Antagonistic interactions between the flavonoids hesperetin and naringenin and β-lactam antibiotics against *Staphylococcus aureus*

B. J. DENNY*, P. W. J. WEST† and T. C. MATHEW†
*Department of Pharmaceutical Chemistry, Faculty of Pharmacy; and
†Department of Medical Laboratory Sciences, Faculty of Allied Health,
Kuwait University, Kuwait

Accepted: 25 April 2008

ABSTRACT

Flavonoids are a group of polyphenolic plant compounds with a range of biological activities. This study shows that the flavonoids hesperetin and naringenin have antibacterial activity against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Minimum inhibitory concentrations for hesperetin were 250 and 500 μg/mL, respectively, and for naringenin were 125 and 250 μg/mL, respectively. This effect was reversed by the β-lactam antibiotics methicillin, penicillin and oxacillin, but not by cefoxitin. For bacteria growing in the presence of these antibiotics, the flavonoids had no effect on the levels of β-lactamase enzymes and PBP-2′ compared to controls. Electron microscopy showed abnormal morphology in bacteria treated with subinhibitory concentrations of flavonoids. These results are interesting because previous studies have reported synergistic interactions between flavonoids and β-lactam antibiotics. It is suggested that an interaction removes both inhibitors from the bacterial growth milieu.

KEY WORDS: Electron microscopy.
Methicillin resistance.
Microbial sensitivity tests.
Staphylococcus aureus.

Introduction

Flavonoids are a group of benzo-γ-pyrone derivatives widely distributed in the plant kingdom. Over 4000 plant-derived flavonoids have been described.[1] The exact function of flavonoids in plants is not known but they are usually synthesised in response to external damage. They have been used for centuries in Chinese medicine in the form of medicinal plants.[2] The compounds have a range of biological effects including antimicrobial,[3] antioxidant,[4] anticancer,[5] anti-inflammatory and antiviral activity: however, the exact mechanisms involved are not fully understood.

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are major nosocomial pathogens. These organisms exhibit resistance to β-lactamase-sensitive and -resistant antibiotics by virtue of a mutation of a penicillin-binding protein.[6] Resistance to β-lactam antibiotics can also result from increased production of β-lactamases.[7] Previous work has shown that flavonoids cause morphological changes in MRSA.[8,9]

The flavonoids hesperetin and naringenin (Fig. 1) are found in high concentrations in citrus fruits such as lemons and grapefruit.[10] This study aims to investigate interactions between hesperetin and naringenin and β-lactam antibiotics against *S. aureus*. In addition, electron microscopy (EM) is used to examine morphological changes caused by the flavonoids in the presence and absence of methicillin.

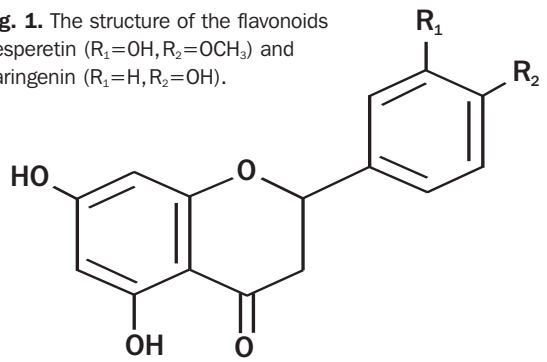

Fig. 1. The structure of the flavonoids hesperetin (R_1=OH, R_2=OCH_3) and naringenin (R_1=H, R_2=OH).

Previous study of flavonoids has shown a synergistic effect with methicillin and other antibiotics,[11] and that hesperetin and naringenin have antibacterial effects which are eliminated in the presence of methicillin, penicillin and oxacillin. Cefoxitin has been recommended for the detection of methicillin resistance and reported to be a better predictor than oxacillin.[12]

Materials and methods

Ten epidemiologically distinct strains of MRSA were obtained from the microbiology department of the Faculty of Medicine, Kuwait University. Nine methicillin-sensitive *S. aureus* (MSSA) strains (Amiri Hospital, Kuwait) plus a control strain (ATCC 25923, Faculty of Medicine, Kuwait University) were also included. The strains were stored at –80°C in 10% skimmed milk (Difco, USA).

Hesperetin, naringenin and other chemicals were obtained from Sigma (USA), and nitrocefin, methicillin strips, penicillin, oxacillin and cefoxitin (30 mg) susceptibility

Correspondence to: Dr Brian Denny
Email: briandenny59@yahoo.ie

disks were obtained from Oxoid (UK). The MRSA monoclonal antibody screen for PBP-2′ (Denka-Seiken, Japan) was used.

Stock solutions of the flavonoids were prepared in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the medium was less than 1%. Inhibitors were added to the agar at 50°C. Strains of MRSA were cultured on Mueller-Hinton agar (Acumedia) in the presence and absence of flavonoids. The cultures were inoculated on the agar according to NCCLS procedures,[13] using bacterial suspensions prepared in peptone water (Oxoid, UK).

Strips containing 25 mg methicillin or disks containing penicillin (10 units) or oxacillin (1 mg) were applied and the plates were incubated in air at 37°C overnight and examined for growth. Disks impregnated with nitrocefin were used to screen for β-lactamase production.

Bacteria grown on Mueller Hinton agar in the presence of either flavonoid or methicillin were transferred to scanning EM studs and air dried. The cells were fixed in 1% osmium tetroxide and then coated with carbon and with gold using a sputter coater. The specimens were examined with a JSM 35 scanning electron microscope.[8]

Results

Minimum inhibitory concentration (MIC) for hesperetin and naringenin against 10 strains of MRSA, nine strains of MSSA and the control strain ATCC-25923 were found to be 250 and 500 mg/mL (hesperetin) and 125 and 250 mg/mL (naringenin), respectively. When inhibitory concentrations of hesperetin and naringenin were incorporated in Mueller-Hinton agar, and a methicillin strip or penicillin or oxacillin disks applied, growth of the bacteria occurred predominantly beside the antibiotic strip or disk (Figs. 1 and 2).

Areas of apparent bacterial growth were Gram-stained to exclude the possibility of artifacts caused by precipitation.

Fig. 2. Growth of MRSA around a methicillin strip on Mueller-Hinton agar in the presence of hesperetin.

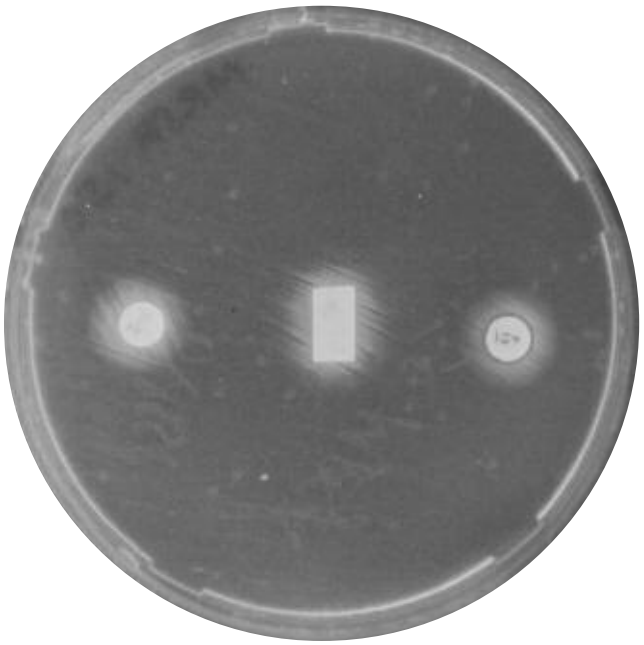

Fig. 3. Growth of MSSA around penicillin (left), methicillin (centre) and oxacillin (right) disks on Mueller-Hinton agar in the presence of naringenin.

Filter paper disks not containing antibiotics did not have this effect. Cefoxitin failed to counteract growth inhibition by hesperetin and naringenin for all the strains of MRSA and MSSA tested.

Bacteria growing beside antibiotic discs in the presence of hesperetin or naringenin were tested qualitatively for the presence of β-lactamase using the chromogenic substrate nitrocefin. β-lactamase breaks down nitrocefin to produce a colour change that was monitored visually. No change in β-lactamase activity was observed between the flavonoid and control groups. Bacteria growing beside antibiotic disks were also tested for PBP-2′ activity (with a monoclonal antibody). No change in PBP-2′ activity was observed between the flavonoid and control groups.

The MRSA cells grown in the presence of subinhibitory concentrations of hesperetin or naringenin showed highly abnormal morphology when viewed using EM (Figs. 4a, b and c). Cells were often smaller and abnormally shaped. When methicillin was added to hesperetin or naringenin, MRSA strains showed a more normal appearance. In addition, particles of white precipitate were seen in association with the bacterial cells (Fig. 4d).

Discussion

To the authors' knowledge this is the first study that has demonstrated antagonism between flavonoids and β-lactam antibiotics, and the first to report the antibacterial properties of hesperetin and naringenin.

The observation that MRSA and MSSA produce the same results indicates an interaction between the flavonoid and the β-lactam antibiotic, resulting in the removal of both from the bacterial growth environment. However, cefoxitin does not remove flavonoids from the growth environment.

Bacteria growing around the antibiotics essentially were

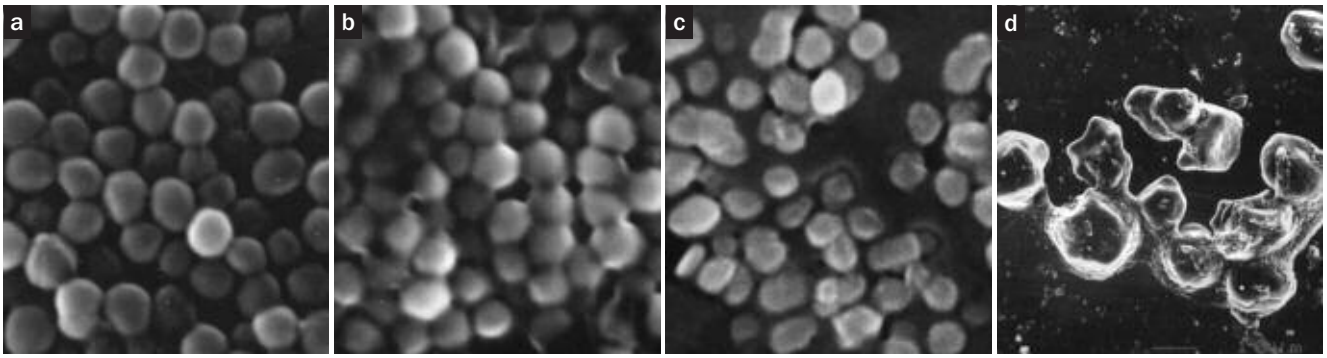

Fig. 4. Scanning electron microscopy of MRSA: a) normal appearance; b) exposed to 500 mg/L hesperetin; c) exposed to 250 mg/L naringenin; and d) embedded in a precipitate of hesperetin (500 mg/L) with methicillin.

normal in morphology and other tested characteristics (β-lactamase and PBP-2′ activity). The flavonoids are always present in large (>100-fold) stoichiometric excess over the antibiotic agent. The EM results indicate that flavonoids probably damage cell walls, which is consistent with results obtained with other flavonoids.[14,15]

Several published studies have shown that flavonoids act synergistically with β-lactam antibiotics to increase bacterial susceptibility,[16–18] but the exact mechanism of this synergy is unclear. Conflicting results may be obtained when a particular flavonoid and antibiotic are assayed against a particular bacterial species. Lack of test method standardisation is probably responsible for many of the discrepancies reported,[3] but application of NCCLS procedures should result in more consistent results.[19]

From a clinical standpoint, the results reported here demonstrate that the effectiveness of antibiotics can be modulated by dietary flavonoids. However, to the authors' knowledge, no epidemiological studies have been conducted to correlate dietary levels of flavonoids with the efficacy of particular antibiotic agents. □

This work was supported by Kuwait University Grant PC02/01. The authors thank Dr. E. Udo (Faculty of Medicine, Kuwait University) for supplying strains of MRSA, and Dr. N. J. Miller (BioLab, London, UK) for useful discussions.

References

1 Herrmann K. Flavonols and flavones in plants: a review. *J Food Technol* 1976; **11**: 433–48.
2 Qin GW, Xu RS. Recent advances on bioactive natural products from Chinese medicinal plants. *Med Res Rev* 1998; *18*: 375–82.
3 Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* 2005; **26**: 343–56.
4 Rice-Evans CA, Miller NJ. Antioxidant activities of flavonoids as bioactive components of food. *Biochem Soc Trans* 1996; **24**: 790–5.
5 Choi SU, Ryu SY, Yoon SK *et al*. Effects of flavonoids on the growth and cell cycle of cancer cells. *Anticancer Res* 1999; **19** (6B): 5229–33.
6 Berger-Bachi B, Rohrer S. Factors influencing methicillin resistance in staphylococci. *Arch Microbiol* 2002; **178**: 165–71.
7 Fluit AC, Visser MR, Schmitz F-J. Molecular detection of antimicrobial resistance. *Clin Microbiol Rev* 2001; **14**: 836–71.
8 West PWJ, Mathew TC, Miller NJ, Electricwala Q. The effect of green tea on the growth and morphology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*. *J Nutr Environ Med* 2001; **11**: 263–69.
9 Stapleton PD, Shah S, Ehlert K, Hara Y, Taylor PW. The β-lactam-resistance modifier (-)-epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus*. *Microbiology* 2007; **153**: 2093–103.
10 Erlund I, Silaste ML, Alfthan G, Rantala M, Kesantemi YA, Aro A. Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets and diets high or low in fruit and vegetables. *Eur J Clin Nutr* 2002; **56**: 891–8.
11 Fujita M, Shiota S, Kuroda T *et al*. Remarkable synergies between baicalein and tetracycline, and baicalein and beta-lactams against methicillin-resistant *Staphylococcus aureus*. *Microbiol Immunol* 2005; **49**: 391–6.
12 Cauwelier B, Gordts B, Descheemaecker P, Van Landuyt H. Evaluation of a disk diffusion method with cefoxitin (30 microg) for detection of methicillin-resistant *Staphylococcus aureus*. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 389–92.
13 National Committee for Clinical Laboratory Standards. Methods for determining bactericidal activity of antimicrobial agents; Approved guideline (M26-A). Wayne PA: NCCLS, 2000: Vol. 19.
14 Park KM, You JS, Lee HY, Baek NI, Hwang JK. Kuwanon G: an antimicrobial agent from the root bark of *Morus alba* against oral pathogens. *J Ethnopharmacol* 2003; **84**: 181–5.
15 Hamilton-Miller JMT, Shah S. Disorganization of cell division of methicillin-resistant *Staphylococcus aureus* by a component of tea (*Camellia sinensis*): a study by electron microscopy. *FEMS Microbiol Lett* 1999; **176**: 463–9.
16 Zhao WH, Hu ZQ, Hara Y, Shimamura T. Inhibition of penicillinase by epicatechin gallate resulting in restoration of antibacterial activity of penicillin against penicillinase-producing *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2002; **46**: 2266–8.
17 Yam TS, Hamilton-Miller JM, Shah S. The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2′ synthesis and β-lactamase production in *Staphylococcus aureus*. *J Antimicrob Chemother* 1998; **42**: 211–6.
18 Zhao WH, Hu ZQ, Okubo, Hara SY, Shimamura T. Mechanism of synergy between epicatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2001; **45**: 1737–42.
19 National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved guideline (M7-A5) Wayne PA: NCCLS, 2000: Vol. 20.