.H. Wright), together with *Scutellaria baicalensis* Georgi (Chinese Pharmacopoeia Committee 1985), have been used in traditional Chinese medicine to treat a wide range of infectious diseases such as respiratory tract infections, pneumonia, scarlet fever, jaundice, hepatitis, and dysentery (Wang 1983). In this study, we have investigated the in-vitro activity of baicalin (**1**; Figure 1), a major constituent of *S. amoena* C.H. Wright, in combination with β-lactam antibiotics against MRSA and other resistant staphylococci.

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Materials and Methods

Chemistry

S. amoena C.H. Wright was collected in Xichang, Sichuan, China and a specimen deposited in the Herbarium, Chengdu University of Traditional Chinese Medicine, China. Dried and powdered root (1 kg) was extracted with hot water at 80°C for 2 h and filtered. Acidifying the filtrate with dilute hydrochloric acid to pH 2 resulted in a precipitate, which was filtered and resuspended in fresh water. The acidity of this suspension was adjusted to pH 7·0 by addition of 40% NaOH. An equal volume of ethanol was added, and after 1 h at room temperature the mixture was filtered to remove insoluble material. The clear filtrate was acidified to pH 2 with dilute hydrochloric acid at 80°C. The precipitate that formed was filtered, washed with

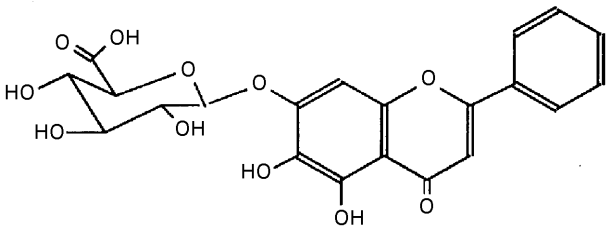

Figure 1. Structure of baicalin (**1**).

water and dried under vacuum in an oven, to obtain a yellow powder (5·2 g). The product was chromatographed over silica (Merck) and eluted with chloroform–methanol–water (65 : 45 : 12) to obtain compound **1** as a yellow crystalline powder (2·2 g). The isolation procedures were monitored to optimize antibacterial activity in the final product.

Compound **1** was characterized as baicalin by spectroscopic and chemical methods, in comparison with a reference sample (Central Drug Control Institute, State Public Health Administration, Beijing, China).

Melting point: 178–197°C (with decomposition) (Mettler FP50 melting point apparatus; uncorrected). IR ($\nu\,cm^{-1}$) nujol: 3400, 1720, 1660, 1060 (Perkin–Elmer 681 spectrometer). UV (λ_{max} nm) methanol: 277, 314 (Shimadzu UV-160A spectrometer). High resolution FABMS (m/z) Na+ matrix: found 447·0940, required 447·0927 for $C_{21}H_{18}O_{11} + H^+$. ^{1}H NMR (250 MHz, DMSO-d_6, δ ppm, TMS = 0): 12·60, 8·69, 8·08, 7·61, 7·05, 5·52, 5·31, 5·25, 4·05. ^{13}C NMR (62·5 MHz, DMSO-d_6, δ ppm, TMS = 0): 93·8, 104·7, 106·2, 126·5, 129·3, 130·6, 130·9, 132·2, 146·8, 149·2, 151·3, 163·6, 182·6 (all flavone), 71·3, 72·8, 75·3, 75·5, 99·9, 170·1 (all sugar).

Microbiology

Staphylococcus aureus strains NCTC 6751, NCTC 11940 (methicillin resistant), NCTC 9968 and NCTC 11561 (both penicillin resistant) were obtained from The National Collection of Type Cultures (NCTC, Colindale, London, UK). β-Lactam antibiotics were obtained from Sigma (Poole, UK). Iso-sensitest broth, agar and nutrient broth were obtained from Oxoid (Basingstoke, UK). Microtitre plates were obtained from Bibby Sterilin Ltd (Stone, UK).

Preparation of test solution and inoculum

Antibiotic test solutions were prepared by dissolving β-lactams (1 mg mL^{-1}) in sterile water.

Baicalin (500 μg mL^{-1}) was dissolved in 1% ammonia solution and diluted with sterile water to the required test concentrations.

Test organisms were incubated in 20 mL Iso-sensitest broth for 18 h at 37°C. The cell cultures were centrifuged at 4000 rev min^{-1} for 15 min, the cell pellets washed with saline, recentrifuged, and resuspended in saline. The cell concentrations were adjusted with saline to give 10^8 colony-forming units (CFU) mL^{-1} using a predetermined calibration curve of absorbance at 500 nm against viable count (Richards & Xing 1993). This cell suspension was diluted with double strength broth to 10^6 CFU mL^{-1}. Overdried Iso-sensitest agar plates were used for determining CFU.

Minimum inhibitory concentration (MICs) determination

MICs were determined using a microtitre method as described in the literature (American National Standards Institute 1991) using an Iso-sensitest broth medium. Antibiotic test solution (100 μL) was added to the first row of wells, then twofold serial dilutions performed by transferring 50 μL to the next well-row and subsequent rows containing 50 μL sterile water to give a final concentration of 8 μg mL^{-1}. An inoculum (50 μL 10^6 cells mL^{-1} in double strength broth containing the required baicalin concentration) was added into each plate well to give a final cell concentration in all wells of 5×10^5 cells mL^{-1}. The plates were covered and incubated at 37°C for 18 h. The MIC was taken as the lowest duplicate concentration of antibacterial at which the test organism did not show visible growth. Positive controls contained corresponding concentrations of solvent.

Checkerboard determinations

Checkerboard determinations in duplicate of β-lactam/baicalin combinations were performed as previously described (Lorian 1991) with modification (Richards & Xing 1991). The inoculum (50 μL 10^6 cells mL^{-1} in double strength medium) was added to each plate well to give a final concentration of the inoculum in all the wells of 5×10^5 cells mL^{-1}. Plates were covered and incubated at 37°C for 18 h before MIC determinations.

Killing curve determinations

Viable counts for the determination of killing-curves were performed as previously described (Richards & Xing 1993) using a culture medium volume of 100 μL. Inocula of 5×10^5 cells mL^{-1} were exposed to the antibacterials either singly or in combination with baicalin at an incubation temperature of 37°C. After contact times of 0, 0·5, 1, 2, 4, 6 and 24 h, a 10-μL sample of each incu-

bated mixture was inactivated by addition of nutrient broth (90 μL) containing 0·125% lecithin (BDH, UK) and 3% (v/v) Tween 80 (ICI, UK). Subsequent dilution plating on overdried Iso-sensitest agar plates in quadruplicate and incubation at 37°C for 18 h allowed counting of growing colonies. The lowest detectable limit for counting was 10^3 CFU mL^{-1}. Positive controls were used containing similar cell and solvent concentrations.

Results

MIC determinations

The MICs for baicalin and β-lactams against four strains of *S. aureus* are shown in Table 1.

Baicalin showed weak activity against all tested strains of *S. aureus* at MICs of 64 μg mL^{-1}. Two penicillin-resistant strains (NCTC 9968 and 11561) were resistant to benzylpenicillin and ampicillin, but susceptible to methicillin, while MRSA (NCTC 11940) showed resistance to all β-lactams tested, both of the penicillins and a cephalosporin, cefotaxime.

Checkerboard determinations

The MICs of baicalin plus β-lactams against MRSA and penicillin-resistant *S. aureus* are shown in Table 2. Baicalin at a concentration 75% lower than its MIC, when in combination with β-lactams significantly reduced their MIC values.

Figure 2 shows the synergistic activity for all combinations of baicalin and tested β-lactams.

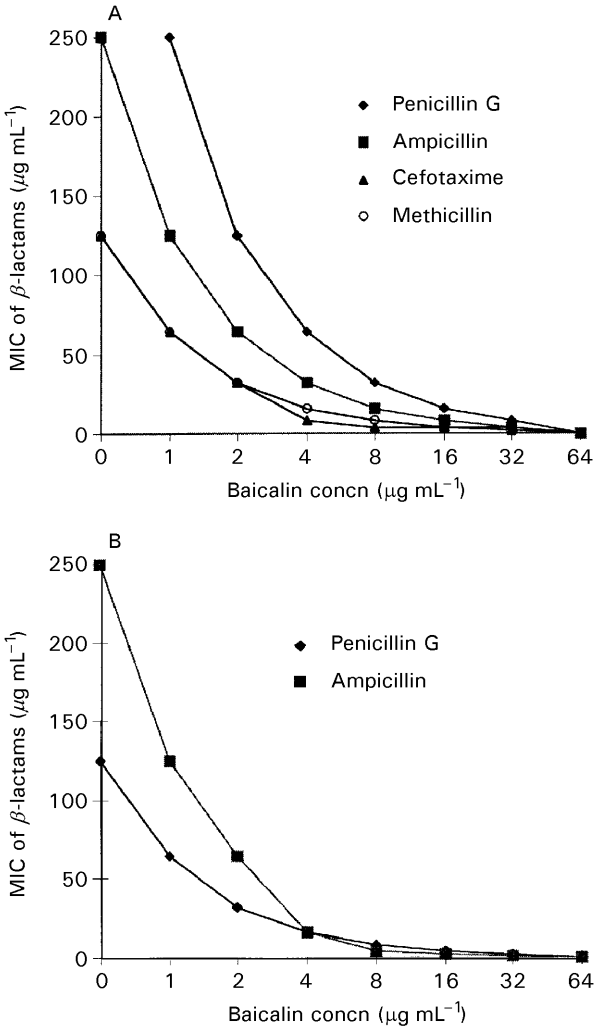

Figure 2. Potentiation of β-lactams by baicalin against (A) methicillin-resistant *Staphylococcus aureus* NCTC 11940 and (B) penicillin-resistant NCTC 9968.

Table 1. MICs (μg mL^{-1}) for baicalin and β-lactams used against *S. aureus* strains.

S. aureus (NCTC) strains	Baicalin	Benzylpenicillin	Ampicillin	Methicillin	Cefotaxime
6571	64	0·032	0·032	< 8	< 8
9968*	64	125	250	< 8	< 8
11940**	64	250	250	125	125
11561*	64	250	500	< 8	< 4

*Penicillin-resistant, **methicillin-resistant.

Table 2. MICs (μg mL^{-1}) for β-lactams used in combination with baicalin (16 μg mL^{-1}) against penicillin and methicillin-resistant strains of *S. aureus*.

Combination of agents	*S. aureus* NCTC 9968*	*S. aureus* NCTC 11940**	*S. aureus* NCTC 11561*
Baicalin/benzylpenicillin	4	16	16
Baicalin/ampicillin	2	8	8
Baicalin/methicillin	< 4	4	< 4
Baicalin/cefotaxime	< 4	4	< 4

*Penicillin-resistant, **methicillin-resistant.

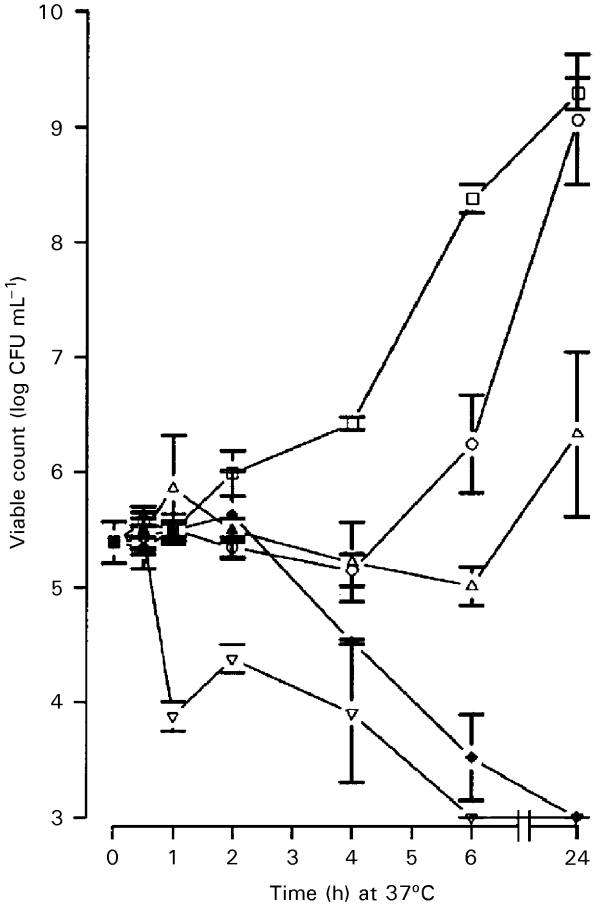

Figure 3. The effect of methicillin combined with baicalin on the viable counts of methicillin-resistant *Staphylococcus aureus* (NCTC 11940). □, Control (bacterial culture with corresponding solvent); ○, baicalin 25 μg mL^{-1}; △, methicillin 50 μg mL^{-1}; ▽, methicillin 50 μg mL^{-1} plus baicalin 25 μg mL^{-1}; ◆, methicillin 12·5 μg mL^{-1} plus baicalin 25 μg mL^{-1}. The values plotted are the means of four observations and the vertical bars indicate the standard errors of the mean.

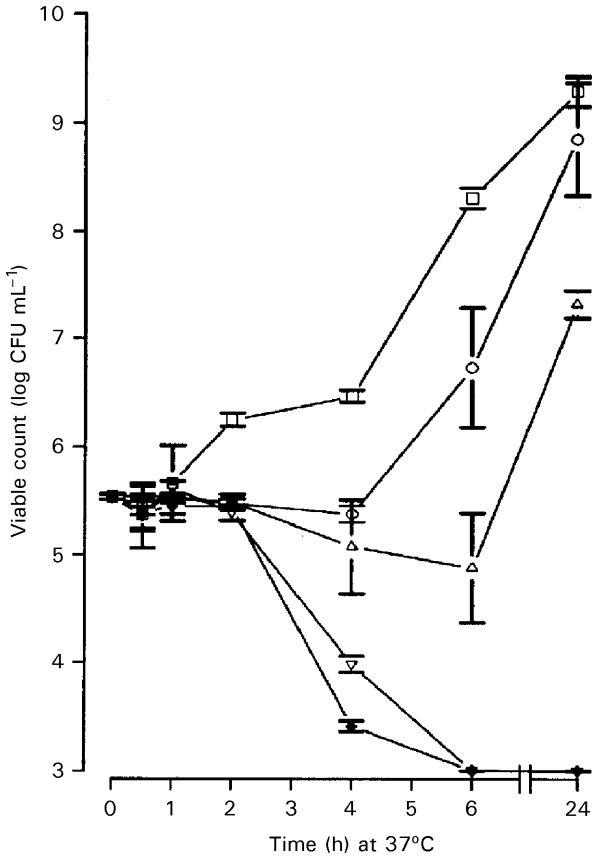

Figure 4. The effect of cefotaxime combined with baicalin on the viable counts of methicillin-resistant *Staphylococcus aureus* (NCTC 11940). □, Control (bacterial culture with corresponding solvent); ○, baicalin 25 μg mL^{-1}; △, cefotaxime 50 μg mL^{-1}; ▽, cefotaxime 50 μg mL^{-1} plus baicalin 25 μg mL^{-1}; ◆, cefotaxime 12·5 μg mL^{-1} plus baicalin 25 μg mL^{-1}. The values plotted are the means of four observations and the vertical bars indicate the standard errors of the mean.

Increasing the concentration of baicalin in the combination resulted in an apparent decrease in the MICs for each β-lactam.

Killing curve determinations

Sample killing curves resulting from baicalin alone and in combination with β-lactams against MRSA and penicillin-resistant *S. aureus* are presented in Figures 3 and 4 . The control showed no reduction in the counts of CFU from control inoculum.

Figure 3 shows that baicalin (25 μg mL^{-1}) combined with methicillin (12·5μg mL^{-1} or higher) caused a reduction of 1×10^2 CFU mL^{-1} for MRSA in 6 h and to below the lowest detectable limit (10^3 CFU mL^{-1}) in 24 h.

Figure 4 shows that viable counts for MRSA were not only reduced by baicalin (25 μg mL^{-1}) in combination with cefotaxime (12·5 μg mL^{-1} or higher), from 5×10^5 to below 10^3 CFU mL^{-1} in 6 h, but that the counts were maintained under the detectable limit for over 24 h.

Similar decreases in resistance of microorganisms to antibiotics were obtained with combinations of baicalin with other β-lactam antibiotics. Against penicillin-resistant *S. aureus* (NCTC 9968), both benzylpenicillin and ampicillin at a concentration of 10 μg mL^{-1} in combination with baicalin at 25 μg mL^{-1} reduced the CFU mL^{-1} count by 1×10^3 over 4 h. The reduced counts did not recover in 24 h. Ampicillin (12·5 μg mL^{-1}) in combination with 25 μg mL^{-1} baicalin reduced the viable counts from 5×10^5 to 1×10^3 CFU mL^{-1} in 4 h and maintained the counts under the detectable limit over 24 h against a penicillin-resistant strain of *S. aureus* (NCTC 11561).

Baicalin (25 μg mL^{-1}) in combination with ampicillin (50 μg mL^{-1}) reduced the viable counts of MRSA over 6 h by more than 5×10^3 CFU mL^{-1} and maintained the reduced counts below 1×10^3 CFU mL^{-1} over 24 h. The same concentra-

tion of baicalin combined with ampicillin ($12{\cdot}5\,\mu g\,mL^{-1}$), while reducing the viable counts of MRSA from 5×10^5 to 5×10^3 CFU mL^{-1} over 6 h, did not maintain this level of inhibition, with counts recovering and increasing to approximately 10^9 CFU mL^{-1} after 24 h.

Discussion

A flavone isolated from the Chinese herb Xi-nan Huangqin has been identified as baicalin by spectroscopic and chemical methods. Although the isolated baicalin used alone showed only moderate antibacterial activity against *S. aureus*, significant synergistic activities against both MRSA (NCTC 11940) and penicillin resistant *S. aureus* in combination with β-lactams were observed.

In checkerboard tests, results showed that for the antibiotics tested, baicalin markedly enhanced the activities of β-lactams against MRSA by reducing MICs (Table 2). Against a β-lactamase-producing *S. aureus* (NCTC 11561) similar synergistic activity was observed. The correlation curves based on the MICs of β-lactams against the concentration of baicalin not only showed marked potentiation of β-lactam-activities by baicalin, but also demonstrated activity–dose relationships for these combinations (Figure 2). The curves indicate that the MICs of β-lactams fell with an increase in the concentration of baicalin. For example, baicalin ($8\,\mu g\,mL^{-1}$) reduced the MIC of methicillin against MRSA to $8\,\mu g\,mL^{-1}$, while baicalin ($16\,\mu g\,mL^{-1}$) reduced the MIC to $4\,\mu g\,mL^{-1}$.

The viable counting results were equally impressive. In combination with baicalin, methicillin ($12{\cdot}5\,\mu g\,mL^{-1}$) killed approximately 99% of MRSA cells in 6 h. The activity of cefotaxime ($12{\cdot}5\,\mu g\,mL^{-1}$) was also enhanced, with approximately 99·9% of MRSA cells killed in 6 h. Ampicillin also showed moderate bactericidal activity in combination with baicalin against MRSA, although it was not as effective as either methicillin or cefotaxime. However, baicalin enhanced the activities of both benzylpenicillin and ampicillin at $10\,\mu g\,mL^{-1}$ against a penicillin resistant strain (*S. aureus* NCTC 9968), killing 99·9% of cells in 4 h. Furthermore, ampicillin ($12{\cdot}5\,\mu g\,mL^{-1}$) killed 99·9% of resistant *S. aureus* NCTC 11561 cells in 4 h when used in combination with baicalin.

Results of viable count determinations were consistent with those of the checkerboard tests. Both sets of results suggested that baicalin combined with a range of β-lactams had synergistic activity by enhancing β-lactam activity in the combination. The enhanced in-vitro activity of β-lactams in combination with baicalin (16–$25\,\mu g\,mL^{-1}$) inhibited and killed MRSA and/or penicillin-resistant *S. aureus* at relatively low ranges of β-lactam concentrations ($2–16\,\mu g\,mL^{-1}$) in-vitro. The concentration of $16\,\mu g\,mL^{-1}$ has been regarded by Livermore (1993) as the breakpoint concentration for clinical susceptibility.

These results indicate that the effects of β-lactam/baicalin combinations against *S. aureus* may arise from contributions from three distinct types of activities. The first of these is due to a weak/moderate direct antibacterial action of baicalin on cell growth. The second mechanism arises from the ability of baicalin to inhibit the β-lactamase hydrolysis of susceptible penicillins and hence restore cell sensitivity to the penicillin. The third mechanism involves an action against MRSA, which is not dependent upon β-lactamase inhibition, but may well be associated with the inhibitory interactions between β-lactams and penicillin-binding proteins.

As a major constituent of the Chinese herb Huangqin, baicalin has been studied extensively. Pharmacological studies have shown that baicalin has a wide range of bioactivity: aldose-reductase inhibition, anti-inflammatory and anti-allergic activity (Williamson & Evans 1988), anti-thrombotic activity (Harborne & Baxter 1993), anti-anaphylactic activity (Abe et al 1990) and antibacterial activity (Tsao et al 1982). The parent herb of baicalin has been in use for more than a thousand years in China and Japan (Huang et al 1994). It has been reported that oral administration of aqueous extracts of the herb (4 or $5\,g\,kg^{-1}$) to dogs three times daily for eight weeks did not produce any significant abnormalities in either blood tests or histology of internal organs. Loose bowel movement occurred in the high dosage group but disappeared upon discontinuation of administration (Wang 1983). The literature-reported value for intravenous LD50 for baicalin in the mouse is $> 3\,g\,kg^{-1}$ (Duke 1998). Although in other animal models the toxicity may be higher, the traditional use of the parent herb Huangqin suggests that the toxicity of baicalin in man is low.

From this study it may be concluded that baicalin has the potential to restore the effectiveness of β-lactam antibiotics against MRSA and other β-lactam-resistant *S. aureus*. In view of its limited toxicity, baicalin offers potential for development as a possible adjunct to β-lactam treatments against otherwise resistant strains of microorganisms.

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References

Abe, K.-I., Inoue, O., Yumioka, E. (1990) Biliary excretion of metabolites of baicalin and baicalein in rats. Chem. Pharm. Bull. 38: 208–211

American National Standards Institute (1991) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 2nd edn, NCCLS Document M7-A2, Vol. 10, No. 8, pp 12–21

Chinese Pharmacopoeia Committee (1985) Chinese Pharmacopoeia I. People's Health Publishing House and Chemical Industry Press, Beijing, pp 271–272

Duke (1998) Duke's Phytochemical and Ethnobotanical Database USAD-ARS-NGRL, Beltsville Agricultural Research Center, Beltsville, Maryland. Available at: http:www.ars-grin.gar/duke/dosage.html [accessed 15 May 1999]

Harborne, J., Baxter, H. (1993) Phytochemical Dictionary, A Handbook of Bioactive Compounds from Plants. Taylor & Francis, London, Washington, DC, pp 392

Huang, H. C., Wang, H. R., Hsieh, L. M. (1994) Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. Eur. J. Pharmacol. 251: 91–93

Livermore, D. M. (1993) Activity of inhibitor combinations. J. Antimicrob. Chemother. 31 (Suppl.): 9–17

Lorian, V. (1991) Antibiotics in Laboratory Medicine.3rd edn, Williams & Wilkins, Baltimore, USA, pp 436–441

Mulligan, M. E., Murray-Leisure, K. A., Ribner, B. S., Standiford, H. C., John, J. F., Korvick, J. A., Kauffman, C. A., Yu, V. L. (1993) Methicillin-resistant *Staphylococcus aureus*: a consensus review of the microbiology, pathogenesis, and epidemiology with implications for prevention and management. Am. J. Med. 94: 313–328

O'Brien, T. F. (1986) The International Survey of Antibiotic Resistance Group. Resistance to antibiotics at medical centre in different parts of the world. J. Antimicrob. Chemother. 1 (Suppl. C): 243–253

Richards, R. M. E., Xing, D. K. L. (1991) Evaluation of synergistic effects of combinations of antibacterials having relevance to treatment of burn wound infections. Int. J. Pharm. 75: 81–88

Richards, R. M. E., Xing, D. K. L. (1993) In vitro evaluation of the antimicrobial activities of selected lozenges. J. Pharm. Sci. 82: 218–220

Tsao, T. F., Newman, M. G., Kwok, Y. Y., Horikoshi, A. K. (1982) Effect of Chinese and western antimicrobial agents on selected oral bacteria. J. Dental Res. 61: 1103–1106

Wang, Y. S. (1983) Pharmacology and Applications of Chinese Herbs. People's Health Publishing House, Beijing, pp 1022–1027

Williamson, E. M., Evans, F. J. (1988) Potter's New Cyclopaedia of Botanical Drugs and Preparations, Revised edn, The C. W. Daniel Co., Ltd, Saffron Walden, Essex, UK, p. 362