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Synthesis and anti Methicillin resistant *Staphylococcus aureus* activity of substituted chalcones alone and in combination with non-beta-lactam antibiotics

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ABSTRACT

A total of 30 chalcone analogues was synthesized via a base catalyzed Claisen Schmidt condensation and screened for their in vitro antibacterial activity against Methicillin-sensitive *Staphylococcus aureus* (MSSA) and Methicillin-resistant *Staphylococcus aureus* (MRSA) alone or in combination with non beta-lactam antibiotics namely ciprofloxacin, chloramphenicol, erythromycin, vancomycin, doxycycline and gentamicin. In the checkerboard technique, fractional inhibitory concentration indices (FICI) show that the following combinations like ciprofloxacin with **25** (4′-bromo-2-hydroxychalcone); doxycycline with **21** (4-hydroxychalcone); doxycycline with **25**; and doxycycline with **4** (2′,2-dihydroxychalcone) were synergistic against MRSA. In term SAR study, the relationship between chalcone structure and their antibacterial activity against *S. aureus* and synergy with tested antibiotics were discussed. Possible mechanisms for antibacterial activity of chalcones alone as well as the synergistic effect in combinations were proposed by molecular modeling studies, respectively. Combinations of chalcones with conventional antibiotics could be an effective alternative in the treatment of infection caused by MRSA.

Currently, more and more pathogenetic bacterial species have been appearing to be resistant to different antibiotics used.[1] The world are facing with antibiotic-resistant bacteria and there are several new antibiotics are accepted each year to help repel the infectious diseases. However, it takes much time and high cost to issue a new antibiotic. Hence, a new trend today is to coordinate the existing antibiotics with antimicrobial agents available in nature as effective combinations against bacteria. Moreover, using natural products also help to diminish the toxicity of the drugs when they are used on humans.[2]

Methicillin-resistant *Staphylococcus aureus* (MRSA), a potentially life-threatening pathogen including pneumonia and cSSSI (complicated skin and skin structure infections), has led to an increasing need for new novel antibiotics for both community-acquired and hospital-acquired bacterial infections. Key antibiotics to treat MRSA are vancomycin, linezolid, daptomycin and tigecycline.[3a] Agents currently recommended for the treatment of MRSA cSSSi include vancomycin, linezolid, daptomycin, tigecycline, telavancin.[3b] For MRSA pneumonia, approved antimicrobials are vancomycin and linezolid in the USA, and vancomycin, linezolid, teicoplanin and quinupristin/dalfopristin in Europe.[3c,d] Currently, pathogens resistant to vancomycin, the centred drug for serious infections due to MRSA, are gradually reported.[3] The problems of resistant Gram-positive bacteria such as *S. aureus* (SA) highlight the urgent need for new drugs with new modes of action or combination of conventional antibiotic and suitable chemical agents to treat infections caused by resistant human pathogens.

Chalcone is an open-chain flavonoid with α,β-unsaturated carbonyl group and is one of the important compound groups of flavonoid derived from nature.[4] Thousands of chalcone derivatives are synthesized in chemical laboratories, up to date. Both natural and synthetic chalcones posses a wide variety of pharmacological activities like antimicrobial,[5] anti-cancer,[6] anti-tuberculosis,[7] anti-inflammatory,[8] antioxidant,[9] and so on. Recently, numerous studies confirm that the activity against MRSA of flavonoid derivatives is generally weak, but when they are in combinations with some antibiotics, they could contribute to increase antibacterial activity of antibiotics used together or to restore the effect of separate invalid antibiotics. This result shows significant contributions of flavonoid on increasing or recovering the effectiveness of specific antibiotics.[10]

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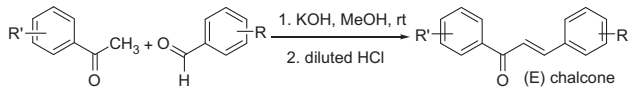


Scheme 1. Synthesis of chalcone analogues via Claisen Schmidt conventional condensation.

In present study, a total of 30 chalcone analogues with different substituents in A and B rings was synthesized and tested for their anti Methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA activity alone and in combination with several conventional antibiotics.

Chalcone derivatives with high purity were synthesized via Claisen–Schmidt condensation (Scheme 1).[11] Thus, the appropriate commercially available aryl aldehydes were reacted with substituted acetophenones in methanol/KOH at room temperature for several hours.[12–15] This process afforded the desired chalcones (**1–30**) with average yield of 45–79% as listed in Table 1.

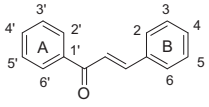
The purity of products was checked by TLC on silica gel plates. The ^{1}H NMR spectrum of synthesized chalcones displayed two doublets at δ 6.2–8.0 ppm with characteristic coupling constant (J) of 15–16 Hz, which confirms the formation of chalcones (possessing a α,β -unsaturated ketone).[16] This higher coupling constant value indicates all synthetic compounds were geometrically pure and were exclusively trans (E) isomers.

Preliminarily identifying antibacterial activity of test antibiotics confirmed that all test antibiotics which had the diameters of antimicrobial circles within the limits following professional documentation (Clinical Laboratory Standards Institute, CLSI)[17] meet the requirements to use in the test (Table 1 and Table 2).[18–21] Results on determining antibacterial susceptibility of test substances showed that of thirty synthesized chalcone analogues, four compounds like **4**, **20**, **21** and **25** were found to be in the range of weak to moderate against two test strains of MRSA and MSSA. The others were inactive both MSSA as well as MRSA.

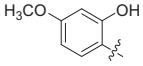
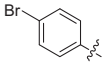
Results on determining antibacterial coordination ability of test substances and antibiotics showed that of thirty synthesized chalcone analogues, only four active compounds exhibited positive interaction with antibiotics like gentamicin (with **4**), doxycycline (with **4**, **20**, and **25**), ciprofloxacin (with **4**).[22,23] Surprisingly, chalcone **20** and **25** showed positive interaction in combination with ciprofloxacin only on MRSA but not on MSSA. It means that gentamicin exhibited positively interaction with only chalcone **4**, meanwhile, ciprofloxacin and doxycycline demonstrated positive interactions with almost above mentioned chalcones. Conversely, some gram-positive antibiotics like clindamycin, erythromycin and vancomycin showed negative interactions with all tested chalcones (Table 3). It was also noted that chloramphenicol a broad-spectrum antibiotic did not have any interaction with synthesized chalcones.

Antibacterial interactions were determined using the Checkerboard method as previously described.[24,25] The results of quantitatively determining antibacterial coordination ability proved that combinations between antibiotics and chalcone derivatives (**4**, **20**, **21,** and **25)** were predominantly synergistic. Among 14 tested

Table 1
Chemical structures of synthesized chalcone analogues and antibacterial activity



(*E*) chalcone

Compound	Formula	A ring structure	Substituents in B ring				Isolated yield (%)	Ref.
			C2	C3	C4	C5		
1	$C_{15}H_{12}O_2$		H	H	H	H	46	12
2	$C_{15}H_{11}ClO_2$		H	H	Cl	H	78	13
3	$C_{15}H_{10}Cl_2O_2$		H	Cl	Cl	H	82	13
4	$C_{15}H_{12}O_3$		OH	H	H	H	56	14
5	$C_{16}H_{14}O_3$		H	H	OCH_3	H	59	12
6	$C_{16}H_{14}O_3$		OCH_3	H	H	H	58	12
7	$C_{17}H_{16}O_4$		OCH_3	H	OCH_3	H	54	12
8	$C_{17}H_{16}O_4$		H	OCH_3	OCH_3	H	57	12
9	$C_{18}H_{18}O_5$		H	OCH_3	OCH_3	OCH_3	53	12
10	$C_{16}H_{14}O_3$		H	H	H	H	61	12
11	$C_{16}H_{13}ClO_3$		H	H	Cl	H	76	12
12	$C_{16}H_{12}Cl_2O_3$		H	Cl	Cl	H	79	12
13	$C_{17}H_{16}O_4$		H	H	OCH_3	H	55	12
14	$C_{18}H_{18}O_5$		OCH_3	H	OCH_3	H	51	12
15	$C_{18}H_{18}O_5$		H	OCH_3	OCH_3	H	49	12
16	$C_{19}H_{20}O_6$		H	OCH_3	OCH_3	OCH_3	48	12
17	$C_{15}H_{12}O$		H	H	H	H	42	12
18	$C_{16}H_{14}O_2$		OCH_3	H	H	H	48	12
19	$C_{16}H_{14}O_2$		H	H	OCH_3	H	52	12
20	$C_{15}H_{12}O_3$		OH	H	H	H	47	12
21	$C_{15}H_{12}O_2$		H	H	OH	H	49	12
22	$C_{17}H_{16}O_3$		OCH_3	OCH_3	H	H	54	13
23	$C_{17}H_{16}O_3$		H	OCH_3	OCH_3	H	58	13
24	$C_{15}H_{11}BrO$		H	H	H	H	62	14a
25	$C_{15}H_{11}BrO_2$		OH	H	H	H	66	14b
26	$C_{17}H_{15}BrO_3$		OCH_3	OCH_3	H	H	51	14c
27	$C_{17}H_{15}BrO_3$		H	OCH_3	OCH_3	H	57	14c
28	$C_{16}H_{13}BrO_2$		OCH_3	H	H	H	63	14d
29	$C_{16}H_{13}BrO_2$		H	OCH_3	H	H	65	14e
30	$C_{16}H_{13}BrO_2$		H	H	OCH_3	H	62	14c

combinations in which six were carried out on MSSA and eight were carried out on MRSA.

The combination of doxycycline with compound **4** had the most synergistic effect against both MSSA and MRSA, in which the rates in increasing susceptibility of bacteria with doxycycline were eight to sixteenfold, respectively. In such combinations, the MICs of doxycycline against MSSA and MRSA were very low about 0.125 and 0.25 μg/mL, respectively. Other combinations, pair of doxycycline with compound **20** was synergistic against both MSSA and MRSA, meanwhile, pair of doxycycline with compound **25** was only additive against MRSA but not effective with MSSA. Ciprofloxacin showed positive interaction with all four chalcones **4**, **20**, **21** and **25**, in which of six tested combinations, one was indifferent, two were additive and the last four were synergistic effect against SA (Table 4). The combinations of ciprofloxacin with **4** and **21,** respectively were demonstrated significantly synergism against MRSA with very low MICs (0.0625 μg/mL) for ciprofloxacin in both combinations. The rates in increasing susceptibility of MRSA with ciprofloxacin were eight-fold.

From the results of bioactivities, some preliminary remarks on structure–activity relationship can be drawn as follow: (i) a free hydroxyl group in position(s) 2 and/or 4 of B ring appears to be very important to anti-MRSA activity alone or in combination with antibiotics (**4** vs **6**; **20** vs **18**; **21** vs **19** and **25** vs **28**); (ii) in the opposite, a free hydroxyl group in position 2′ of A ring, like 2′-hydroxychalcone analogues (**1**–**9**) seems to be unnecessary; (iii) methylation to hydroxyl group might also be responsible for the abolishment in the anti SA activity; (iv) chlorine substitution seems to be unnecessary for the anti SA activity.

The chalcone derivatives such as 3-hydroxy-4′-methoxy-chalcone was found to act by damaging the cell wall of SA which clearly similar to the observed mechanism of a well-known cell membrane permeabilizer, polymyxin B, a cationic peptide antibiotic shows 78–82% membrane damaging activity against SA.[26] Hence, our synthesized chalcones which have the same structures could effect on SA and MRSA by damaging the bacterial cell wall in the presence of chalcones alone.

Recently, the use of efflux pump inhibitors (EPIs) may be a powerful strategy to overcome transporter-mediated bacterial multidrug resistance and also tumor cell multidrug resistance.[27,28] Drug efflux transporter has attracted considerable interest also with respect to its role in absorption-distribution and elimination of drugs. Multidrug resistance efflux pumps are recognized as an important component of resistance in both Gram-positive and Gram-negative bacteria.[29] Tariquidar and elacridar, the third-generation P-glycoprotein inhibitors in clinical development, have potent inhibitory effect against certain bacterial efflux pumps in vitro and indicated the potency overcome bacterial multidrug resistance towards ciprofloxacin by increasing intracellular drug concentration.[29] Tariquidar had no intrinsic activity against any strain tested but tariqudar (as elacridar) showed dose-dependently increased susceptibility towards ciprofloxacin and resulted in a 10-fold reduction of the ciprofloxacin MIC in SA.[29] In cancer cell, flavonoids were reported as bifunctional modulators binding to a site partly overlap the ATP site and vicinal hydrophobic region interacting with the cytosolic domain of P-glycoprotein (P-gp or ABCB1).[30] Moreover, flavonoids are considered as the potent inhibitors of mouse and human P-gp targeting the nucleotide binding domain

Table 2
In vitro antibacterial activity of synthesized chalcone and test antibiotics by disc diffusion method and diameter of zone of inhibition (mm)

Compounds[a]	MSSA		MRSA	
	Zone of inhibition (mm)	MICs (μg/ml)	Zone of inhibition (mm)	MICs (μg/ml)
4	14	64	14	64
20	11	32	11	64
21	10	64	9	32
25	13	32	13	64
Vancomycin	17	4	16	8
Doxycycline	27	8	27	4
Ciprofloxacin	27	0.25	21	0.5
Gentamicin	27	1	23	4
Chloramphenicol	25	ND	26	ND
Clindamycin	24	ND	–	ND
Erythromycin	25	ND	–	ND

(–): not inhibition at test concentrations: erythromycin at 15 μg/disc; clindamycin at 2 μg/disc.
ND = not determined.
[a] Other chalcones: not inhibition at tested concentration of 256 μg/mL.

Table 3
Qualitative determination of the interaction between the synthesized chalcones and known-antibiotics

Antibiotic	Synthesized chalcones							
	4		20		21		25	
	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA
Vancomycin	+	+	–	–	–	–	–	–
Doxycycline	+	+	+	+	–	–	+	+
Ciprofloxacin	–	–	–	–	–	–	–	–
Gentamicin	–	–	–	–	–	–	–	–
Chloramphenicol	+	+	+	–	+	+	+	–
Clindamycin	–	–	–	–	–	–	–	–
Erythromycin	–	–	–	–	–	–	–	–

(+): positive interaction; (–): negative interaction.

Table 4
Antibacterial results from the combinations between synthesized chalcones and known-antibiotics

Mix[a]	MSSA ATCC 25923					MRSA ATCC 43300				
	MICs (μg/mL)		FICI	Interpretation	Increasing rate (fold)[b] Antibiotic/chalcone	MICs (μg/mL)		FICI	Interpretation	Increasing rate (fold)[b] Antibiotic/chalcone
	Alone	Mix[a]				Alone	Mix[a]			
4	64	8	0.25	Synergy	8/8	64	2	0.15	Synergy	16/8
Doxy	1	0.125				4	0.25			
4	64	64	1.5	Indifference	2/1	64	32	0.62	Additive	8/2
Cipro	0.25	0.125				0.5	0.0625			
4	64	1	0.36	Synergy	4/64	64	16	0.50	Synergy	4/4
Genta	1	0.25				4	1			
20	32	2	0.31	Synergy	4/16	64	2	0.15	Synergy	16/32
Doxy	1	0.25				4	0.25			
20	ND	ND				64	32	0.75	Additive	4/2
Cipro	ND	ND				0.5	0.125			
21	64	4	0.31	Synergy	4/16	32	4	0.25	Synergy	8/8
Cipro	0.25	0.0625				0.5	0.0625			
25	32	4	0.37	Synergy	4/8	64	8	0.62	Additive	4/8
Doxy	1	0.25				4	1			
25	ND	ND				64	2	0.28	Synergy	4/32
Cipro	ND	ND				0.5	0.125			

ND = not determined. Genta: gentamicin; Doxy: doxycycline; Chloram: chloramphenicol; Vanco: vancomycin; Cipro: ciprofloxacin; Clin: clindamycin; Ery: erythromycin.
[a] Mix = Combination.
[b] Rate in increasing antibacterial activity of antibiotic/chalcone in combination compared to that of in alone (fold).

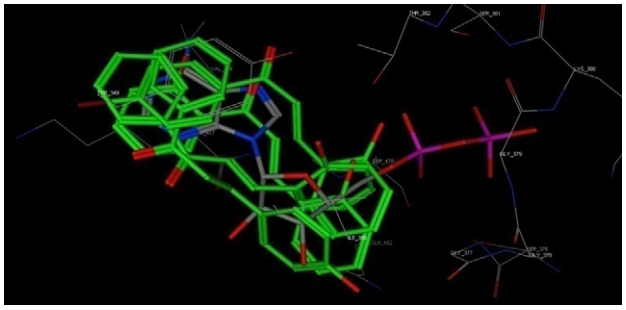


Figure 1. Docked alignment of ADP (gray carbon) and chalcones **4**, **20**, **21**, **25** (green carbon) within the nucleotide binding domain (Sav1866 of *S. aureus*) generated by MOE docking. Colors of other atoms are red (oxygen), blue (nitrogen), and magenta (phosphorus).

(NBD).[30] Activity of chalcones increased susceptibility towards non beta-lactam antibiotics may be explained by the inhibitory of efflux pump. To get insight the interaction of chalcones and multi-drug transporter Sav1866 of SA, molecular docking studies are performed. The X-ray structure of the multi-drug transporter Sav1866 of SA in complex with adenosine-5′-diphosphate ADP (pdb code: 2hyd, resolution: 3.00 Å) was used for molecular docking study.[31] The ligands, **4, 20, 21**, and **25** were flexibly docked into the NBD of Sav1866, respectively, by using the MOE dock program.[32,33] The multi-drug transporter Sav1866-bound conformation of those chalcones are partially overlapped with ADP position with A ring superimposed onto the adenine and B ring positioned in the space of ribose of ADP (Fig. 1). Compounds **4, 20, 21**, and **25** also showed the π–π stacking between A ring and Tyr349 that similar observed of purine ring of adenosine in the

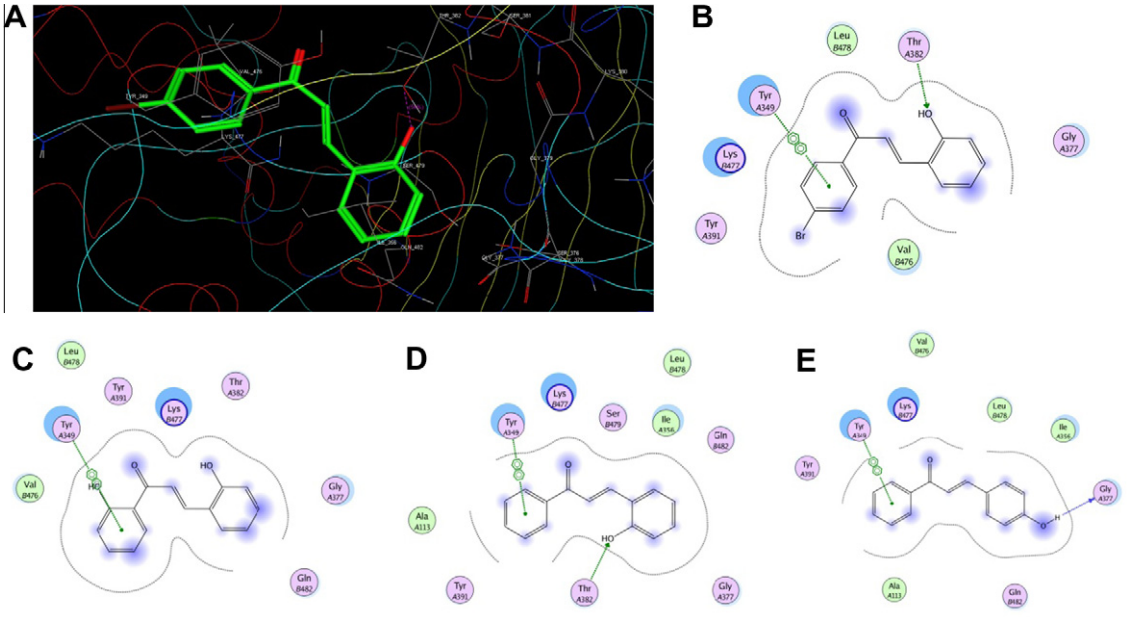


Figure 2. Docked conformation of chalcone **25** (green carbon) in ADP binding site of Sav1866 of *S. aureus* (A). In right side (B), 2D ligand-interactions between this chalcone and multi-drug transporter Sav1866 is also showed. A ring of **25** interacts with Tyr349 by π- π stacking and hydrogen presented in B ring contributed the hydrogen bond (magenta dotted lines) with Thr382. In lower side C-E, 2D ligand-interactions between this chalcones **4**, **20**, **21** and Sav1866 are indicated, respectively.

active site. Moreover, B rings of docked chalcones formed various hydrogen bonds with Thr382 and Gly377 (Fig. 2). Recently, the combination between in silico approach (docking, 3D-QSAR) and experimental results indicated the important amino acid in NBD crucial for flavonoids derivatives interacted with human P-gp binding affinity[30] and this hydrophobic site is related to NBD of Sav1866 of *S. aureus*. The resulting tight interactions between chalcones**4, 20, 21**, **25** and Sav1866 may used to explain and would be related to the synergistic mechanism on non beta-lactam antibiotics.

In the previous reports, the NorA efflux pump in SA extrudes a variety of structurally unrelated antibacterial like berberine, an cationic antimicrobial, from bacterial cells.[2b,34] Hence, a test combined activity between berbeine and a synthetic chalcone against MRSA may be a positive proof to prove the above proposed hypothesis. Resulting from our experiments indicated that synergistic effect was obtained for the combination of chalcone **21** and berberine. The MIC values of berberine decreased in 32-fold from 128 μg/mL (berberine alone) to 4 μg/mL (berberine with chalcone **21**). However, the proposed mechanism of synergistic activity of chalcones still needs more experiments to confirm. Since flavonoids have potential without toxicity, this class of drugs is promising for future applications in infectious diseases as well as in tumor diseases.

In conclusion, the present investigation has clearly shown that certain hydroxylated chalcone derivatives moderately inhibited against MSSA and MRSA activities when using alone and they proved to have significantly synergistic effect with non beta-lactam antibiotics against MRSA. These potential analogues are 2′,2-dihydroxychalcone (**4**); 2-hydroxychalcone (**20**); 4-hydroxychalcone (**21**) and 4′-bromo-2-hydroxychalcone (**25**). The potential combinations such as (i) ciprofloxacin with 4-hydroxy-chalcone **(21)**; (ii) ciprofloxacin with 4′-bromo-2-hydroxychalcone (**25**); (iii) doxycycline with 4′-bromo-2-hydroxychalcone (**25**); (iv) doxycycline with 2′,2-dihydroxychalcone (**4**) and (v) gentamicin with 2′,2-dihydroxychalcone (**4**) could be investigated further to achieve new combinations in treatment infectious diseases caused by MRSA. Related to the antibacterial mechanisms, our synthesized chalcones could effect on SA and MRSA by damaging the bacterial cell wall. Possible mechanism for the synergistic effect of chalcones in combinations with non-beta-lactam antibiotics were also proposed as the efflux pump inhibitors and discussed via molecular docking studies.

Acknowledgments

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15. *General procedure for the synthesis of chalcones:* a solution of substituted acetophenones (5 mM) and aromatic aldehyde (5 mM) in methanol (15 mL) was cooled to 5–10 °C in an ice bath. The cooled solution was treated with adding a small portion of pulverized KOH (10 mM). The reaction mixture was magnetically stirred for 60 min and then left overnight or longer, monitored by thin layer chromatography using developing solvent *n*-hexane–acetone (5–1). The resulting dark solution was diluted with ice water and carefully acidified using dilute hydrochloric acid. The chalcone which separated as a yellow solid was collected by filtration after washing with water and further purified by crystallization from methanol or by silica gel column chromatography.
16. *Chemical physical properties:* Melting points (mp, °C) were recorded on a Gallenkamp apparatus and were uncorrected. IR spectra were recorded in KBr on Shimadzu FTIR 8201 PC. ^{1}H NMR spectra were measured on Bruker Avance 500 MHz spectrometer. Chemical shifts are reported in ppm. Coupling constants *J* are reported (Hz).
17. CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Test; Approved Standard*, 9th ed.; CLI document M2–A9.26:1; Clinical Laboratory Standards Institute: Wayne, PA, 2006.
18. *Antibiotics and antibacterial agents:* Standard powder forms of vancomycin and ciprofloxacin (Sigma Chemical Co., Ltd), chloramphenicol, doxycycline and gentamicin (Shanghai Fine Chemicals Co., Ltd), were stored at 2 to 8 °C until use. Antibiotics discs like gentamicin (10 μg), chloramphenicol (30 μg), vancomycin (30 μg), clindamycin (2 μg), doxycycline (30 μg), ciprofloxacin (5 μg) and erythromycin (15 μg) were supplied by Nam Khoa Co., Ltd, Vietnam. Two standard spices of bacteria like *Staphylococcus aureus* ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* ATCC43300 preserved and activated at our Department of Microbiology were used for this study. The bacterial culture media were purchased from Merck, in which Tryptic Soy Agar (TSA) was used to isolate and preserve bacterial species, Tryptic Soy Broth (TSB) was used as bacteria-activating medium and Mueller-Hinton Agar (MHA) was used for testing antibiotics activity.
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20. *Antibacterial susceptibility testing:* The disc diffusion method was carried out for the antimicrobial tests. Briefly: Suspension of bacterial strains (100 μL) containing 108 cfu/mL of bacteria was spread on Mueller Hinton agar (MHA) medium. The disc (6 mm in diameter), impregnated with 10 μl of the test compound at the concentration of 102.4 mg/mL were placed on the inoculated agar. Negative control was prepared using the same solvent (DMSO), which was employed to dissolve the test compounds. Antibiotic (2–30 μg/disc, 6 mm in diameter) were used. The inoculated plates were incubated at 37 °C for 24 h. Measure the diameter (mm) of inhibitory zones (Table 2).
21. *Measurement of MIC values:* The MICs of antibiotics and selectively active chalcone were determined by the microdilution method as described by The National Committee for Clinical Laboratory Standard (NCCLS). Each test compound was run in duplicate. The test plates were incubated at 37 °C for 24 h. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism. The concentration of the solvents used in the following assays was maintained at less than 2% so that no inhibition of organisms or interference occurred.
22. (a) National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 6th ed.; Approved standard M7-A6; National Committee for Clinical Laboratory

Standards: Wayne, PA, 2003; (b). National Committee for Clinical Laboratory Standards. *Performance Standards for Antimicrobial Susceptibility Testing*, 12th ed.; Informational supplement M100-S13; National Committee for Clinical Laboratory Standards: Wayne, PA, 2003.

23. *Qualitative determination of the interaction between the chalcones and antibiotics:* A routine Kirby–Bauer antibiotics susceptibility test was performed to determine the inhibitory zone of selected compounds. Briefly: sterile MHA medium is melted and poured into Petri boxes to get the agar layer about 3–4 mm thick. Dip sterile cotton stick into prepared bacterial suspension (prepared to a final concentration of approximately 108 cfu/mL), then press it on the tube wall to drain, then spread bacteria on the agar surface evenly. Put opened boxes in the incubator in 3–5 min to drain. Wells (6 mm in diameter) were punched in the agar. The distances from the hole (synthesized chalcone analogues) and disc (antibiotics) were measured so that margins of two antibiotic inhibitory zones would meet or slightly overlap. The chalcones samples prepared in solutions at rational concentration (1024 g/mL) with not more than 2% DMSO were filled into the wells. After allowing the test compounds to diffuse into agar (15 min at 37 °C) the plates were further incubated at 37 °C in the period of 18 h (for MSSA), 24 h (for MRSA). There is a positive interaction between a specific antibiotic and a test substance when antibacterial circle of each extend towards the other.
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25. *Evaluation of combined activity:* Antibacterial interactions were determined using the Checkerboard method. The range of tested compounds concentration used in the checkerboard analysis was such that the dilution range encompassed the MIC for each drug used in the analysis. The first compound of the combination was serially diluted along the ordinate, while the second compound was diluted along the abscissa. Broth microdilution plates were inoculated with each SA strains to yield $\sim 5 \times 105$ cfu/ml in a 100 μl final volume, and incubated for 18–24 h at 37 °C. Synergy has been defined as requiring a fourfold reduction in the MIC of both antibiotics in combination, compared with each used alone, measuring the fractional inhibitory concentration index (FICI). The FICI was calculated for each combination (A and B as tested compounds) using the following formula: $FICI = \frac{MIC_A(\text{combination})}{MIC_A(\text{alone})} + \frac{MIC_B(\text{combination})}{MIC_B(\text{alone})}$. The FICI was interpreted as follows: synergy, FICI $\leq$0.5; additive, 0.5< FICI 1; indifference, 1 <FICI $\leq$2; antagonism, FICI >2. Each test was run in duplicate.
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33. *Molecular modeling and docking study: Preparation of molecular structures.* The 3D structure of chalcone derivatives were prepared using the build molecule module in MOE. The structures of molecules are optimized by energy minimization until converged to a maximum derivative of 0.001 kcal mol^{-1} $Å^{-1}$. The lowest-energy conformer of each molecule was selected and stored in mdb database. *Preparation of target enzyme structure and docking.* The X-ray crystal structure of bacterial multidrug ABC transporter SAV1866:ADP complex (pdb 2hyd) was retrieved from the RCSB Protein Data Bank (www.rcsb.org). The active site was defined as all the amino acid residues enclosed within 4.5 Å radius sphere centered by the bound ligand, ADP and 'site finder' in MOE was used to determine the binding site. The docking and subsequent scoring were performed using the MOE docking programs. The final of 30 docked conformations per ligand were analyzed and used to create the illustrative figures.
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