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โดย

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มหาวิทยาลัยเทคโนโลยีสุรนารี

remedies (Teubert *et al.*, 1977; H' aznagy *et al.*, 1976), against β-lactam-resistant *S. aureus* and MRSA when used alone and in combination with β-lactam antibiotics.

Materials and Methods

Flavonoids Sources and Structures

Baicalin (the 7-glucuronide of baicalein) was isolated from the Chinese herb *Scutellaria amoena* C.H. Wright and identified by chemical and spectroscopic methods and compared with a reference sample (The Cenural Drug Control Institute, State Public Health Administration, Beijing). The structure was confirmed by x-ray crystallographic analysis of the methyl ester derived from the isolate. The crystallographic data are filed in the Cambridge Structural Databasc (CSD) and will be published elsewhere. Other flavonoids were obtained from Sigma-Aldrich (Gillingham-Dorset, UK), Lancaster Synthesis (Morecambe, UK) and Apin (Abingdon, UK).

Minimum Inhibitory Concentration (MIC) Derterminations

MIC determinations were carried out using a microtiter method as described in the literature (American National Standards Institute, 1991) using Iso-sensitest broth (Oxoid). The test strains included *S. aureus* NCTC 11,940 (MRSA); 6 fresh Clinical MRSA (from Diagnostic Department, Edinburgh Royal Infirmary) which were also ceftazidime-resistant (MICs > 32 μg/ml); *S. aureus* NCTC 9,968 and 11,561, both penicillin-resistant; 25 recent clinical strains of penicillin-resistant *S. aureus* (from Microbiology Department, Aberdeen Royal Infirmary) and 2 recent clinical *Staphylococci* (from Microbiology Department, Aberdeen Royal Infirmary) which were β-lactamase producers and coagulase negative. The bacterial inoculum used in these tests was 2.5×10^5 CFU/ml and the concentration of flavonoid was 25 μg/ml unless otherwise specified. Incubation was at 32°C for 24 h for MRSA and 37°C for 24 h for the other strains. Ceftazidime was obtained from Glaxo Welicome and all other antibiotics were from Sigma Co.

Viable Counts

Viable counts were performed using the microtiter method described in Richards and Xing (1993).

Electronmicroscopy

Galangin dramatically decreased the MICs of selected β-lactam antibiotics when used in combination. Therefore, galangin was chosen for electronmicroscopy study when used singly and in combination.

Subculture of *S. aureus* NCTC 11,940 was incubated at 37°C in fresh Iso-sensitest broth in 250 ml conical flasks with shaking at 100 oscillation/min for 18 h This culture was further incubated in fresh Iso-sensitest broth for 4 h, incubation with shaking in a water bath at 100 oscillation/min. Then 40 ml of the log phase culture was removed and inoculated separately into 360 ml of prewarmed Iso-sensitest broth and the same broth containing, galangin, benzylpenicillin alone and in combination, respectively. After 4 h incubation with shaken in a water bath at 100 oscillation/min at 37°C, the cell pellet was collected, treated and examined under electronmicroscope as described by Richards *et al.* (1995).

Enzyme Assays

The β-lactamases of *Bacillus cereus (B. cereus)* and *Enterobacter cloacae (E. cloacae)* were obtain from Sigma (Poole, England). Enzymes activities were adjusted to concentrations sufficient to hydrolyse 50 - 60% substrate in 5 min. Flavonoids were pre-incubated with enzyme in 50 mM sodium phosphate buffer (pH 7) at 37°C for 5 min prior to substrate addition. Time-course assays were carried out using methanol/acetic acid (100:1) as stopping reagent. The analyses of the remaining substrate were determined by reverse-phase HPLC (Reading and Farmer, 1983) using acetronitrile/acetate as a mobile phase.

Results and Discussion

MIC Derterminations

Thirty six flavonoids were tested for activity and their structures are shown in

Table 1. Minimum inhibitory concentration (μg/ml) of amoxicillin alone and in *combination with galangin 12.5 μg/ml against clinical isolates of Staphylococci.*

Organism	Strain lab. No.	Amoxicillin alone	Amoxicillin plus galangin 12.5 μg/ml
Penicillin-resistant	321	2	< 0.25
	108	250	< 0.25
	141	> 250	< 0.25
	296	16	< 0.25
	684	64	< 0.25
	352	125	<0.25
	543	250	< 0.25
	975	125	0.5
	593	125	< 0.25
	718	250	< 0.25
	349	64	< 0.25
	360	64	2
Methicillin-resistant	588	32	< 0.25
S. aureus (MRSA)	68-15	64	< 0.25
	71-16	250	< 0.25
	70-15	> 250	< 0.25
Coagulase-negative Staphylococci	428,605	16	< 0.25*

* galangin at 25 μg/ml

Table 2. Minimum inhibitory concentration (μg/ml)* of β-lactams used alone and in combination with 25 μg/ml of the following flavonoids againsts *S. aureus* NCTC11940 (MRSA).

	β-lactam alone	β-lactam plus 25 μg/ml flavonoids			
Compound		Baicalin	Apigenin	Luteolin	Galangin
Methicillin	210	6	0.2	0.1	0.1
Amoxicillin	250	3	45	0.2	0.1
Ampicillin	350	3	16	0.1	0.1
Cefotaxime	150	2	0.1	0.1	0.1
Cloxacillin**	1,000	1	0.5	N/D	2

* MIC presented as Geomean of 3-5 observations

** data obtained from cloxacillin-resistant strain induced in this lab

Figure 1. All flavonoids tested were described in the International Patent Application (PCT/GB98/00512) (Richards *et al.*, 1998). The twelve fresh clinical isolates of penicillin-resistant *S. aureus,* four isolates of methicillin-resistant *S. aureus* (MRSA) and a clinical isolates of coagulase-negative staphylococci tested were made sensitive to amoxicillin by galangin 12.5 μg/ml and had their Minimum Inhibitory Concentrations (MICs) reduced from an initial range of 2- >250 μg/ml to a range of < 0.25-2 μg/ml (Table 1).

In addition, six clinical isolates of ceftazidime-resistant *S. aureus* strains with MICs 32 - 250 μg/ml had their resistance to ceftazidime reversed by galangin 25 μg/ml to MICs of < 0.25 μg/ml, while the MICs for galangin alone were > 250 μg/ml. The highest fractional inhibitory concentration (FIC) for these ceftazidime plus galangin combinations was only marginally over 0.1. An FIC of 0.1 indicates a high level of synergistic activity since values below 0.5 are widely accepted as representing synergism between two antibacterials (Sabath, 1967). A type strain of MRSA (NCTC 11,940) also had its resistance to methicillin, cloxacillin, amoxicillin, ampicillin and cefotaxime reversed when any of these β-lactams was combined with 25 μg/ml of baicalin, apigenin, luteolin or galangin (Table2).

Viable Counts

An example of a typical killing curve obtained with penicillin-resistant *S. aureus* (NCTC 9,968) using viable counts is given in Figure 2. MICs for benzylpencillin and baicalin against this strain were 125 and 64 μg/ml, respectively. The *S. aureus* strain was tested using the flavonoid alone and in combination. Baicalin at 25 μg/ml had little effect on the bacterial growth rate compared with the control. Benzylpenicillin at 50 μg/ml reduced the viable counts by 1.25 log cycles after about 2 h but then the viable counts recovered so that after 24 h they were 2 log cycles greater than the concentration of cells produced by the initial inoculum. Baicalin at 25 μg/ml plus either benzylpenicillin at 50 or 10 μg/ml reduced the viable counts by 3 log cycles within 2 and 4 h, respectively and maintained that reduction in over 24 h (The lower limit of the counting technique was a suspension of 103 CFU/ml).

Electronmicroscopy

Electronmicroscope investigations clearly showed that the combination of β-lactam antibiotic with galangin caused damage to the ultrastructures of MRSA cells. Figure 3 indicates that galangin 25 μg/ml reduced the thickness of the cell walls compared with the cell walls of the control cells and also apparently delayed cell division. The galangin treated cells were considerably larger than the normal cells. Benzylpenicillin at 25 μg/ml alone apparently had no effect on the cell wall structure but the combination of the antibacterial agents is observed to have affected the integrity of the cell walls and led to an increase in cell size. This latter effect is apparently due to inhibition of cell division.

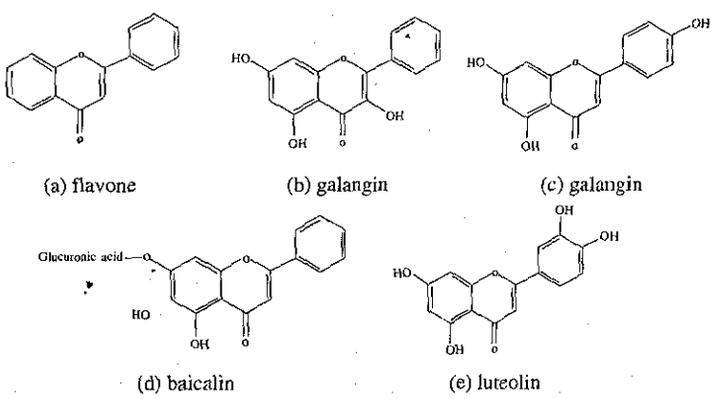

Figure 1. Structure of example flavonoids tested (Source: Indofine chemical company, 2002).

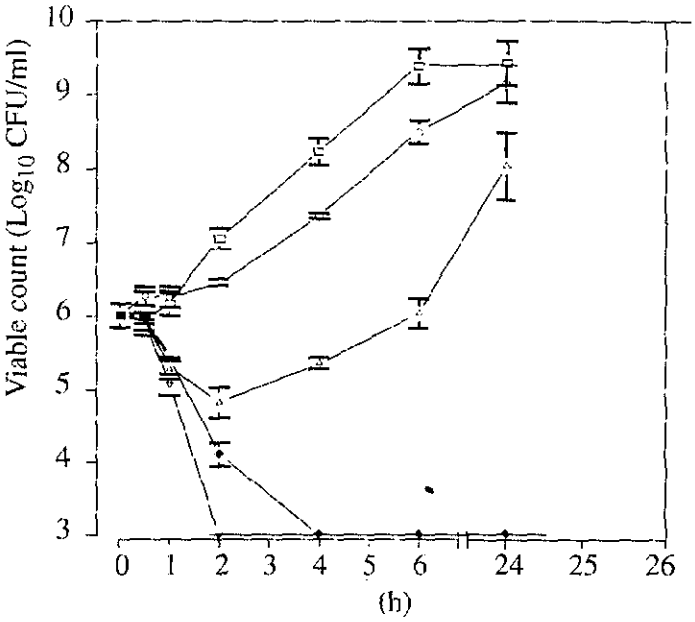

Figure 2. **The effect of benzylpenicillin combined with baicalin on the viable counts of penicillin-resistant *Staphylococcus aureus* (NCTC 9968). □, control (bacterial culture with corresponding solvent); ○, baicalin 25 μg/ml; △, benzylpenicillin 50 μg/ml; ◆, benzylpenicillin 10 μg/ml plus baicalin 25 μg/ml; ▽, benzylpenicillin 50 μg/ml plus baicalin 25 μg/ml; the values plotted are the means of 4 observations, and the vertical bars indicate the standard errors of the means.**

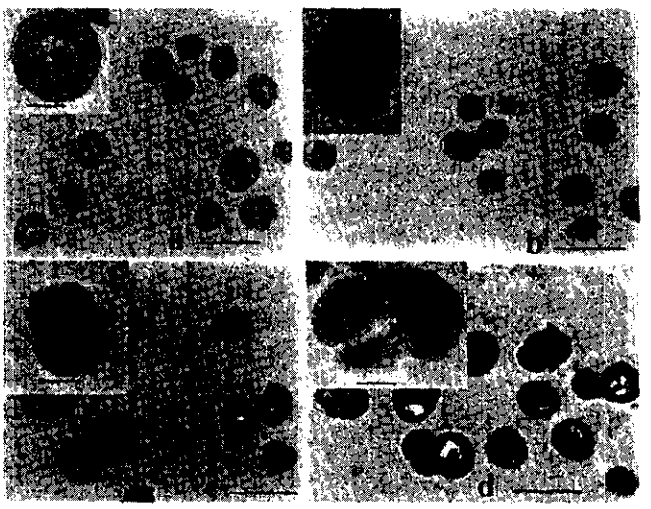

Figure 3. **Ultrathin sections of log phase *S. aureus* NCTC 11,940 (MRSA) grown in Iso-sensitest broth containing: (a) drug-free (control); (b) 25 μg/ml benzylpenicillin; (c) 25 μg/ml galangin; (d) 25 μg/ml benzylpenicillin plus 25 μg/ml galangin (a, b, c, d, original magnification, x 17,480; bar, 1 μm; *Inset*, a, b, d, original magnification, x 42,800; c, x 32,500; bar, 0.25 μm).**

Enzyme Assays

The ability of flavonoids to inhibit the *in vitro* activity of β-lactamases varied considerably. Figure 4 indicates that galangin has an inhibitory activity against β-lactamaseI from *B. cereus*. Galangin had some activity and tectochrysin and 6-chloro-7-methylflavone showed greater activity. Against penicillinase type IV from *E. cloacae*, apigenin showed marked inhibitory activity but none of other flavonoids tested showed appreciable activity. These results indicate that in addition to the direct effect on cell structure and cell division, the resistance reversing activity of flavonoids against bacteria might also include inhibition of β-lactamase activity.

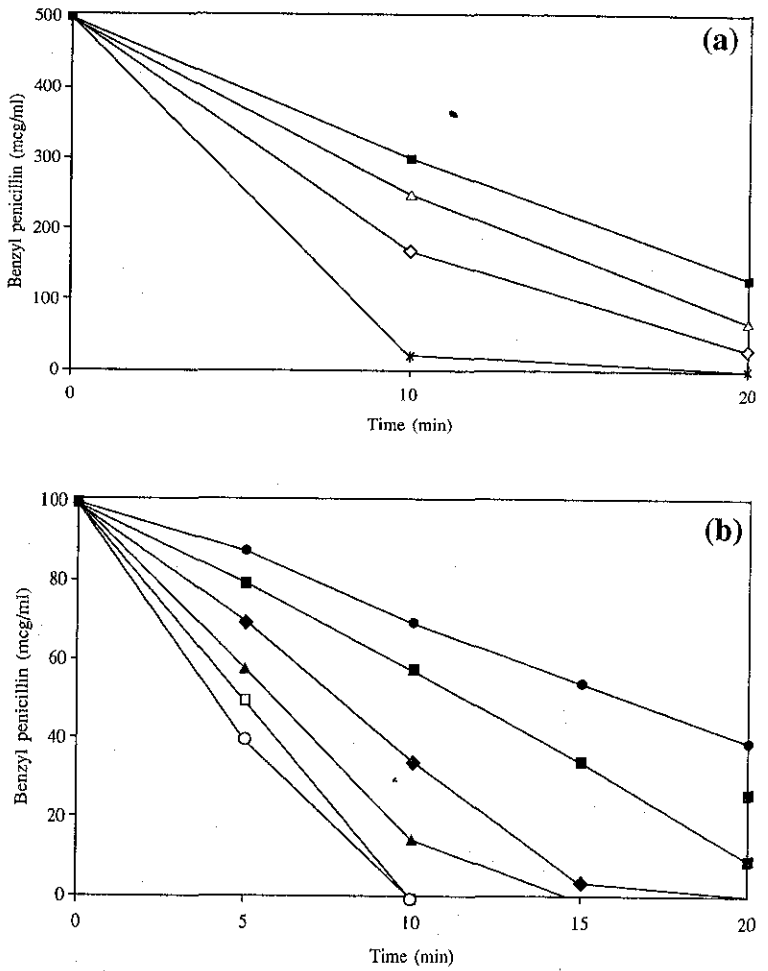

Figure 4. The inhibitory activity of flavonoids against β-lactamase in hydrolyzing benzylpenicillin. (a) β-lactamase used from *B. cereus*; symbol represents flavonoids (200 μg/ml); *, control (without flavonoids); ◇, galangin; Δ, 6-chloro-7-methylflavone; ■, tectochrysin. (b) β-lactamase used from *E. cloacea*; symbol represent concentrations (μg/ml) of apigenin; o, control (without apigenin); □, 20; ▲, 40; ◆, 60; ■, 80; ●, 100.

Discussion

The results indicate that flavonoids not only have an activity of their own against β-lactam-resistant staphylococci but also have the ability to reverse the resistance of such bacterial strains to the activity of the primary antibiotics. This may involve two mechanisms of action by the flavonoids. The first is on the integrity of the cell wall and on septum formation prior to cell division. This implies an effect on protein synthesis including an effect on penicillin-binding proteins. The second mechanism of β-lactam activity is via inhibition of the activity of certain β-lactamase enzymes. The first action could also include an effect on the production and/or release of β-lactamase enzymes within and from the cell walls (Yam *et al.*, 1998). In the last two decades, β-lactamase inhibitors like clavulanic acid have played an important role in fighting β-lactam-resistant bacteria. These inhibitors work as suicide compounds to react with the enzymes since they share the same key structure with β-lactam antibiotics (Coulton and Francois, 1994). Recent studies demonstrated that clavulanate caused a considerable induction of β-lactamase expression and an increase of clavulanate concentration was followed by an elevation in β-lactamase production (Stapleton *et al.*, 1995; Tzouvelekis *et al.*, 1997). This indicates that the presently available β-lactamase inhibitors can also lose their activity by the same mechanism as the β-lactam antibiotics. Our research provides an unique example that flavonoids without a β-lactam structure can reverse bacterial resistance to β-lactams via multiple mechanisms. Because of this structural dissimilarity these compounds are unlikely to induce β-lactamase production. It should also be remembered that conventional β-lactamase inhibitors, unlike flavonoids, cannot reverse the resistance of MRSA, which is one of the most dangerous bacterial pathogens.

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