AGGREGATION OF *STAPHYLOCOCCUS AUREUS* FOLLOWING TREATMENT WITH THE ANTIBACTERIAL FLAVONOL GALANGIN

T.P.T. Cushnie [1],

V.E.S. Hamilton [1],

D.G. Chapman [1],

P.W. Taylor [2],

A.J. Lamb [1]

[1] School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen. AB10 1FR. UK.

[2] Microbiology Group, Department of Pharmaceutics, School of Pharmacy, University of London, 29-39 Brunswick Square, London. WC1N 1AX. UK.

Abbreviated running headline

Aggregation of *S. aureus* by galangin

Correspondence

A.J. Lamb, School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen. AB10 1FR. UK. E-mail: a.lamb@rgu.ac.uk Telephone: +44 1224 262526 Fax: +44 1224 262555

ABSTRACT

Aim: The flavonol galangin, an antimicrobial constituent of the traditional medicines propolis and *Helichrysum aureonitens*, is being assessed as part of an ongoing investigation into the antibacterial activity of flavonoids. The present study sought to establish whether galangin has an aggregatory effect upon bacterial cells.

Methods and results: In preparatory time-kill assays, 50 μg ml^{-1} galangin was found to reduce colony counts of ~5 x 10^7 cfu ml^{-1} *Staphylococcus aureus* NCTC 6571 by approximately 15,000-fold during 60 min incubation. Subsequent light microscopy studies demonstrated significant increases in the number of large clusters of bacterial cells in populations treated with the flavonol.

Conclusion: Data presented here shows that galangin causes aggregation of bacterial cells.

Significance and impact of study: The finding that galangin causes bacterial cells to clump together may implicate the cytoplasmic membrane as a target site for this compound's activity. More importantly, this observation indicates that decreases in CFU numbers detected in time-kill and minimum bactericidal concentration (MBC) assays in previous investigations were at least partially attributable to this aggregatory effect. This raises the possibility that galangin is not genuinely bactericidal in action, and calls into question the suitability of time-kill and MBC assays for determining the nature of activity of naturally occurring flavonoids.

Keywords

galangin; antibacterial; flavonoid; aggregation; time-kill; MBC; bactericidal; bacteriostatic

INTRODUCTION

Flavonoids are a group of heterocyclic organic compounds that occur widely in the plant kingdom and possess a diverse range of pharmacological properties (Middleton and Chithan 1993; Harborne and Williams 2000). Preparations containing these compounds as the principal physiologically active constituents have been employed extensively in the treatment of human disease (Havsteen 1983; Cushnie and Lamb 2005a). The flavonol galangin (3,5,7-trihydroxyflavone) is an antimicrobial component of at least two such preparations. Galangin is present in propolis (Pepeljnjak *et al.* 1982; Grange and Davey 1990; Bosio *et al.* 2000; Hegazi *et al.* 2000), a resinous substance sometimes referred to as 'bee glue' because of its function in nature, or 'Russian penicillin' following its clinical success as a wound ointment in the USSR (Fearnley 2001). Galangin is also reported to be one of the major constituents of *Helichrysum aureonitens* Sch. Bip. (Asteraceae), a perennial herb used by South African indigenes to treat infection (Afolayan and Meyer 1997).

Recent investigations have shown that galangin has antistaphylococcal activity, with MICs of 25 to 50 μg ml^{-1} against antibiotic sensitive *Staphylococcus aureus* (Cushnie *et al.* 2003; Cushnie and Lamb 2005b), MICs of 50 μg ml^{-1} against penicillin and methicillin resistant *S. aureus* (Cushnie *et al.* 2003), and MICs of 50 μg ml^{-1} or less against quinolone resistant *S. aureus* (Cushnie and Lamb 2006). Inhibitory activity against enterococcal species and strains of *Pseudomonas aeruginosa* has also been reported but at quite high concentrations, approximately 240 μg ml^{-1} and 170 μg ml^{-1} respectively (Pepeljnjak and Kosalec 2004). The mechanism(s) of action underlying this antibacterial activity is not clear, but data is available to indicate that inhibition of topoisomerase enzymes (Cushnie and Lamb 2006) and cytoplasmic membrane perturbation (Cushnie and Lamb 2005b) are both possibilities.

In addition to the above work, time-kill studies with galangin have shown that MIC levels of the flavonol are capable of causing decreases of 1000-fold and more in colony counts of antibiotic sensitive *S. aureus* NCTC 6571 and β-lactamase producing *S. aureus* NCTC 11561 populations (Cushnie *et al.* 2003). Recent MBC assays by Pepeljnjak and Kosalec also indicate that the flavonol reduces colony counts of *S. aureus* below minimum detectable levels (~100 cfu ml^{-1}) when used at concentrations of one to two times the MIC (Pepeljnjak and Kosalec 2004). Viable count reductions

of this magnitude (NCCLS 1999) at one to two times the MIC (Prescott *et al.* 1999) would immediately appear to suggest that galangin activity is bactericidal. Ikigai and colleagues report that the flavonoid (-)-epigallocatechin gallate induces aggregation of bacterial cells however (Ikigai *et al.* 1993). If this effect is also caused by galangin, then it raises the possibility that the decreases in colony counts noted in time-kill and MBC assays may have been caused by bacterial cells clumping together rather than flavonol-induced cell death. In order to address this uncertainty, and gain insight into the antibacterial mechanism(s) of action of galangin, the present study examined flavonol-treated populations of *S. aureus* for aggregation.

MATERIALS AND METHODS

Chemicals

Galangin was purchased from Aldrich (Sigma-Aldrich Company Ltd., Gillingham, UK.), Iso-sensitest nutrient broth and agar were from Oxoid Ltd. (Basingstoke, UK.), and sodium chloride was from Fisher Scientific UK Ltd. (general purpose grade; Loughborough, UK.). Phosphate buffered saline (PBS) tablets were obtained from Sigma (Sigma-Aldrich Company Ltd., Poole, UK.), as was sodium carbonate (anhydrous), the reagent employed for dissolution of galangin. Ammonium oxalate was from May and Baker Ltd. (Dagenham, UK.), crystal violet was from Thornton and Ross Ltd. (Huddersfield, UK.) and ethanol was from Hayman Ltd. (Witham, UK.).

Bacteria

S. aureus NCTC 6571 was sub-cultured and maintained on Iso-sensitest nutrient agar. Bacterial cells in exponential growth phase, prepared in Iso-sensitest nutrient broth, were harvested, washed and resuspended in aqueous 0.9 % (w/v) sodium chloride as described previously (Cushnie *et al.* 2003).

Examination of *S. aureus* populations for decreases in colony forming unit numbers during incubation with galangin

Time-kill assays were performed with galangin so that a suitable flavonol concentration and treatment time could be identified for subsequent light microscopy studies. A variation of the method described

previously was used (Cushnie *et al.* 2003). In brief, experiments were carried out as before but flasks of Iso-sensitest broth supplemented with 50 μg ml^{-1} galangin were prepared after *S. aureus* NCTC 6571 had been harvested and enumerated in order to minimise any flavonol degradation. Also, the cell density of *S. aureus* was elevated from ~5 x 10^5 to ~5 x 10^7 cfu ml^{-1} so that bacterial numbers would be at a readily detectable level in the work that followed. Time-kill assays were performed in duplicate to verify the reproducibility of results.

Examination of *S. aureus* populations for galangin-induced aggregation using light microscopy

Two sterile 100 ml glass flasks (Pyrex; Fisher Scientific UK Ltd.) were prepared containing 10 ml of distilled water and 10 ml of aqueous 0.05 % (w/v) sodium carbonate solution respectively. Following bacterial harvest, washing and enumeration, 10 ml of 100 μg ml^{-1} galangin solution was prepared in 0.05 % (w/v) sodium carbonate solution and pipetted into a third sterile flask. At this point, 35 ml of sterile double strength Iso-sensitest nutrient broth was measured out and inoculated so as to contain ~1 x 10^8 cfu ml^{-1} *S. aureus* NCTC 6571. Ten ml aliquots of this bacterial suspension were then pipetted into the three flasks (bringing the total volume in each to 20 ml), and the flasks were shaken in order to mix the contents and evenly distribute the bacteria. Four 25 μl samples were removed from the flask containing broth only, and applied to the surface of two clean and suitably labelled glass microscope slides (Blue star; Chance Propper Ltd., Smethwick, UK.). In advance of the experiment, these slides had been washed with detergent (Instrument washing detergent; DiverseyLever, Northampton, UK.) and 70 % (v/v) ethanol, then rinsed with water and allowed to dry, before being marked on their undersides with two 1 cm (diameter) circles and labelled appropriately. Following 60 min orbital incubation at 37°C and 100 rpm (IOX400.XX2.C; Sanyo Gallenkamp PLC, Loughborough, UK.), four 25 μl samples were also removed from the flask containing broth and 0.025 % (w/v) sodium carbonate and the flask containing broth, 50 μg ml^{-1} galangin and 0.025 % (w/v) sodium carbonate. Slides were then allowed to air dry at room temperature, before being heat fixed in a Bunsen flame and stained with oxalate crystal violet [100 ml of aqueous 2 % (w/v) crystal violet and 19 % (v/v) ethanol solution mixed with 100 ml of aqueous 1 % (w/v) ammonium oxalate]. Stain was left in contact with bacterial films for 75 seconds before being poured off and carefully rinsed away

with water. When dry, the slides were examined by light microscopy (Unilux-11; Kyowa, Tokyo, Japan.) at x 1000 magnification using immersion oil (Type A; Cargille Laboratories Inc., Cedar Grove, New Jersey, USA.). In order to detect galangin-induced aggregation, the number of bacterial clusters present in each of the four films of untreated, solvent only-treated and galangin-treated bacteria were counted. Tallies were performed for clusters with a visible surface size of 21 to 40 cells, 41 to 100 cells and greater than 100 cells. It should be noted that a thin, darkly stained region around the circumference of each of the bacterial films was ignored during this tallying process, as it was impossible to unambiguously distinguish between bacterial cells and extracellular material / detritus in these areas. Also, to prevent duplicate counting of clusters, slides were examined by panning from left to right and right to left, counting those clusters protruding through the bottom of a field of view, and ignoring those clusters extending through the top of a field of view. In addition to performing these counts, representative photographs were taken of untreated, solvent only-treated and galangin-treated bacteria, using a Leitz DMR microscope and DMRD camera system (Leica UK Ltd., Milton Keynes, UK.). The above study was performed in duplicate to verify the reproducibility of results.

RESULTS

Examination of *S. aureus* populations for decreases in colony forming unit numbers during incubation with galangin

Time-kill assays were initially conducted using galangin at a concentration of 50 μg ml^{-1} [the MIC for ~5 x 10^5 cfu ml^{-1} *S. aureus* NCTC 6571 (Cushnie and Lamb 2005b)]. At this concentration, galangin was found to reduce numbers of ~5 x 10^7 cfu ml^{-1} *S. aureus* NCTC 6571 by 15,000-fold within 60 min incubation (Fig. 1). In light of these results, 50 μg ml^{-1} was selected as a suitable test concentration, and 60 min an appropriate treatment time, for subsequent aggregation studies. Such a short incubation period, it was rationalised, would minimise *S. aureus* cell growth and the incidence of naturally occurring clusters in treated and untreated flasks.

Examination of *S. aureus* populations for galangin-induced aggregation using light microscopy

When samples of untreated and sodium carbonate-treated *S. aureus* were examined by light microscopy, bacteria were found to be quite evenly distributed across the surface of each film, and typically occurring as single cells, cell pairs and to a lesser extent small clusters (Figs. 2a and 2b). Large clusters of bacterial cells were observed infrequently in these samples. Of those large clusters that were seen, many had an elongated appearance and occurred in close proximity to each other in 'veins' running through the film (Fig. 2c). Given their appearance and location it is thought that such clusters represent artefacts of sample preparation, probably formed as a consequence of cells pooling together during evaporation of moisture from bacterial suspensions. Samples of galangin-treated *S. aureus* had a very different appearance when examined by light microscopy. Instead of single cell, cell pair and small cluster formations, the majority of cells observed in galangin-treated samples were in large clusters (Fig. 2d). These clusters did not resemble the elongated cell associations seen in untreated and sodium carbonate-treated samples. They tended to be rounder in shape and larger in size. In addition, clusters observed in galangin-treated samples were quite evenly distributed across the film (not in 'veins') and much more frequent in occurrence (Fig. 3).

DISCUSSION

Time-kill assays conducted in the present study indicate that 50 μg ml^{-1} galangin is capable of causing a 15,000-fold reduction in colony forming unit numbers of ~5 x 10^7 cfu ml^{-1} *S. aureus* NCTC 6571, during 60 min incubation in Iso-sensitest nutrient broth (Fig. 1). From these results, 50 μg ml^{-1} was identified as a suitable flavonol concentration, and 60 min an appropriate treatment time, for discerning whether or not galangin induces aggregation of bacterial cells. In the light microscopy studies that followed, it was found that the general appearance of samples of untreated and sodium carbonate-treated *S. aureus* differed greatly from those of galangin-treated *S. aureus*. Bacteria from untreated and sodium carbonate-treated samples were typically observed as single cells, cell pairs and small clusters (Figs. 2a and 2b). Bacteria from galangin-treated samples, by sharp contrast, were almost exclusively seen in large cell clusters (Fig. 2d). The results of tallies indicate that large cell clusters present in flavonol-treated samples occurred in much greater numbers than the 'background'

levels of clusters detected in untreated and sodium carbonate-treated control samples (Fig. 3). Results also show that cell clusters present in galangin-treated samples tended to be larger in size than those in untreated and sodium carbonate-treated samples (Fig. 3). Data presented here strongly suggests that galangin causes aggregation of *S. aureus* cells.

Findings from the present study lend support to Ikigai's argument that some flavonoids induce bacterial aggregation (Ikigai *et al.* 1993), and raise interesting questions about the antibacterial activity of galangin. With regard to whether galangin's activity is bacteriostatic or bactericidal, this remains unclear for now because the 1000-fold reductions seen in viable counts of antibiotic sensitive and β-lactamase producing *S. aureus* (Cushnie *et al.* 2003), and the decreases in viable counts noted in MBC assays (Pepeljnjak and Kosalec 2004), may have been caused by bacterial aggregation rather than cell death. Future studies might find it possible to determine whether galangin's antistaphylococcal activity is bacteriostatic or bactericidal by assessing the viability of bacterial cells present in aggregates. The apparent ability of galangin to cause aggregation of cells warrants further investigation in itself though. It would, for example, be useful to establish whether or not aggregated cells are capable of separating. If treated bacterial cells are found to be incapable of separation, galangin's aggregatory effect may prove to be an efficient means of controlling staphylococcal infections. It is also conceivable that aggregates of bacterial cells would have increased virulence compared to groups of individual cells however. The effect of galangin-induced aggregation upon susceptibility to antibiotic treatment and phagocytosis should therefore be investigated too.

The findings of the present study may also provide clues as to galangin's antibacterial mechanism(s) of action. Given that cell clusters had the appearance of large clumps of individual cocci, and that they formed during the relatively short incubation period of 60 min [approximately 3 cell cycles under optimum growth conditions (Singleton 1999)], it is unlikely that they occurred as a consequence of topoisomerase enzyme inhibition (Cushnie and Lamb 2006) or that they represent dividing cells which have failed to separate [pseudomulticellular bacteria (Hamilton-Miller and Shah 1999; Stapleton *et al.* 2004)]. If, as suspected, the clusters are aggregates of bacterial cells, then this may tie in with the hypothesis that galangin causes damage to the cytoplasmic membrane (Cushnie and Lamb 2005b). Ikigai and colleagues postulated that antibacterial catechins cause membrane

fusion, and that this leads to leakage of intramembranous material (Ikigai *et al.* 1993). It may be that galangin exerts its antibacterial effect in a similar manner. Alternatively, it may be that aggregation of bacterial cells is an effect of cytoplasmic membrane damage. If galangin penetrates and exposes the hydrophobic regions present within the phospholipid bilayer of bacterial membranes, aggregation could be occurring as a result of hydrophobic regions from different cells forming associations with each other. Interestingly, a recent publication by Nilsson and colleagues reports that cells of *Escherichia coli* transformed with the inducible cyclic peptide LNO5 also exhibit extensive aggregation (Nilsson *et al.* 2005). It is not clear for what length of time bacterial cells were incubated before aggregation was noted though. It may be that LNO5 prevents dividing cells from separating, rather than causing individual cells to clump together. Any comparisons between the antibacterial mechanisms of action of the flavonol galangin and the cyclic peptide LNO5 should therefore be made with caution.

In summary, results from the present study indicate that galangin causes aggregation of bacterial cells, a finding which may provide clues as to the flavonol's antibacterial mechanism(s) of action, but which also calls into question the nature of this activity. As well as having a specific bearing upon galangin research, results presented here may have wider implications. The apparent ability of flavonoids from two completely different classes (flavonol and catechin) to induce bacterial aggregation raises the possibility that flavonoid-induced decreases in colony counts of bacteria are caused in part, or perhaps even wholly, as a consequence of bacterial cell aggregation. This should be taken into account when interpreting results from time-kill (Sakanaka *et al.* 1989; Kono *et al.* 1994; Xu and Lee 2001; Verdrengh *et al.* 2004; Navarro-Martínez *et al.* 2005; Lin *et al.* 2005) and MBC assays (Tsao *et al.* 1982; Mirzoeva *et al.* 1997; Wahdan 1998; Bosio *et al.* 2000; Koo *et al.* 2000; Basile *et al.* 2000) with natural flavonoids and flavonoid-rich phytochemical preparations. On their own, one thousand-fold decreases in colony forming unit numbers can no longer be used as a reliable indicator of bactericidal activity for this group of natural products.

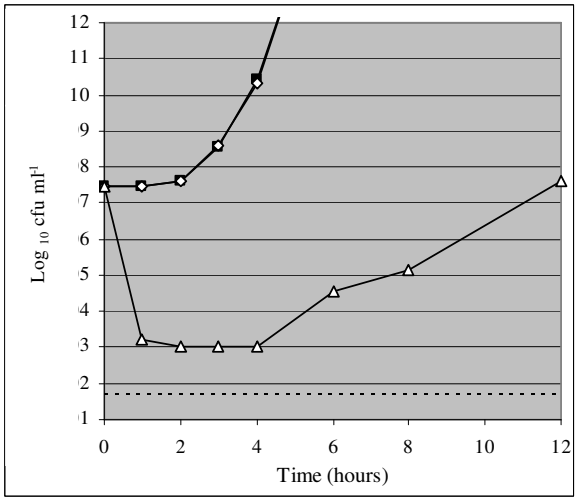

Figure 1 The effect of galangin on a population of ~5 x 10^7 cfu ml^{-1} *S. aureus* NCTC 6571. ■: Broth only (control); ◇: Broth with Na_2CO_3 (control); △: Broth with 50 µg ml^{-1} galangin; ------: minimum detectable number of cfu ml^{-1}

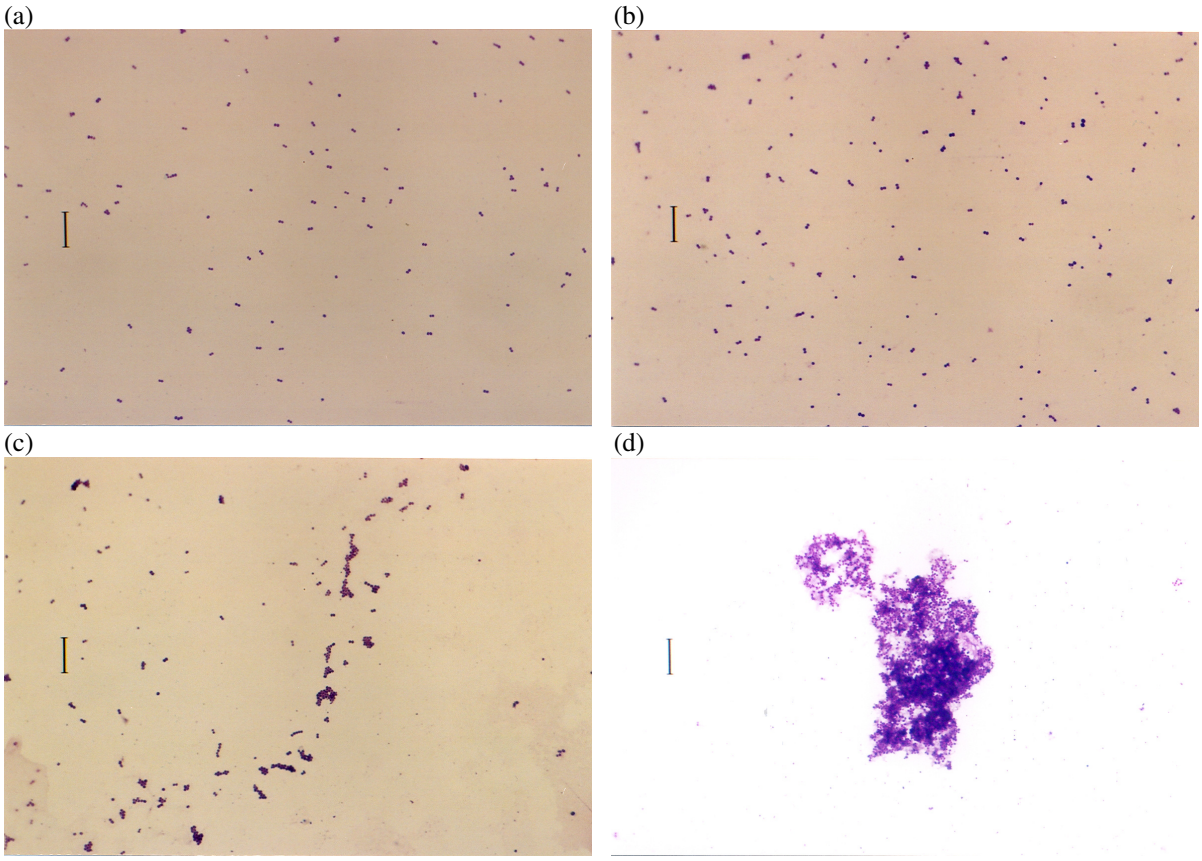

Figure 2 Light microscopy images of (a) untreated cells of *S. aureus* NCTC 6571 (control), (b) cells of *S. aureus* NCTC 6571 incubated in broth supplemented with 0.025 % (w/v) sodium carbonate for 60 min (control), (c) a 'vein' of bacterial clusters running through one of the control samples and (d) cells of *S. aureus* NCTC 6571 incubated in broth supplemented with 50 μg ml^{-1} galangin for 60 min (bar = 10 μm).

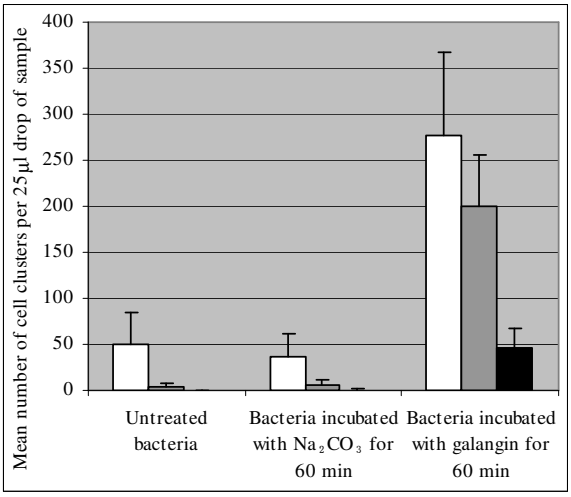

Figure 3 Examination of a population of ~5 x 10^7 cfu ml^{-1} *S. aureus* NCTC 6571 for increases in the frequency of occurrence of large cell clusters following 60 min incubation with 50 µg ml^{-1} galangin [error bars represent standard deviation from mean for four 25 µl samples]. □: 21-40 cell cluster; ■: 41-100 cell cluster; ■: cluster of more than 100 cells

ACKNOWLEDGEMENTS

The authors are very grateful to Mr Brian Stewart (University of Aberdeen) for assistance with the Leitz DMR microscope and DMRD camera system.

REFERENCES

Afolayan, A.J. and Meyer, J.J.M. (1997) The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. *J Ethnopharmacol* **57,** 177-181.

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Castaldo Cobianchi, R., Vuotto, M.L. and Ferrara, L. (2000) Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia* **71 Suppl 1,** S110-S116.

Bosio, K., Avanzini, C., D'Avolio, A., Ozino, O. and Savoia, D. (2000) *In vitro* activity of propolis against *Streptococcus pyogenes*. *Lett Appl Microbiol* **31,** 174-177.

Cushnie, T.P.T., Hamilton, V.E.S. and Lamb, A.J. (2003) Assessment of the antibacterial activity of selected flavonoids and consideration of discrepancies between previous reports. *Microbiol Res* **158,** 281-289.

Cushnie, T.P.T. and Lamb, A.J. (2005a) Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* **26,** 343-356.

Cushnie, T.P.T. and Lamb, A.J. (2005b) Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. *J Ethnopharmacol* **101,** 243-248.

Cushnie, T.P.T. and Lamb, A.J. (2006) Assessment of the antibacterial activity of galangin against 4-quinolone resistant strains of *Staphylococcus aureus*. *Phytomedicine* **13,** 187-191.

Fearnley, J. (2001) *Bee propolis.* London: Souvenir Press Ltd.

Grange, J.M. and Davey, R.W. (1990) Antibacterial properties of propolis (bee glue). *J R Soc Med* **83,** 159-160.

Hamilton-Miller, J.M.T. and Shah, S. (1999) Disorganization of cell division of methicillin-resistant *Staphylococcus aureus* by a component of tea (*Camellia sinensis*): a study by electron microscopy. *FEMS Microbiol Lett* **176,** 463-469.

Harborne, J.B. and Williams, C.A. (2000) Advances in flavonoid research since 1992. *Phytochemistry* **55,** 481-504.

Havsteen, B. (1983) Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol* **32,** 1141-1148.

Hegazi, A.G., Abd El Hady, F.K. and Abd Allah, F.A.M. (2000) Chemical composition and antimicrobial activity of European propolis. *Z Naturforsch [C]* **55,** 70-75.

Ikigai, H., Nakae, T., Hara, Y. and Shimamura, T. (1993) Bactericidal catechins damage the lipid bilayer. *Biochim Biophys Acta* **1147,** 132-136.

Kono, K., Tatara, I., Takeda, S., Arakawa, K. and Hara, Y. (1994) Antibacterial activity of epigallocatechin gallate against methicillin-resistant *Staphylococcus aureus*. *Kansenshogaku Zasshi* **68,** 1518-1522.

Koo, H., Rosalen, P.L., Cury, J.A., Ambrosano, G.M.B., Murata, R.M., Yatsuda, R., Ikegaki, M., Alencar, S.M. and Park, Y.K. (2000) Effect of a new variety of *Apis mellifera* propolis on mutans streptococci. *Curr Microbiol* **41,** 192-196.

Lin, R.-D., Chin, Y.-P. and Lee, M.-H. (2005) Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*. *Phytother Res* **19,** 612-617.

Middleton, E., Jr. and Kandaswami, C. (1993) The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In *The Flavonoids: Advances in research since 1986* ed. Harborne, J.B. pp.619-652. London: Chapman and Hall.

Mirzoeva, O.K., Grishanin, R.N. and Calder, P.C. (1997) Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. *Microbiol Res* **152,** 239-246.

Navarro-Martínez, M.D., Navarro-Perán, E., Cabezas-Herrera, J., Ruiz-Gómez, J., García-Cánovas, F. and Rodríguez-López, J.N. (2005) Antifolate activity of epigallocatechin gallate against *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* **49,** 2914-2920.

NCCLS (1999) *Methods for determining bactericidal activity of antimicrobial agents; Approved guideline (M26-A)*. Wayne, Pennsylvania: National Committee for Clinical Laboratory Standards.

Nilsson, L.O., Louassini, M. and Abel-Santos, E. (2005) Using siclopps for the discovery of novel antimicrobial peptides and their targets. *Protein Pept Lett* **12,** 795-799.

Pepeljnjak, S., Jalšenjak, I. and Maysinger, D. (1982) Growth inhibition of *Bacillus subtilis* and composition of various propolis extracts. *Pharmazie* **37,** 864-865.

Pepeljnjak, S. and Kosalec, I. (2004) Galangin expresses bactericidal activity against multiple-resistant bacteria: MRSA, *Enterococcus* spp. and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **240,** 111-116.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1999) *Microbiology.* London: WCB / McGraw-Hill.

Sakanaka, S., Kim, M., Taniguchi, M. and Yamamoto, T. (1989) Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agric Biol Chem* **53,** 2307-2311.

Singleton, P. (1999) *Bacteria, in biology, biotechnology and medicine.* Chichester: John Wiley and Sons, Ltd.

Stapleton, P.D., Shah, S., Hamilton-Miller, J.M.T., Hara, Y., Nagaoka, Y., Kumagai, A., Uesato, S. and Taylor, P.W. (2004) Anti-*Staphylococcus* aureus activity and oxacillin resistance modulating capacity of 3-*O*-acyl-catechins. *Int J Antimicrob Agents* **24,** 374-380.

Tsao, T.-F., Newman, M.G., Kwok, Y.-Y. and Horikoshi, A.K. (1982) Effect of Chinese and Western antimicrobial agents on selected oral bacteria. *J Dent Res* **61,** 1103-1106.

Verdrengh, M., Collins, L.V., Bergin, P. and Tarkowski, A. (2004) Phytoestrogen genistein as an anti-staphylococcal agent. *Microbes Infect* **6,** 86-92.

Wahdan, H.A.L. (1998) Causes of the antimicrobial activity of honey. *Infection* **26,** 26-31.

Xu, H.-X. and Lee, S.F. (2001) Activity of plant flavonoids against antibiotic-resistant bacteria. *Phytother Res* **15,** 39-43.