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Additive antibacterial activity of naringenin and antibiotic combinations against multidrug resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* has been causing numerous problems in the health care sector. This is mainly due to its ability to develop resistance to a number of antibiotics used to treat staphylococcal infections. Medicinal plants have been used to treat various ailments over the years and are generating a lot of interest as alternative treatment options. Naringenin is a plant derived flavonoid that possesses antibacterial properties, among others. This study assessed the effect of combinations of naringenin and four antibiotics against two *Staphylococcus aureus* strains. The minimum inhibitory concentrations were determined using the disk diffusion and broth microdilution assays. In the disk diffusion assay, naringenin did not inhibit bacterial growth, nor did it enhance the antibacterial activity of the antibiotics in the combination study. This was attributed to its slow rate of diffusion out of the disks. On the contrary, in the broth microdilution assay, naringenin exhibited additive effects when combined with the antibiotics (at sub-inhibitory concentrations). These results show the potential of naringenin as an antibacterial agent. Furthermore, the additive effects observed at low naringenin concentrations showed that it can potentially be used in combination with antibiotics against multidrug resistant bacteria.

Key word: Staphylococcus aureus, MSSA, MRSA, antibiotics, flavonoids, disk diffusion assay, broth dilution assay, MIC.

INTRODUCTION

Premature deaths from infectious diseases are a global problem (Ahmad and Beg, 2001). Moreover, as a result of the widespread overuse of antimicrobial agents to treat these infectious diseases, bacteria have developed ways to minimise the effects of antibiotics through evolutionary adaptations, leading to the emergence of antibiotic resistant pathogens (Fielding et al., 2012). *Staphylococcus aureus* (*S. aureus*) is the leading cause of both nosocomial and community acquired infections (Lowy, 1998) and is often isolated from the bloodstream, skin and soft tissue infections (Bernards et al., 1998; Pfaller et al., 1999; Deresinski, 2005). Disconcertingly,

because methicillin-resistant *S. aureus* (MRSA) strains carry multiple genes of antibiotic resistance, many have developed resistance to a variety of commonly used antimicrobials and may, in future, become resistant to newly developed antimicrobial agents as well (Vijaya et al., 2013; Xia et al., 2013; Medeiros et al., 2014; Szweda et al., 2014). Hence, alternative methods, such as natural plant products, are being studied for their potential to alleviate the burden created by the multidrug resistant bacteria such as MRSA.

Flavonoids are plant-derived compounds reported to possess numerous therapeutic properties which include direct antibacterial activity, as well as synergistic activity when used in combination with antibiotics (Cushnie and Lamb, 2005). Historically, flavonoids are used to treat and prevent various infectious and toxin-mediated diseases, including sores, wound infections (Cushnie and Lamb, 2005), acne, respiratory infections (Gutierrez et al., 2008), gastrointestinal disease (Shan et al., 2007) and urinary tract infections (Ngueyem et al., 2009), among others. For this reason, flavonoids have been extensively studied for their antibacterial properties (Cushnie et al., 2003; Cushnie and Lamb, 2005; Mehndiratta et al., 2010; Cushnie and Lamb, 2011). Naringenin is a flavanone, a type of flavonoid, found in grapefruit and tomatoes. It contains two benzene rings linked together with a heterocyclic pyrone ring (a class of cyclic compounds) (Tripoli et al., 2007). It is believed to possess antibacterial (Celiz et al., 2011), antioxidant, anticancer anti-inflammatory and immunomodulatory properties (Goldwasser et al., 2011; Khachatoorian et al., 2012; Lee et al., 2013). The purpose of this study was to evaluate the antibacterial activities of naringenin on its own, as well as assess the combinatory antibacterial effects of naringenin and antibiotics against methicillin-sensitive and methicillin-resistant strains of *S. aureus* (*i.e.* MSSA and MRSA, respectively).

MATERIALS AND METHODS

Naringenin and the antibiotics (ampicillin, methicillin, tetracycline and vancomycin) used in this study were purchased from Sigma-Aldrich (USA). *S. aureus* ATCC 25923 (MSSA) and ATCC 33591 (MRSA) were obtained from the American Type Culture Collection (ATCC). Antibacterial activity and determination of minimum inhibitory activity was determined as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.

In the disk diffusion assay, naringenin was made to yield twofold serial dilutions (0.002 to 2 $mg.ml^{-1}$); the antibiotic concentrations used were 1.25, 2.5 5, 12, 25 and 50 $\mu g.ml^{-1}$. Whereas the naringenin concentrations were made up in dimethyl sulfoxide (DMSO), the antibiotic dilutions were made up in distilled water. 9 mm sterile filter disks (Lasec) were impregnated with 40 µl of naringenin or antibiotic and allowed to dry at 37°C; this was done in triplicate. DMSO control disks were also included to assess its effect on bacterial growth. Bacterial suspensions were prepared by culturing an inoculum of each strain in 5 ml Tryptone soy broth (TSB) and incubated overnight at 37°C. Next, 100 µl of each overnight suspension was re-cultured in 5 ml fresh broth and incubated for 1-2 h at 37ºC and then adjusted to a bacterial density of 1×10^8 to 2×10^8 $CFU.ml^{-1}$. 100 µl of each suspension was spread onto Tryptone soy agar (TSA) plates. The disks impregnated with naringenin or antibiotics, as well as DMSO, were placed on the plates and incubated at 37ºC for 18-24 h. For the combination studies, the antibiotic concentrations chosen were; 1.25, 2.5, 5, and 12 $\mu g.ml^{-1}$ for tetracycline, ampicillin, methicillin and vancomycin, respectively. These concentrations were chosen because they produced zones that were big enough to show antagonism but also small enough to show synergy. These antibiotic concentrations were individually combined with the naringenin concentrations (0.002 to 2 $mg.ml^{-1}$) and loaded onto disks as described above. Zones of inhibition were measured and recorded.

The broth microdilution assay was carried out in 96 well microtitre plates. Twofold serial dilutions of the antibiotics were made to yield concentrations ranging from 0.0156 to 1 $\mu g.ml^{-1}$ for ampicillin, 0.0313 to 16 $\mu g.ml^{-1}$ for methicillin, 0.0625 to 32 $\mu g.ml^{-1}$ for tetracycline and 0.0039 to 5 $\mu g.ml^{-1}$ for vancomycin, which were made up in distilled water. Naringenin concentrations remained the same as those used in the disk diffusion assay. The solvent of choice for naringenin, in the microdilution assay, was methanol. The other solvents, such as DMSO, caused naringenin to precipitate when added to the wells while methanol did not. The naringenin experimental wells contained 10 µl naringenin and 90 µl of TSB (this was done to ensure that the final methanol concentration in the wells was at 5%), the antibiotic experimental wells contained 20 µl antibiotic and 80 µl TSB. 100 µl of either MSSA or MRSA culture was added to obtain a final volume of 200 µl. Control wells containing a final volume of 5% methanol, distilled water, bacteria alone and TSB alone were also included and the experiments were done in triplicate. The plates were then sealed and incubated at 37ºC with continuous shaking for 18 h to allow detection of viability. The naringenin and antibiotic experimental plates were then read at 620 nm in a plate reader. The MIC was determined as the lowest concentration that inhibited bacterial growth. For the combinations studies, the antibiotic concentrations chosen were 0.0625 $\mu g.ml^{-1}$ for both ampicillin and methicillin, 0.25 $\mu g.ml^{-1}$ for tetracycline and 0.0039 $\mu g.ml^{-1}$ for vancomycin. These concentrations were selected because they did not have any inhibitory activity against the bacterial strains (results not shown). Naringenin was serially diluted in TSB (0.002 to 2 $mg.ml^{-1}$) and 20 µl of each antibiotics were added to the wells followed by 100 µl of the two bacterial strains as outlined earlier.

Mathematical synergistic ratios (SR) for the combination of naringenin and antibiotics were calculated using the Abbott formula $\%C_{exp}=A+B-(AB/100)$, where A was the control level of the antibiotic, B the control level of naringenin and C_{exp} represented the expected efficacy of the mixture (naringenin and antibiotic combination) (Levy et al., 1986). After calculating the $\%C_{exp}$ (expected efficacy), the SR was obtained using the formula $SR=C_{obs}/C_{exp}$, with C_{obs} representing the observed efficacy. The ratios obtained after these calculations enabled determination of

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Table 1. Resistance (R) and susceptibility (S) of *S. aureus* strains to antibiotics in the disk diffusion assay.

Antibiotic		MIC (µg.ml^{-1}) / Zone of inhibition (mm)			
		MSSA ATCC 25923		MRSA ATCC 33591	
Ampicillin	R	2.5	19±0.9	50	12±0
	S	5	31±1.2	-	-
Methicillin	R	1.25	-	50	-
	S	5	19±0.9	-	-
Tetracycline	R	5	11±0	50	10±0
	S	12	21±0.5	-	-
Vancomycin	R	-	-	-	-
	S	12	15±0.9	12	15±0.2

CLSI breakpoints for susceptibility and resistance criteria for zone diameter; ampicillin (10 µg) ≤28=R, ≥29=S; methicillin (5 µg) ≤ 9 = R, 10-13 = I, ≥ 14 = S; tetracycline (30 µg) ≤ 14 = R, 15-18 = I, ≥ 19 = S; vancomycin (30 µg) ≥ 15 =S (no breakpoints for R or I for vancomycin). R = resistant; I = intermediate; S = susceptible. Zones of inhibition expressed as mean of three replicates ± standard deviation (SD).

synergistic interaction in the mixture. If SR is greater than 1.5, then synergistic interactions are present; if between 0.5-1.5 then the interaction is additive; and if the SR is below 0.5, then the effect is antagonistic (Gisi and Binder et al., 1985; Gisi, 1996). This enabled the evaluation of the level of interaction between naringenin and antibiotics.

RESULTS AND DISCUSSION

In the disk diffusion assay, naringenin did not show any inhibitory activity against the *S. aureus* strains, neither did naringenin enhance the antibacterial activities of the antibiotics. However, lack of inhibition zones does not always mean the compound does not possess antibacterial activities. It could be attributed to its slow diffusion ability (Moreno et al., 2006). The various antibiotic concentrations exhibited some antibacterial activity against the MSSA ATCC 25923 strain. The MRSA ATCC 33591 strain was only susceptible to vancomycin with it being resistant even at concentrations of 50 µg.ml^{-1} for the other antibiotics (Table 1). The growth of MSSA was inhibited by 5 µg.ml^{-1} ampicillin, 5 µg.ml^{-1} methicillin and 12 µg.ml tetracycline though inhibitory activity was lost at 5 µg.ml^{-1}. MSSA ATCC 25923 and MRSA ATCC 33591 were both inhibited by a vancomycin concentration of 12 µg.ml^{-1} (as seen by the inhibition zones of ≥ 15 mm, as outlined by the CLSI breakpoints for sensitivity). The MIC breakpoints for resistance and susceptibility were determined according to those outlined by the CLSI (CLSI, 2007).

Although DMSO has some antibacterial activity (Basri and Zin, 2008), it did not inhibit the growth of the bacterial strains when used as control. Previous studies show that flavonoids diffuse slowly out of disks, thereby affecting their antibacterial activities (Zheng et al., 1996). This could explain why naringenin did not have any inhibitory effect or enhance the antibacterial effect of the antibiotics in the disk diffusion assay.

However, in the broth dilution assay, naringenin enhanced the antibacterial activities of the four antibiotics at concentrations that were sub-inhibitory as seen by the synergistic ratios (SR) obtained in Table 2. This was despite naringenin showing only minimal antibacterial effect on its own against *S. aureus*. The results obtained in this study showed that naringenin had an additive effect on the antibacterial activity of the antibiotics (as determined by the calculated SR of between 0.66 and 0.95 for the different combinations. These values depict the lowest and highest SR obtained). Studies have shown that naringenin has an additive effect when combined with vancomycin and oxacillin against vancomycin-intermediate *S. aureus* (VISA) (Bakar et al., 2012). In the naringenin-antibiotic combinations, an increase in antibiotic antibacterial activity was seen at naringenin concentrations of; 0.063 to 0.25 mg.ml^{-1} for ampicillin, 0.25 to 1 mg.ml^{-1} for methicillin, 0.125 to 5 mg.ml^{-1} for tetracycline and 0.016 to 1 mg.ml^{-1} for vancomycin (with the greatest effect observed at 0.016, 0.063 and 0.125 mg.ml^{-1}) for the MSSA strain. With the MRSA strain, an increase in antibiotic antibacterial activity was seen at naringenin concentrations of; 0.25 mg.ml^{-1} for ampicillin, 0.25 to 1 mg.ml^{-1} for methicillin, 0.25 to 0.5 mg.ml^{-1} for tetracycline and 0.125 to 0.25 mg.ml^{-1} for vancomycin. The increased antibacterial effect

Table 2. Antibacterial activity of naringenin in combination with antibiotics against methicillin-sensitive (MSSA) and –resistant (MRSA) *S. aureus*

Drugs (µg.ml^{-1})	Naringenin (mg.ml^{-1}) + antibiotic combinations						
MSSA ATCC 25923							
Ampicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	86	82	**72**	**69**	**73**	83	82
SR	0.87	0.83	0.73	0.67	0.74	0.84	0.83
Methicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	92	93	92	90	**84**	**82**	**82**
SR	0.93	0.94	0.93	0.91	0.85	0.83	0.83
Tetracycline (0.25)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	92	92	91	**88**	**84**	**84**	90
SR	0.93	0.93	0.92	0.89	0.85	0.85	0.91
Vancomycin (0.0039)	0.019	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	**66**	82	**70**	**71**	77	78	85
SR	0.66	0.83	0.70	0.72	0.78	0.80	0.86
MRSA ATCC 33591							
Ampicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	95	95	93	**89**	91	92
SR	0.96	0.96	0.96	0.94	0.90	0.92	0.93
Methicillin (0.0625)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	93	93	94	92	**88**	**89**	**89**
SR	0.93	0.93	0.94	0.92	0.89	0.90	0.90
Tetracycline (0.25)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	95	94	94	**89**	**87**	93
SR	0.95	0.95	0.94	0.94	0.89	0.87	0.94
Vancomycin (0.0039)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	94	**88**	**77**	**81**	84	84
SR	0.95	0.94	0.88	0.77	0.81	0.85	0.85

The numbers in bold represent points at which the greatest reduction in bacterial % growth was observed. SR = synergistic ratio. Naringenin exhibited an additive effect when combined with the antibiotics.

was accompanied by a decrease in bacterial growth at the concentrations outlined above (Table 2). Studies have shown that naringenin, as well as its derivatives, exhibit increased antibacterial activities. In fact, data demonstrate that an increase in chain length (10-12 carbon atoms) and modifying its structure increased the antibacterial activities. This shows that the structure of naringenin is important for interaction with the antibiotics in combination studies (Celiz et al., 2011; Lee et al., 2013).

Synergy studies of natural products and antibiotics are increasing in order to assess their combination effects (Olajuyigbe and Afolayan, 2013). Studies of natural plant products such as *Salvia afficinalis* (*S. afficinalis*) oils, reveal that they effectively inhibited the growth of *S. aureus* and *Streptococcus* group D at a 20 µl/ml concentration and show enhanced antibacterial activity when compared to most known antibiotics in multidrug resistant bacteria (Khalil and Li, 2010). Extracts of *Rehum palmatum*, *Cassia angustifolia* and *Glycyrrhiza glabra* also exhibited antibacterial activity against antibiotic-resistant bacteria. These extracts enhanced the antibacterial activities of the antibiotics used hence showing their synergistic effect (Dawoud et al., 2013). Liu and colleagues showed that despite numerous *S. aureus* clinical isolates being resistant to fluroquinolones, they can be combined with the flavonoid biochanin A (BCA) creating a potent antimicrobial agent (Liu et al., 2011). Analysis of the ethanol bark extract of *Ziziphus mucronata* in combination with antibiotics against clinically important bacteria revealed both additive and synergistic effects. These results further agree with other published data stating that natural plants and plant products enhance antibacterial activities of antibiotics.

The data generated in this study demonstrated the flavonoids possess the ability of enhancing the antibacterial activities of antibiotics to which bacteria are resistant. However, further studies explaining the precise mode of action of flavonoids, including other plant extracts, against multidrug resistant pathogens will be

beneficial in their overall treatment. Despite the availability of various methods for evaluating the MIC of plants extracts or plant compounds, not all produce similar results (Jorgensen and Ferraro, 2009). For instance, the disk diffusion assay does not allow for natural antimicrobial compounds that are barely soluble or insoluble in water to diffuse uniformly from the disks. Consequently, these compounds also do not diffuse uniformly through agar media when the agar diffusion assay is used (Mann and Markham, 1998). A study evaluating the disk diffusion and broth microdilution assays showed that the broth microdilution assay was a better technique for assessing the antibacterial activity of plant extracts or plant compounds (Klancnik et al., 2010). Another study showed that results obtained with the broth microdilution assay were more reproducible (99%) when compared to the macrodilution technique (89%) (Murray and Hospenthal, 2004). This study further showed that the MICs obtained in the microdilution assay were lower than those obtained in the macrodilution assay (Murray and Hospenthal, 2004). Another advantage of the broth microdilution assay is that it produces quantitative results and also allows for the compounds to interact with the bacterial strains in suspension. This could explain why the MICs generated in the broth microdilution assay were lower than those in the disk diffusion assay.

In conclusion, the burden created by multidrug resistant bacteria, which is worsened by the lack of new therapeutic drugs, is mainly brought about by the duration it takes for new therapeutic agents to be tested and released on the market. It is for this reason that alternative treatment options, such as plants and plant products, are generating a tremendous amount of interest. Antimicrobial combinations are currently used in medicine to try and limit the rate of antibiotic-resistance and should be encouraged. This study showed that naringenin displayed additive effects when combined with the different antibiotics at sub-inhibitory concentrations. These results further showed that naringenin has the capacity to be used as a therapeutic agent, or to enhance the antibacterial effects of various antibiotics. Further research to determine its exact mechanism of action would be an added advantage in understanding how naringenin interacts with these antibiotics.

Conflict of interests

The authors did not declare any conflict of interest.

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