Original Research

Synergistic antibacterial effects of herbal extracts and antibiotics on methicillin-resistant *Staphylococcus aureus*: A computational and experimental study

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Impact statement

Antibiotic resistant is a well-known threat to global health and methicillin-resistant *Staphylococcus aureus* is one of the most significant ones. These resistant bacteria kill thousands of people every year and therefore a new effective antimicrobial treatment is necessary. This study identified the herbs and their associated bioactive ingredients that can potential the effects of current antibiotics. These herbs have long history of human usage in China and have well-defined monograph in the Chinese Pharmacopeia. These indicate their relatively high clinical safety and may have a quicker drug development process than that of a new novel antibiotic. Based on the results of this study, the authors will perform further *in vitro* and animal studies, aiming to accumulate significant data for the application of clinical trial.

Abstract

Antibiotic resistance has become a serious global concern, and the discovery of antimicrobial herbal constituents may provide valuable solutions to overcome the problem. In this study, the effects of therapies combining antibiotics and four medicinal herbs on methicillin-resistant *Staphylococcus aureus* (MRSA) were investigated. Specifically, the synergistic effects of *Magnolia officinalis*, *Verbena officinalis, Momordica charantia*, and *Daphne genkwa* in combination with oxacillin or gentamicin against methicillin-resistant (ATCC43300) and methicillin-susceptible (ATCC25923) *S. aureus* were examined. *In vitro* susceptibility and synergistic testing were performed to measure the minimum inhibitory concentration and fractional inhibitory concentration (FIC) index of the antibiotics and medicinal herbs against MRSA and methicillin-susceptible *S. aureus*. To identify the active constituents in producing these synergistic effects, *in silico* molecular docking was used to investigate the binding affinities of 139 constituents of the four herbs to the two common MRSA inhibitory targets, penicillin binding proteins 2a (PBP2a) and 4 (PBP4). The physicochemical and absorption, distribution, metabolism, and excretion properties and drug safety profiles of these compounds were also analyzed. *D. genkwa* extract potentiated the antibacterial effects of oxacillin against MRSA, as indicated by an FIC index value of 0.375. *M. officinalis* and *V. officinalis* produced partial synergistic effects when combined with oxacillin, whereas *M. charantia* was found to have no beneficial effects in inhibiting MRSA. Overall, tiliroside, pinoresinol, magnatriol B, and momorcharaside B were predicted to be PBP2a or PBP4 inhibitors with good drug-like properties. This study identifies compounds that deserve further investigation with the aim of developing therapeutic agents to modulate the effect of antibiotics on MRSA.

Keywords: Antibiotic resistance, methicillin-resistant *Staphylococcus aureus*, synergistic effects, penicillin binding proteins, traditional Chinese medicines, molecular docking

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of multidrug resistance infections with significant morbidity and mortality.[1] In 2011, 80,461 invasive MRSA infections were recorded in the US,[2] causing substantial economic and logistical problems.[3] The rates of MRSA infections have been stabilized in certain developed countries, but those in many developing regions continue to increase rapidly.[4] More importantly, MRSA infections previously occurred exclusively in hospitals, but in the past decade, the number of community-acquired infections has significantly increased.[4] Presently, most of the MRSA infections that occur in communities affect the skin, whereas life-threatening infections such as bacteremia, endocarditis, and pneumonia still occur in medical facilities.[5] No one knows whether these less severe community-acquired MRSA infections will ultimately prove as life threatening as those occurring in hospitals, but it is safe to assume

that hospital infection prevention is no longer the only consideration in reducing MRSA rates, and that more effective treatments are urgently needed.

Over the past few decades, several antibiotics, including vancomycin and daptomycin, have been considered drugs of choice for treating and preventing severe MRSA infections.[6] However, their effectiveness is declining.[5] Much evidence has shown that the increased resistance of vancomycin has led to poor therapeutic outcomes and increased patient mortality.[7] More importantly, another strong resistance strain of *S. aureus*, called heterogeneous vancomycin-resistant *S. aureus* (hVRSA) has been detected globally. hVRSA is closely related to MRSA and clinical study has demonstrated that hVRSA phenotype was detected in 50% of clinical MRSA in certain countries.[8] In addition, vancomycin dosing that achieves an appropriate trough serum level (15–20 mg/L) for serious infection is difficult and has become associated with significant increased risk of toxicity, mainly nephrotoxicity.[9] Daptomycin is generally considered to be clinically effective, with less risk of nephrotoxicity than vancomycin in treating MRSA-based endocarditis and bacteremia infections.[10] However, an increasing number of cases of daptomycin treatment failure due to resistance have been reported,[11,12] and the typical minimum inhibitory concentration (MIC) has been raised from 0.5 to 4–8 mg/L in the past few decades.[13] Thus, scientists are always seeking new ways to counter the effects of MRSA antibiotic resistance.

The most common ways to overcome antibiotic resistance are to develop new drugs or via combined treatment therapy.[14] The latter method is generally considered to be less complicated because much of the necessary scientific data may already exist, especially when the drugs being combined are already marketed for human use. Examples of recent studies include the combination of clarithromycin and daptomycin[15] and rifampicin and daptomycin.[16] Both studies revealed potentiated antimicrobial efficacy against different strains of *S. aureus*. However, combining antibiotics may also make their adverse effects more pronounced.[17]

Apart from the combined use of antibiotics, the synergistic effects of combining phytopharmaceuticals and antibiotics have been demonstrated in various studies,[18,19] particularly in relation to traditional Chinese medicines (TCMs),[20] which has flourished for thousands of years in clinical practice. Many TCMs have been documented as effective in treating various infectious diseases. Thus, we performed *in vitro* studies of four traditional Chinese medicinal plants to investigate their anti-MRSA activity – alone and in combination with oxacillin or gentamicin. The four plants studied – *Daphne genkwa*, *Magnolia officinalis*, *Verbena officinalis*, and *Momordica charantia* – are commonly used in China as anti-infection treatments (Additional file 1: Table S1).

Hundreds of studies have verified that active compounds present in medicinal plants can be used as therapeutic agents, and that *in silico* methods are effective approaches to screening out these lead molecules for further investigation.[21] In this study, 139 constituents of the four anti-infection medicinal plants were identified using the TCM Database@Taiwan.[22] Molecular docking was used to investigate their binding affinities to the two common MRSA inhibitory targets, Penicillin binding proteins 2a (PBP2a) and 4 (PBP4).[23,24] This helped us identify the anti-MRSA compounds and may explain any inhibitory or synergistic effects reported from the *in vitro* synergy testing. The physicochemical and absorption, distribution, metabolism, and excretion (ADME) properties and drug safety of these compounds were also analyzed to estimate their drug-likeness and reduce the failure rate among further drug discovery processes.[25] Hence, this study uses *in vitro* and *in silico* methods to investigate the anti-MRSA properties of four medicinal herbs and their 139 constituents (Table S1).

Methods

Reagents and consumables for in vitro studies

Mueller–Hinton broth and agar were purchased from Hope Biol-Technology Company (Qingdao, China). Trypticase soy agar (TSA), brain heart infusion broth (BHIB), 30 μg cefoxitin filter paper discs, and strains of MRSA (ATCC43300) and methicillin-susceptible *S. aureus* (MSSA, ATCC25923) were commercially purchased from Compass Biotechnology Company (Shenyang, China). Extracts of *M. officinalis* and *V. officinalis* were from Ruikang Biological Company (Shanxi, China). Extracts of *M. charantia* and *D. genkwa* were from Hengdetang Biological Company (Zhengzhou, China) and Runxue Biological Company (Xi'an, China), respectively. These suppliers characterized all the extracts with HPLC fingerprints. The oxacillin and gentamicin were from Yuanye Bio-Technology Company (Shanghai, China).

Strains of ATCC43300 (methicillin resistant) and ATCC25923 (methicillin susceptible) were grown on TSA and BHIB, respectively, at 37°C for 24 h. Both strains were then stored at −80°C for future experiments. A Vitek 2 microbial identification system (bioMérieux, Marcy l'Etoile, France) was used to identify the *S. aureus* strain. The Kirby–Bauer disk diffusion method with 30 μg cefoxitin filter paper discs was used to investigate the drug resistance of both strains.

Susceptibility testing

The MIC is the lowest concentration of the control antibiotics and herbal extracts that will inhibit the visible growth of the ATCC43300 and ATCC25923 after 24 h of incubation at 37°C. MIC is one of the most common measurements of antibacterial activity against MRSA and MSSA.[26] Here, tube dilation methods were used to determine the MIC of oxacillin and gentamicin against both MRSA and MSSA. The MICs of the extracts of *M. officinalis*, *V. officinalis*, *M. charantia*, and *D. genkwa* were determined by plate dilation methods with concentrations ranging from 0.00625 to 0.4 g/ml for both MRSA and MSSA strains. The gentamicin was tested with concentrations ranging from 1 to 32 mg/L for both strains, and the oxacillin was tested from 0.25 to 8 mg/L for MSSA and 8 to 256 mg/L for MRSA. These experiments were performed according to

the guidelines from the Clinical and Laboratory Standards Institute.[27]

Synergistic testing

The fractional inhibitory concentration (FIC) index was used to quantify the synergistic interactions between the plant extracts and antibiotics against both MRSA and MSSA. The antimicrobial assays were performed using the checkerboard method[28] with oxacillin or gentamicin in combination with the extracts of *M. officinalis*, *V. officinalis*, *M. charantia*, and *D. genkwa*. Both the MRSA and MSSA cultures were grown in the presence of one of the four herbal extracts with the following concentrations: $1/8 \times$ MIC, $1/4 \times$ MIC, $1/2 \times$ MIC, $1 \times$ MIC, $2 \times$ MIC, and $4 \times$ MIC in combination with either oxacillin or gentamicin, with concentrations ranging from $1/8 \times$ MIC to $4 \times$ MIC. These experiments were conducted in the same manner as for the MIC determination in the susceptibility testing.

The FIC index was calculated with the following formulas

$$FIC_{antibiotic} = \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone}$$

$$FIC_{plant\ extract} = \frac{MIC\ of\ plant\ extract\ in\ combination}{MIC\ of\ plant\ extract\ alone}$$

$$\text{FIC index} = \text{FIC}_{\text{antibiotic}} + \text{FIC}_{\text{plant extract}}$$

where FIC index values of less than 0.5 indicated synergy, 0.5–0.75 indicated partial synergy, 0.76–1 indicated an additive effect, and >2 indicated antagonism.[28,29]

In silico molecular docking studies

Molecular docking has been successfully used in many studies to investigate the binding potential between proteins and ligands. It is popular for screening active compounds from medicinal plants for inhibition against pharmacological receptors.[30,31] Here, the crystal structures of two common MRSA inhibitory targets, PBP2a and PBP4, were obtained from the Protein Data Bank (PDB) with PDB codes 4CJN[32] and 3HUM,[33] respectively. The constituents of *M. officinalis*, *V. officinalis*, *M. charantia*, and *D. genkwa* were downloaded from the TCM Database@Taiwan (http://tcm.cmu.edu.tw/), which covers more than 20,000 pure compounds isolated from 453 TCMs ingredients.[22] The downloaded structures were built by ChemBioOffice 2008 (CambridgeSoft, Cambridge, MA) and energy minimized in a MM2 force field. Here, 139 constituents were identified and downloaded for *in silico* docking to both the PBP2a and PBP4 crystal structures.

The Surflex-Dock module of the SYBYL-X 1.3 molecular modeling suite was used to perform the molecular docking simulations. The accuracy of Surflex-Dock in scoring protein–ligand interactions has been successfully validated for large and diverse sets of target protein structures, demonstrating a high screening enrichment performance.[34–36] In this study, the 139 constituents in the crystal structures of 4CJN and 3HUM were docked into the PBP2a and PBP4 independently. Apart from the 139 constituents, the binding scores of the original ligands bound to each of the PBP2a and PBP4 crystal structures were also calculated (Figure 1). These ligands were confirmed as PBP2a and PBP4 inhibitors from experimental results downloaded from the PDB, and their binding scores of the two ligands were compared with those of the top-ranked herbal constituents. The structures of all the molecules were minimized using the standard tripos force field[37] before the docking simulations. The docking parameters were set to the default in Surflex-Dock; for instance, the protomol threshold and bloat were set at 0.5 and 0.0, respectively. The protomols were generated by removing the ligands of the co-crystallized compounds and defined by the residues surrounding the active site. This method gives a fixed degree of coverage against the residues proximal to the specified ligands or explicit residues. The water molecules in the PBP2a and PBP4 crystal structures were retained for docking because water molecules may have cross-linkages between proteins and ligands that help to stabilize the protein–ligand complex.[38] More importantly, the presence of water molecules may increase docking accuracy.[39] All of the atoms were allowed to move during the simulations because flexible docking is generally more accurate than rigid docking.[40] Finally, the resulting binding modes of the top-scoring constituents were analyzed using SYBYL-X 1.3 and PyMOL. The docking procedures and calculations were the same as in the following evaluation of docking methods, which would ensure that the results were consistently reliable.

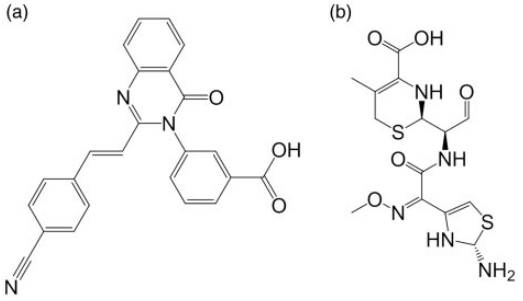


Figure 1 Two-dimensional structures of the two ligands bound to the crystal structures of PBP2a (a) and PBP4 (b). PBP2a: penicillin binding protein 2a; PBP4: penicillin binding protein 4

Evaluation of docking methods

Surflex-Dock has been successfully validated on large and diverse sets of target proteins and has produced valuable results in investigating PBP inhibition.[41] We further evaluated its specific performance in simulating PBP2a–ligand interactions using receiver operating characteristic (ROC) analysis. Docking was performed on the X-ray crystallography structure of PBP2a (PDB: 4CJN) and the 11,421 ligands from the Zinc In Man database,[42] plus eight experimentally approved PBP2a inhibitors with IC_{50} less than 100 μM.[43] These eight approved inhibitors served as the ''true'' hits in this validation. Added together, a ligand library of 11,429 was formed. ROC analysis has previously been used to evaluate the accuracy of docking algorithms in

various protein–ligand systems.[36,44] For PBP4, only five experimentally proved inhibitors were identified from our literature search,[45] which covered various databases including Medline, Embase, Cochrane Library, and the China National Knowledge Infrastructure database. These five inhibitors are in the same series and have very similar structures. Thus, using these compounds exclusively would not provide acceptable statistical power to evaluate the docking accuracy for this particular protein.

Prediction of drug-like properties

The structures of the 139 herbal constituents downloaded from the TCM Database@Taiwan database were applied to ACD/Percepta 14.0 software[46] for drug-like properties evaluation. The drug profiler module in ACD/Percepta uses high-quality predictive methods to screen compounds with three types of profiler parameters[46]: physicochemical, ADME, and drug safety profiling. The physicochemical profiler evaluates an array of physicochemical properties including log P, molecular weight, H-bond donors, H-bond receptors, rotatable bond, solubility, Lipinski's Rule-of-5, and predefined lead-like categories. Based on these properties, each constituent is classified as having favorable or unfavorable drug-like physicochemical properties.[47] The ADME profiler predicts P-glycoprotein specificity, passive permeability across Caco-2 cell monolayers, and human intestinal absorption (HIA), all of which are important factors that affect oral bioavailability.[48] The drug safety profiler includes the prediction of Cytochrome P450 (CYP) regioselectivity (CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2), probability of producing positive Ames (mutagenicity prediction), and human ether-a-go-go-related gene (hERG) (cardiotoxicity prediction).[49,50] The accuracy and sensitivity of all the above methods were successfully evaluated by the supplier, ACD/Labs. All the data sheets are available on the company website (http://www.acdlabs.com).

Table 1 Minimum inhibitory concentration (MIC) of test samples for MRSA ATCC43300 and MSSA ATCC25923

	MIC (mg/L)	
Test samples	**MRSA ATCC43300**	**MSSA ATCC25923**
Oxacillin	128	1
Gentamicin	16,000	32
D. genkwa extract	35,000	60,000
M. officinalis extract	35,000	40,000
V. officinalis extract	6250	6250
M. charantia extract	70,000	90,000

Results

In this study, *in vitro* susceptibility and synergistic testing were conducted to investigate the inhibitory effects of *M. officinalis*, *V. officinalis*, *M. charantia*, and *D. genkwa* on MRSA. *In silico* work was also performed to predict the active ingredients that are responsible for the inhibitory effects.

In vitro susceptibility and synergistic studies

Our *in vitro* susceptibility testing found that the extracts of all four TCMs had antibacterial effects on both MRSA and MSSA strains, with *V. officinalis* performed the best (Table 1). The MIC of *V. officinalis* was lower than that of gentamicin for MRSA (Table 1), which indicated the strong inhibitory effect of *V. officinalis* on MRSA. In terms of the synergistic testing, the extract of *D. genkwa* obtained an FIC index value of 0.375 (Table 2), which demonstrated a potentiated antibacterial effect of oxacillin against MRSA. The extract of *D. genkwa* partially potentiated the effect of gentamicin, as indicated by an FIC index value of 0.750 (Table 2). The extracts of *M. officinalis* and *V. officinalis* demonstrated partial synergistic effects in combination with the antibiotics (Table 2).

In silico docking simulations

The docking accuracy on PBP2a was successfully validated using an ROC analysis (Figure 2), where a calculated area under the curve (AUC) value indicated the high predictive power. An AUC value of 0.5 indicates a random result with no predictive power and 1.0 indicates a perfect prediction. In general, a model with an AUC value of 0.7 or above is considered reliable.[36,44] Here, the obtained AUC value was 0.72, showing that the accuracy of our docking methods was reliable.

The docking results of PBP2a revealed that 25 of the 139 constituents had advanced scores than the crystallographic derived PBP2a inhibitor, quinazolinone (PDB: 4CJN).[32] The first ranked constituent was tiliroside, which obtained a Surflex score of 10.5 (Table 3). This score is superior to that of the experimentally proved inhibitor, quinazolinone, which obtained a score of 7.6. The binding mode analysis revealed that the tiliroside interacted with the residues Tyr105, Gly106, Ile144, Asn146, Lys273, Asp275, Asp295,

Table 2 Fractional inhibitory concentration (FIC) index of test samples for MRSA ATCC43300 in combination with oxacillin or gentamicin

	Oxacillin		Gentamicin	
Test samples	**FIC index**	**Outcome**	**FIC index**	**Outcome**
D. genkwa extract	0.375	Synergy	0.750	Partial synergy
M. officinalis extract	0.625	Partial synergy	0.750	Partial synergy
V. officinalis extract	0.625	Partial synergy	1.125	No effect
M. charantia extract	>2	Antagonism	1.200	No effect

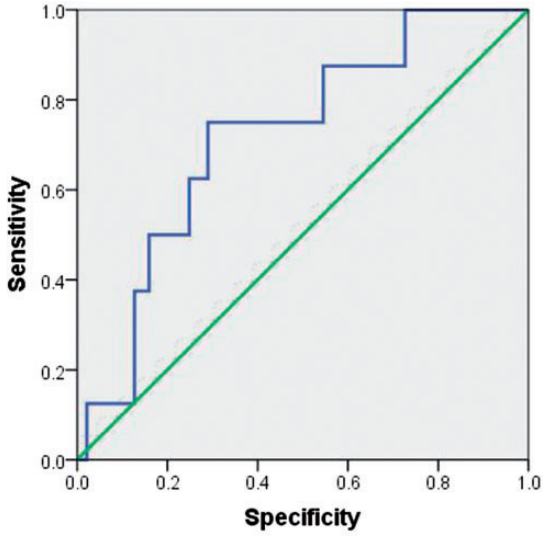
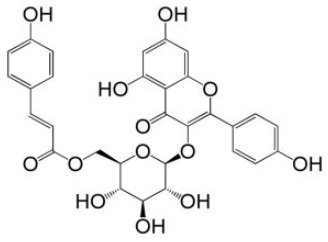
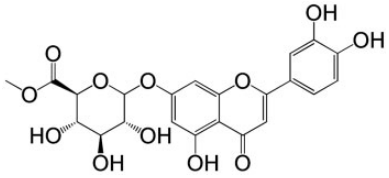
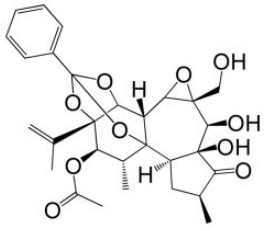
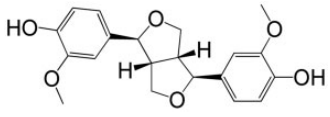


Figure 2 Receiver operating characteristic (ROC) curves of the docking results for PBP2a and the ligands from the ZIM database. The green reference line indicates random results, with an AUC value of 0.50. An AUC value of 1.0 indicates a perfect prediction and one of 0.7 or above is considered reliable. AUC: area under the curve; PBP2a: penicillin binding protein 2a. (A color version of this figure is available in the online journal.)

and Tyr297 in the PBP2a binding pocket. In contrast, quinazolinone demonstrated significantly fewer interactions in the same binding pocket (Figure 3). Tiliroside also achieved a high score in binding to the PBP4 (Table 4), as indicated by a score of 12.3. The binding mode analysis showed that tiliroside formed interactions with the residues Glu114, Ser116, Ser139, Phe241, Gly261, and Ser263. These interactions appeared to be stronger than those between cefotaxime and PBP4 (Figure 4). Luteolin 7-diglucuronide of *V. officinalis* and yuanhuapine of *D. genkwa* achieved the second and third highest score in the PBP2a docking simulation (Table 3). Other highly ranked compounds against PBP2a were (-)-pinoresinol, neo-chlorogenic acid, momorcharaside B, 3,4-dihydroverbenalin, magnatriol B, and daucosterol (Table 3).

The docking results of PBP4 and herbal constituents showed that 40 compounds have advanced binding score than the experimentally proven PBP4 inhibitor, cefotaxime. The binding scores of these constituents ranged from 12.5 to 8.8, whereas cefotaxime obtained a score of 8.7. The top-ranked constituent was apigenin,7-*O*-beta-D-glucopyranoside (Table 4), which is a constituent of *D. genkwa*.

Table 3 Surflex scores and structural properties of the top 10 ranked constituents against PBP2a

Constituents		Botanical source	Surflex score
Tiliroside		*D. genkwa*	10.5
Luteolin-7-diglucuronide		*V. officinalis*	10.1
Yuanhuapine		*D. genkwa*	9.3
(-)-pinoresinol		*D. genkwa*	9.0

(continued)

Table 3 Continued

Constituents	Botanical source	Surflex score
Neo-chlorogenic acid	*V. officinalis*	8.9
Momorcharaside B	*M. charantia*	8.8
3,4-dihydroverbenalin	*V. officinalis*	8.7
Magnatriol B	*M. officinalis*	8.7
Daucosterol	*V. officinalis*	8.7
5beta-hydroxyresiniferonol-6alpha, 7alpha-epoxy-12beta-acetoxy-9, 13,14-ortho-2E-decenoate	*D. genkwa*	8.4

PBP2a: penicillin binding protein 2a.

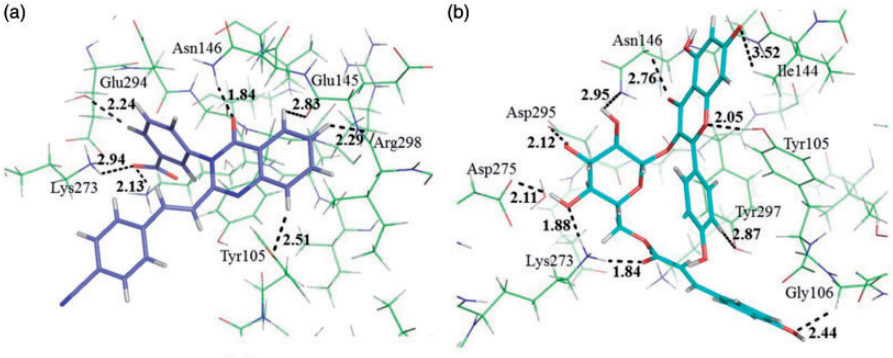
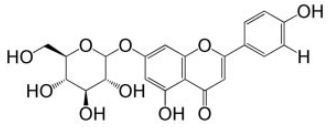
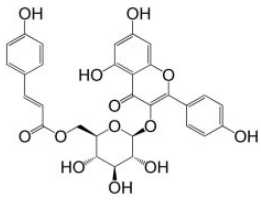
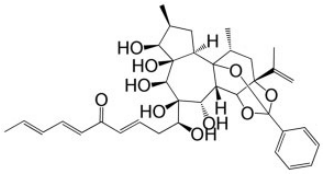
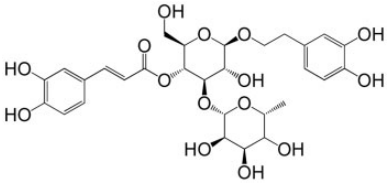
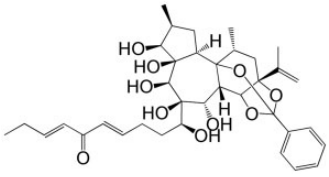
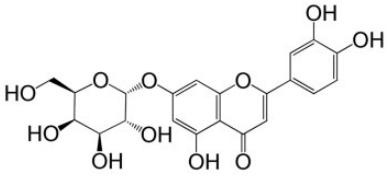
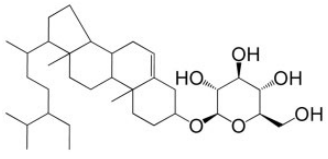
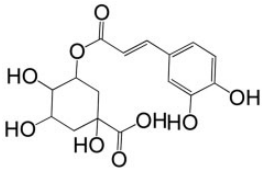


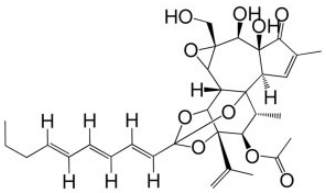
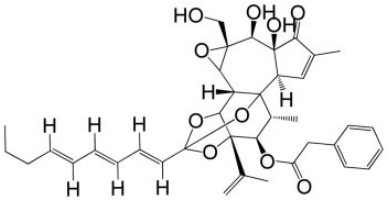
Figure 3 (a) Crystallographic binding mode of quinazolinone in the PBP2a active site. (b) Surflex-Dock predicted binding mode of tiliroside in the PBP2a active site. All distances are measured in Angstroms (Å). PBP2a: penicillin binding protein 2a. (A color version of this figure is available in the online journal.)

Table 4 Surflex scores and structures of the top 10 ranked constituents against PBP4

Constituents		Botanical source	Surflex score
Apigenin-7-*O*-beta-D-glucopyranoside		*D. genkwa*	12.5
Tiliroside		*D. genkwa*	12.3
Genkwanine E		*D. genkwa*	12.1
Jionoside D		*V. officinalis*	11.5
Genkwanine F		*D. genkwa*	11.2
Luteolin-7-*O*-beta-D-galactopyranoside		*V. officinalis*	11.0
Daucosterol		*V. officinalis*	10.9
3-caffeoylquinic acid		*V. officinalis*	10.7

(continued)

Table 4 Continued

Constituents		Botanical source	Surflex score
Yuanhuagine		*D. genkwa*	10.5
Yuanhuajine		*D. genkwa*	10.4

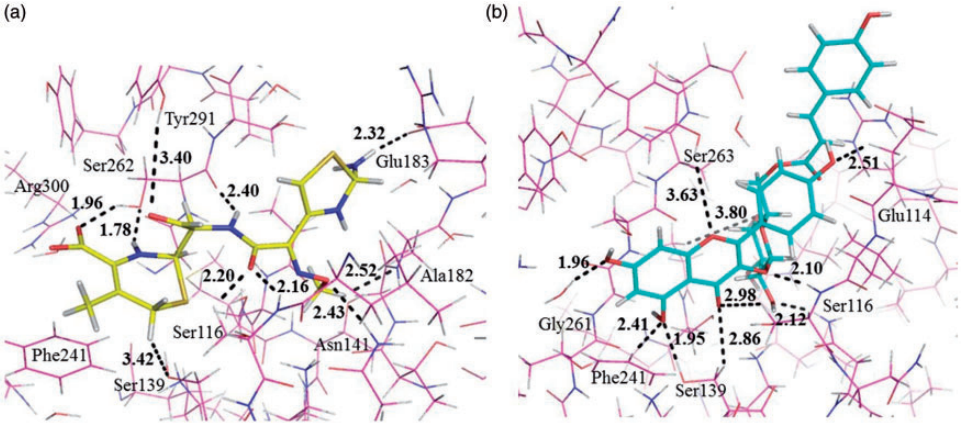


Figure 4 (a) Crystallographic binding mode of cefotaxime in the PBP4 active site. (b) Surflex-Dock predicted binding mode of tiliroside in the PBP4 active site. All distances are measured in Angstroms (Å). PBP4: penicillin binding protein 4. (A color version of this figure is available in the online journal.)

Discussion

In vitro studies

The results of the *in vitro* susceptibility testing revealed that the MICs of oxacillin and gentamicin for MRSA were 128 and 500 times than that of MSSA (Table 1). This clearly showed a high level of antibiotic resistance to MRSA but interestingly, the MIC values of all four TCMs for MRSA were lower or equal to that of MSSA. This indicated that the TCMs produced stronger inhibitory effects on MRSA than on MSSA, which led to a theory that the inhibitory mechanisms are different between the antibiotics and TCMs. Future research on this mechanism could lead to a novel pathway for designing anti-MRSA agents. For the synergistic testing, the extract of *D. genkwa* potentiated the antibacterial effects of oxacillin against MRSA (Table 2); this synergistic effect indicated that the efficacy of *D. genkwa* in combination with oxacillin was greater than the sum of their individual use. It also suggested that the extract of *D. genkwa* somehow enhanced the oxacillin pharmaceutical actions, inhibiting the synthesis of a peptidoglycan layer of bacterial cell walls by binding to PBPs. The extract of *D. genkwa* partially potentiated the effect of gentamicin, which is an aminoglycoside antibiotic, has a different pharmacological mechanism than oxacillin. Gentamicin irreversibly binds to the 30S subunit of the bacterial ribosome and disrupts protein synthesis. There are different opinions about the mechanism of aminoglycoside resistance on MRSA, and the widely accepted hypotheses are ribosomal mutations, active efflux, and aminoglycoside modifying enzymes.[51] Thus, further investigations of these synergistic effects caused by *D. genkwa* on oxacillin and gentamicin should focus on areas relating to these resistance mechanisms.

The extracts of *M. officinalis* and *V. officinalis* demonstrated partial synergistic effects in combination with the antibiotics (Table 2), indicating that the active constituents of these plants must somehow overcome the resistance properties of MRSA. The lower synergistic effects of *M.*

officinalis and *V. officinalis* than *D. genkwa* may be due to the lower efficiency and/or concentrations of these constituents. To further investigate, we performed *in silico* studies on PBP2a and PBP4.

In silico studies

Molecular docking analyses were performed to predict the binding affinities among the 139 herbal constituents of the four herbs against PBP2a and PBP4. Drug-like physicochemical properties, ADME, and drug safety were also predicted to increase the chance of success in developing a drug treatment for clinical use.

PBP2a

The first ranked constituent against PBP2a was tiliroside, which obtained a higher binding score than that of the experimentally proved PBP2a inhibitor, quinazolinone (Table 3). This indicates that the binding of tiliroside to PBP2a will from a stable complex and the defined binding site of PBP2a is an appropriate target of tiliroside. Expressly, this high affinity molecule is being predicted as a PBP2a inhibitor. Tiliroside is a glycosidic flavonoid and a kaempferol derivative and its antibacterial properties have been controversially documented in various studies. For example, tiliroside was found to be a significantly active compound against *Candida albicans* (MIC value = 1 μg/mL), *Escherichia coli* (2 μg/mL), and *Bacillus subtilis* (8 μg/mL).[52] However, the findings of Zhang *et al*.[53] suggested that tiliroside had no relevant antibacterial activity on *E. coli*, *B. subtilis*, or *C. albicans*. The reasons for these diverse results are still uncertain. In terms of the inhibition of *S. aureus*, tiliroside has not been found to have substantial antibacterial activity, but it was shown to somehow modulate the activities of other conventional antibiotics against the resistant strain of *S. aureus*. It increased the activity of norfloxacin 16 times, ciprofloxacin 16 times, lomefloxacin 4 times, and ofloxacin by 2 times.[54] Thus, tiliroside could be a constituent of *D. genkwa* capable of producing a synergistic effect with oxacillin against MRSA in this study. The potential mechanism could be related to the binding of PBP2a or PBP4, as indicated by the high binding scores calculated in this study. In terms of ADME and drug safety, no significant information was found in the literature. Here, the *in silico* methods predicted that tiliroside would have an optimal log P value and good HIA properties (Tables 5 and 6). It was also predicted to be a non-penetrant of the central nervous system (CNS) and a non-inhibitor of cardiac hERG channels (Table 7), which indicate a low risk of CNS adverse effects and hERG-related cardiotoxicity when used clinically. However, the plasma protein binding (PPB) value indicated that a high percentage (94%) of administrated tiliroside will bind with blood plasma protein (Table 6). This means that only 6% of tiliroside may reach the target receptor and produce pharmaceutical effects. This high PPB value may reduce the efficiency and delay the excretion of tilrioside.[55] Moreover, a high PPB value indicates a high potential for producing drug–drug interactions, especially with other highly protein-bound drugs. In general, drugs

Table 5 Physicochemical drug-like properties of the top-ranked herbal constituents of PBP2a and PBP4

	log P	MW	H-bond donors	H-bond acceptors	Rotatable bonds	Rings	Lipinski	Lead like	Aqueous solubility
Tiliroside	2.26	594.5	7	13	8	5	3	3	0.03
LD	0.44	476.4	6	12	5	4	2	3	2.68
Yuanhuapine	4.32	542.6	3	10	5	7	1	3	2.42
(-)-pinoresinol	1.90	358.4	2	4	4	4	0	0	0.06
Neo-chlorogenic acid	−0.33	354.3	6	9	5	2	1	1	1000
Momorcharaside B	2.64	654.9	8	10	8	5	2	3	0.002
3,4-dihydroverbenalin	−2.53	390.4	5	10	5	3	1	2	1000
Magnatriol B	2.59	242.3	3	3	3	2	0	0	0.32
Daucosterol	7.18	576.9	4	6	9	5	2	2	0.0001
5HAD	7.13	588.7	3	10	11	6	2	3	0.003
AG	0.18	432.4	6	10	4	4	1	2	1.38
Genkwanine E	4.59	638.7	6	10	8	6	2	4	0.05
Jionoside D	0.75	624.6	9	15	11	4	3	3	15.7
Genkwanine F	4.68	640.8	6	10	9	6	2	4	0.04
LG	0.13	448.4	7	11	4	4	2	2	1.86
3-caffeoylquinic acid	−2.53	390.4	5	10	5	3	1	2	1000
Yuanhuagine	5.78	584.7	3	10	9	6	2	3	0.07
Yuanhuajine	7.34	660.8	3	10	11	7	2	3	0.02

5HAD: 5beta-hydroxyresiniferonol-6alpha,7alpha-epoxy-12beta-acetoxy-9,13,14-ortho-2E-decenoate; LD: luteolin-7-diglucuronide; LG: luteolin-7-*O*-beta-D-galactopyranoside; PBP2a: penicillin binding protein 2a; PBP4: penicillin binding protein 4; 3AG: apigenin-7-*O*-beta-D-glucopyranoside.

[1]og P = octanol–water partition coefficient under standard conditions at 25°C (optimal value: −1.00 to 4.20); MW: molecular weight (values for optimal drug-like properties are <=460.00); optimal numbers of H-bond donors, H-bond acceptors, rotatable bonds and rings are <=5, <=10, <=10, and <4, respectively; optimal numbers of violations for both Lipinski and lead like are <1; aqueous solubility was calculated at pH 6.4 and in mg/mL (<=0.01 indicates highly insoluble, 0.01–0.1 is insoluble, >0.1 is soluble).

Table 6 ADME drug-like properties of the top 10 ranked herbal constituents of PBP2a and PBP4

	Caco-2	PPB (%)	CNS	HIA (%)
Tiliroside	0.3×10^{-6}	94	−5.78	85
LD	0.1×10^{-6}	84	−5.98	23
Yuanhuapine	1.5×10^{-4}	8	−1.64	100
(-)-pinoresinol	1.2×10^{-4}	81	−2.70	100
Neo-chlorogenic acid	0.0×10^{-6}	76	−6.25	1
Momorcharaside B	2.0×10^{-6}	93	−4.37	96
3,4-dihydroverbenalin	0.0×10^{-6}	51	−6.98	1
Magnatriol B	130×10^{-6}	78	−2.24	100
Daucosterol	0.4×10^{-6}	99	−4.38	100
5HAD	1.0×10^{-6}	13	−2.73	100
AG	0.0×10^{-6}	74	−5.68	18
Genkwanine E	1.6×10^{-5}	55	−2.44	100
Jionoside D	0.1×10^{-6}	53	−5.22	9
Genkwanine F	1.4×10^{-5}	47	−2.36	100
LG	0.0×10^{-6}	64	−5.89	7
3-caffeoylquinic acid	0.0×10^{-6}	51	−6.98	1
Yuanhuagine	2.3×10^{-5}	4	−1.82	100
Yuanhuajine	0.7×10^{-6}	17	−2.92	100

5HAD: 5beta-hydroxyresiniferonol-6alpha,7alpha-epoxy-12beta-acetoxy-9,13,14-ortho-2E-decenoate; LD: luteolin-7-diglucuronide; LG: luteolin-7-*O*-beta-D-galactopyranoside; PBP2a: penicillin binding protein 2a; PBP4: penicillin binding protein 4; 3AG: apigenin-7-*O*-beta-D-glucopyranoside;.
Caco-2 predicts passive intestinal permeability and was measured in cm/s (<=1.00 indicates poorly permeable, 1.00–7.00 is moderately permeable, >7.00 is highly permeable); plasma protein binding estimates the overall percentage bound in blood plasma protein; CNS values of <=−3.50 indicates non-penetrant to central nervous system, −3.50 to −3.00 is weak penetrant, and >−3.00 is penetrant; HIA: human intestinal absorption (<=30% indicates poorly absorbed, 30–70% is moderately absorbed, >70% is highly absorbed).

with less than an 80–85% PPB value have appeared to be of slight clinical importance.[55] Nevertheless, there are still many highly protein-bound drugs with >95% PPB being used, especially for severe illness and serious infections. Classic examples are dicloxacillin and flucloxacillin.[56]

Luteolin 7-diglucuronide of *V. officinalis* achieved the second highest score in the PBP2a docking simulation (Table 3). Its antibacterial activity was uncertain in our literature search. However, its structurally related compounds, luteolin and a series of its derivatives are well-documented antibacterial agents against various species, including *S. aureus*.[57] Luteolin was also characterized as a selective inhibitor to the growth of *S. aureus*, including MRSA and MSSA, as indicated by an MIC value of 62.5–125 μg/mL.[58] It has also been found to have synergetic effects on MRSA in combination with several quinolones and aminoglycosides antibiotics.[59] Thus, as a derivative of luteolin, luteolin 7-diglucuronide may also have anti-MRSA effects. It may have produced the partial synergistic effects of *V. officinalis* observed in this study. Luteolin 7-diglucuronide achieved an optimal log P value (Table 5), which indicated an optimal balance of lipophilicity and hydrophilicity for oral absorption. However, as a more accurate parameter, this compound appeared to have poor HIA properties (Table 6),[60] suggesting that luteolin 7-diglucuronide may only be administered by injection or using costly formulation strategies to improve its bioavailability, such as self-emulsifying systems.[61] In terms of causing CNS adverse reactions, luteolin 7-diglucuronide is a proven moderate $GABA_A$ inhibitor (IC_{50} 40 μM).[62] $GABA_A$ inhibitors produce stimulant effects and are mainly located in the

Table 7 Drug safety profiles of the top 10 ranked herbal constituents of PBP2a and PBP4

	Ames	hERG	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP4A4
Tiliroside	0.64	0.25	NI	U	U	NI	U
LD	0.50	0.34	NI	NI	NI	NI	U
Yuanhuapine	0.36	0.44	NI	U	U	NI	U
(-)-pinoresinol	0.41	0.42	U	U	U	U	U
Neo-chlorogenic acid	0.22	0.27	NI	NI	NI	NI	NI
Momorcharaside B	0.27	0.33	NI	NI	NI	NI	NI
3,4-dihydroverbenalin	0.38	0.29	NI	NI	NI	NI	NI
Magnatriol B	0.30	0.38	U	U	U	NI	U
Daucosterol	0.23	0.38	NI	NI	NI	NI	NI
5HAD	0.37	0.42	NI	U	U	NI	U
AG	0.70	0.30	NI	NI	NI	NI	NI
Genkwanine E	0.40	0.40	NI	NI	U	NI	U
Jionoside D	0.44	0.28	NI	NI	NI	NI	NI
Genkwanine F	0.41	0.40	NI	NI	U	NI	U
LG	0.52	0.30	NI	NI	NI	NI	NI
3-caffeoylquinic acid	0.38	0.29	NI	NI	NI	NI	NI
Yuanhuagine	0.52	0.39	NI	U	U	U	U
Yuanhuajine	0.37	0.43	NI	U	U	U	U

CYP: Cytochrome P450; 5HAD: 5beta-hydroxyresiniferonol-6alpha,7alpha-epoxy-12beta-acetoxy-9,13,14-ortho-2E-decenoate; I: inhibitor; LD: luteolin-7-diglucuronide; LG: luteolin-7-*O*-beta-D-galactopyranoside; 3AG: apigenin-7-*O*-beta-D-glucopyranoside; NI: non-inhibitor; PBP2a: penicillin binding protein 2a; PBP4: penicillin binding protein 4; U: undefined.
Ames test predicts mutagenic potential (<=0.33 indicates non-mutagenic, 0.33–0.67 is undefined, >0.67 is mutagenic); hERG estimates cardiotoxicity of compounds related to drug interactions with the hERG channel (<=0.33 indicates non-inhibitor, 0.33–0.67 is undefined, >0.67 is inhibitor).

CNS.[63] This *in silico* work predicted that luteolin 7-diglucuronide would be a CNS non-penetrant (Table 6), making the CNS effect minimal. Luteolin 7-diglucuronide was also predicted to be a non-inhibitor of four of the most significant Cytochrome P450s (CYPs) (Table 7), indicating a low likelihood of causing drug–drug and drug–food interactions. Thus, further experimental investigations of this compound, especially on the inhibition of PBP2a, would be truly worthwhile.

The third ranked compound against PBP2a was yuanhuapine (Table 3), which had no significant MRSA-related data in our literature search. Its structurally related compound, yuanhuacine, has been identified as a DNA topoisomerase I inhibitor based on an animal study.[64] This type of inhibitor has also been suggested for use in treating cancer and antibacterial activity.[65] Its effect on MRSA is unknown. In terms of ADME and drug safety, yuanhuapine is a very lipophilic compound (log P = 4.32) with 100% HIA (Tables 5 and 6). Its binding ability to most CYP and hERG is undefined, as indicated by a borderline score between inhibitor and non-inhibitor. Thus, the probability of obtaining a correct prediction is low.

Other highly ranked compounds against PBP2a were (-)-pinoresinol, neo-chlorogenic acid, momorcharaside B, 3,4-dihydroverbenalin, magnatriol B, and daucosterol (Table 3). All of them had no significant MRSA-related data in our literature search. Regarding ADME, (-)-pinoresinol and magnatriol B had excellent physicochemical drug-like properties and were predicted as having high HIA properties (Tables 5 and 6). However, they were both CNS penetrant and undefined in most CYP profiling (Tables 6 and 7). Neo-chlorogenic acid and 3,4-dihydroverbenalin were highly hydrophilic compounds, both with poor absorption (<1% HIA) (Tables 5 and 6). Daucosterol was also highly ranked in the docking simulations for PBP4, suggesting that it may also bind tightly to PBP4. However, it is highly lipophilic (log P = 7.18) with 99% of PPB (Tables 5 and 6). The log P value of momorcharaside B was optimal (2.64) and predicted to have 96% HIA (Tables 5 and 6). It was a non-inhibitor to all five common CYP and hERG (Tables 6 and 7). It was also non-mutagenic, as indicated by a low Ames value (0.27) (Table 7). Further *in vitro* investigation on momorcharaside B against PBP2a would be worthwhile.

PBP4

The docking simulations of PBP4 and 139 herbal constituents suggested that 40 of the latter may bind to PBP4 in a more stable mode than the experimentally proven PBP4 inhibitor, cefotaxime.[66] The top-ranked constituent was apigenin,7-*O*-beta-D-glucopyranoside (Table 4), a constituent of *D. genkwa*. According to our literature search, the effect of this compound on MRSA is unknown. However, based on its structurally related compounds, apigenin was found to be selectively toxic to *S. aureus*, including the MRSA and MSSA strains (MIC 3.9–15.6 μg/mL).[58] Thus, apigenin,7-*O*-beta-D-glucopyranoside could be another active constituent of *D. genkwa*, which produced a synergistic anti-MRSA effect in combination with oxacillin in this study. Our docking simulations suggested that its mechanism is PBP4 inhibition. In terms of ADME, apigenin,7-*O*-beta-D-glucopyranoside was predicted to be poorly absorbed (HIA = 18%) (Table 6), such that a specific formulation would be necessary to improve its oral absorption. It was also predicted as a non-inhibitor for all five CYPs and hERG. However, it appeared to be mutagenic (Table 7).

In our literature search, we found no MRSA-related articles on the other top-ranked constituents, including genkwanine E, jionoside D, genkwanine F, luteolin-7-*O*-beta-D-galactopyranoside, 3-caffeoylquinic acid, and yuanhuagine. None of these compounds had excellent ADME or drug-safety properties. Genkwanine E, genkwanine F, jionoside D, and yuanhuagine were predicted to have poor physiochemical drug-like properties but high HIA (Tables 5 and 6). Luteolin-7-*O*-beta-D-galactopyranoside was poorly absorbed and mutagenic. 3-caffeoylquinic acid had acceptable physiochemical drug-like properties and a good drug-safety profile, but poor absorption. Again, absorption of this constituent can be modified by drug formulation technologies.

Conclusions

In vitro susceptibility testing revealed that the extracts of the four TCMs – *M. officinalis*, *V. officinalis*, *M. charantia*, and *D. genkwa* – had anti-MRSA and anti-MSSA effects, with *V. officinalis* exhibiting advanced performance in comparison to gentamicin. All four TCMs produced stronger inhibitory effects on MRSA than on MSSA. The synergistic testing results demonstrated the potentiated antibacterial effects, when combined with antibiotics, on MRSA of *M. officinalis*, *V. officinalis*, and *D. genkwa*. To detect which active constituents were responsible for producing those effects, validated *in silico* simulations were performed on 139 constituents of the four herbs. The results indicated that some of the constituents had the ability to binding more strongly to PBP2a and PBP4 than their experimentally proven inhibitors, indicating their potential to inhibit MRSA. However, most of the constituents had poor physicochemical drug-like properties, ADME, and drug safety profiles. Tiliroside achieved excellent scores for binding to both PBP2a and PBP4, further supporting current scientific evidence of its synergistic inhibition effects on MRSA when combined with antibiotics. This study revealed that tiliroside's mechanism could be the inhibition of PBP2a and PBP4. Pinoresinol, magnatriol B, and momorcharaside B bind to both PBP2a and PBP4 in a stable mode and are thus potentially strong inhibitors with acceptable drug-like properties and drug safety profiles. Further investigation and optimization of these compounds could assist in developing therapeutic agents to modulate the effect of antibiotics on MRSA for use as adjuvant therapy.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript; SOH, CFH, CHC, IHF, CKN, and PF conducted the experiments, LRM provide technical advices and lead the studies, and PF and CFK wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTAL MATERIAL

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