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Computer aided screening of potent inhibitor compounds against inhibitor resistant TEM β-lactamase mutants from traditional Chinese medicine

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Abstract:
Inhibitor-resistant TEM (IRT) type β-lactamase mutation is largely known. Therefore, it is of interest to identify new yet improved leads against IRT from traditional Chinese medicine. Hence, we screened more than 10,000 compounds from Chinese medicine (tcm@taiwan database) with mutant molecular IRT models through docking techniques. This exercise identified compounds affeic acid, curcumin, salvianolic acid E, ferulic acid and p-coumaric acid with high binding score with the mutants. This was further validated in vitro where salvianolic acid E combined with cefoperazone and sulbactam effectively inhibit the R244S mutant.

Keywords: β-lactamase, mutants, docking, Chinese traditional medicines

Background:
β-lactam antibiotics are the most wieldy used in antibiotics because of their wide spectrum of efficiency, bactericidal activity, and low toxicity. But, the drastic emergence of resistance has become a serious problem **[1].** The major resistance mechanism of bacteria is the production of β-lactamase, which can hydrolyze the amide bond of the β-lactam ring, leading to antibiotic inactivation **[2].** Class A β-lactamases, which are considered to be responsible for many failures in the treatment of infectious diseases, are most widespread enzymes. TEM β-lactamase is one type of class A type β-lactamase, it is commonly found in Escherichia coli, which is one of the most common pathogens in community-acquired and nosocomial infections **[3].** To overcome the problem of β-lactamase, one of the effective methods is the combination of β-lactamase inhibitor and β-lactam antibiotic. Beta-lactamase inhibitors can protect β-lactam antibiotics by inhibiting β-lactamase. Their antimicrobial activities are low, but they can be irreversible bound with the β-lactamase, in order to prevent the hydrolysis reaction of β-lactam antibiotics **[4].** However, the emergence of inhibitor resistance strains aggravated the problem. Mutation in some positions can lead to high catalytic activity and resistance to β-lactamase inhibitors, turning the wild type β-lactamases into extended spectrum β-lactamase (Esbls) or inhibitor resistant β-lactamases(IRTs) **[5, 6].** M69I, S130G and R244S are three clinical inhibitor resistant TEM β-lactamase mutants which have been reported **[7, 8, 9].** Studies focused on them showed that Met69, Ser130 and Arg244 were three active residues close to Ser70, the active site of TEM-1 β-lactamase, and played very important roles in the hydrolysis reaction of antibiotics **[10, 11].** Therefore, it is necessary for developing inhibitors against these resistant mutants. The study mainly focused to screen new compounds against IRTS based on docking studies. The compounds screened out from database were later used for in vitro studies.

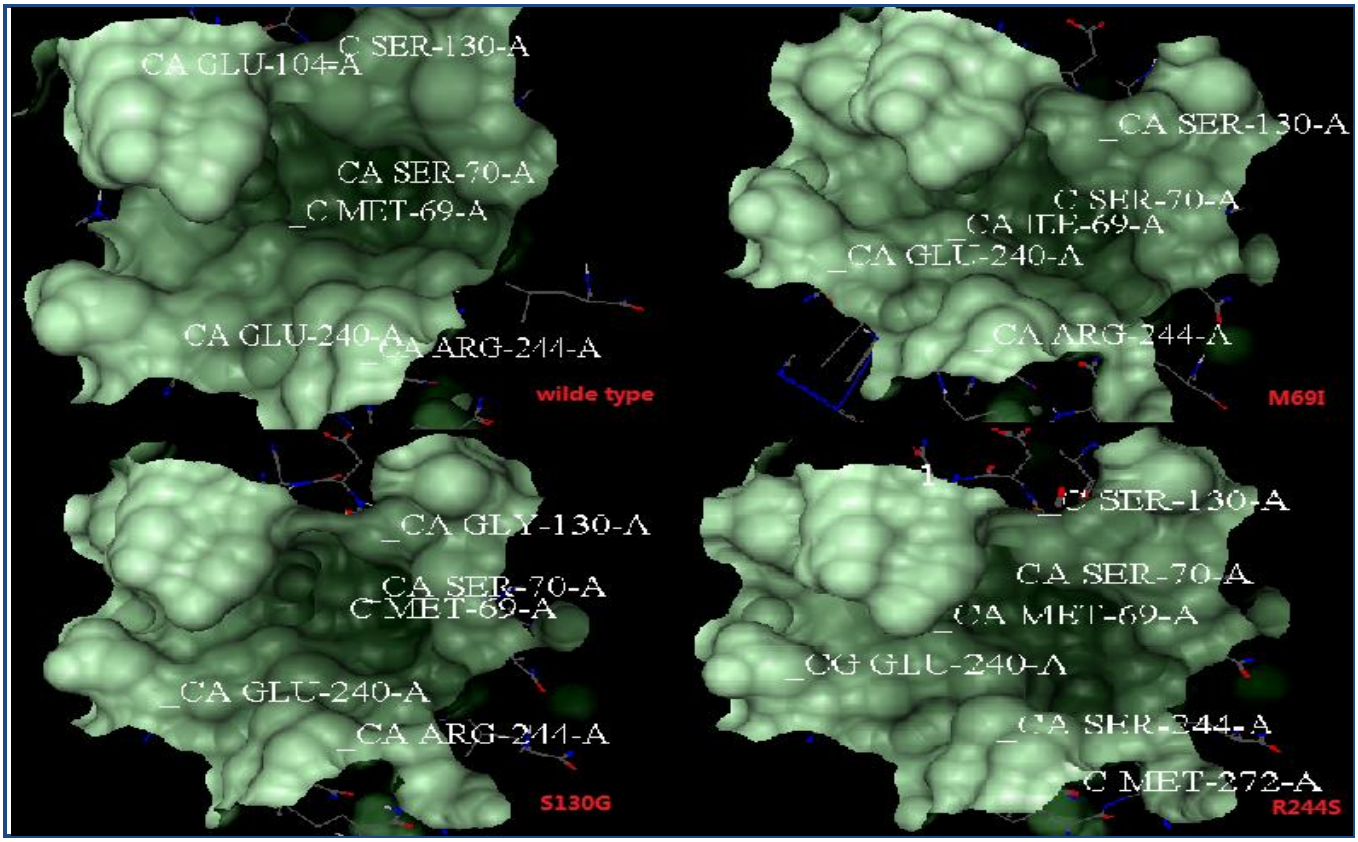

Figure 1: The accessible surface areas of wide type and three mutanted β-lactamases.

Methodology:

Proteins preparation

The crystal structure of tem-1 β-lactamase (pdbid: 1bt5) was downloaded from RSCB website **[12]**, then the software Discovery Studio 3.5 was used for design the mutants of tem-1 β-lactamase. The crystal structure of M69I was constructed by the amino acid substitution Met-69→Ile, and the S130G was constructed by the amino acid substitution Ser-130→Gly, and the R244S was constructed by the amino acid substitution Arg-244→Ser. Each structure was refined by removing the heteroatoms. These structures of the proteins would be used as drug targets in molecular docking.

Preparation of ligand library

It was reported that many compounds based herbal had high medicinal values against many pathogens **[13].** Hence, it was possible to find out the inhibitors against the IRTs by computer aided screening and the data would be useful to screen best lead molecules. A library of about 10,000 compounds from 300 different Chinese medicinea was prepared for molecular docking. All the 3D structures of ligands were retrieved from Traditional Chinese Medicine Database@Taiwan (http://tcm.cmu.edu.tw/) **[14].**

Molecular docking

The software FlexX (a component of LeadIT) was used for molecular dock in this study. In the docking, three mutants and the tem-1 β-lactamase from wild type stain were treated as receptor protein, which were docked with the ligand library receptor respectively. The docking was semi-flexible, and receptor residue bond angles were immutable, furthermore ligands were variable, finally the accessible surface area was within radius 6.5 A. Results are **Table 1 to Table 3 (see supplementary material).**

In vitro studies

The fabrication of inhibitor-resistant β-lactamase mutants

The mutation of the single amino acid would lead to the drug resistance **[15].** According to the mechanism, and the ampicillin resistance genes from PAMP was used as template, the No.139-141 loci of tem-1 β-lactamase are GCG, the No.141 locus G was changed to T, it would lead the arginine of No.244 mutate into serine, and this mutant protein was R244S. In a similar way, the No.478-480 loci ACT were changed to ACC, leading to the serine of No.130 was mutated into glycine, and this mutant protein was S130G. And the No.661-663 loci CAT were changed to AAT, leading to the methionine of No.69 was mutated into isoleucine, this Mutant protein was M69I. The mutated plasmids were transformed into BL21 competent cells in order to get three mutated strains.

The experiment of mutant strains resistant to enzyme inhibitors

The MIC was determined by the doubling dilution method. On the sterile microplate, 200ul LB liquid medium were added into each hole, and the cefperazone-sulbactam was added into the first hole of each row with the final concentration of 1024mg/ml, then stepwise double dilute it to the next holes of the row. At last, three mutant strains and the primordial strain were inoculated in the microplate respectively and the microplate was incubated at 37℃ for 24 hours. Result is shown in **Table 4 (see supplementary material).**

Invitro studies of combination of cefperazone-sulbactam and traditional Chinese medicine ingredient

Based on the docking studies, five Chinese herbal medicinal ingredients were identified for in vitro testing. The Chinese herbal medicinal ingredients used in the study are caffeic acid(ligand id:1066):, curcumin(ligand id:9863), Salvianolic acid E(ligand id:6013), ferulic acid(ligand id:3070) and p-coumaric acid(ligand id:1680) **[16,17,18,19].** Similar to the above, the MIC was determined by the doubling dilution method,but the Chinese medicinal ingredients were mixes into Cefperazone-Sulbactam with the final concentration of 20% respectively. The blank control groups without inoculation were seted up, as well as the control groups which added no Cefperazone-Sulbactam but only Chinese medicine ingredients. **Result is shown in Table 5 (see supplementary material).**

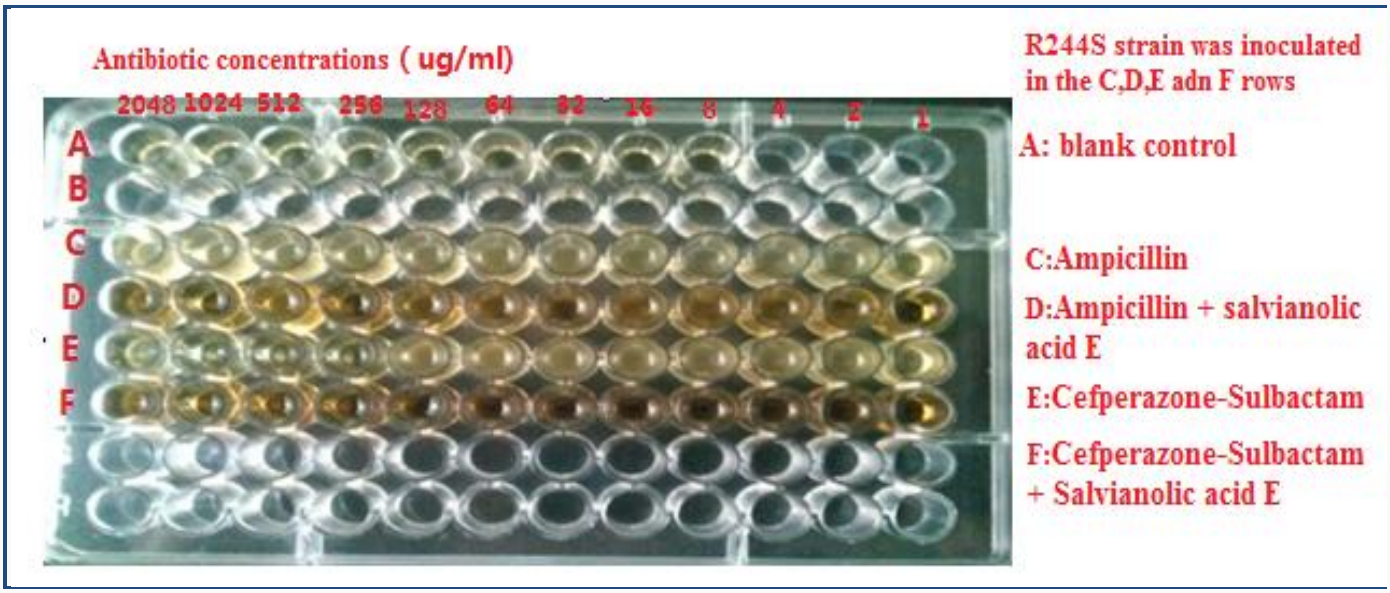

Figure 2: The combination of antibiotics and Salvianolic acid E flight the R244S mutant. The MIC was determined by the doubling dilution method in the microplate. A row was the blank control, C row was Ampicillin, D row was Ampicillin and salvianolic acid E, E row was Cefperazone-Sulbactam, F row was Cefperazone-Sulbactam and Salvianolic acid E. The turbid holes indicate the growth of bacteria, and the limpid ones indicate the bacteria were killed. (Please cite figure 2 in main text)

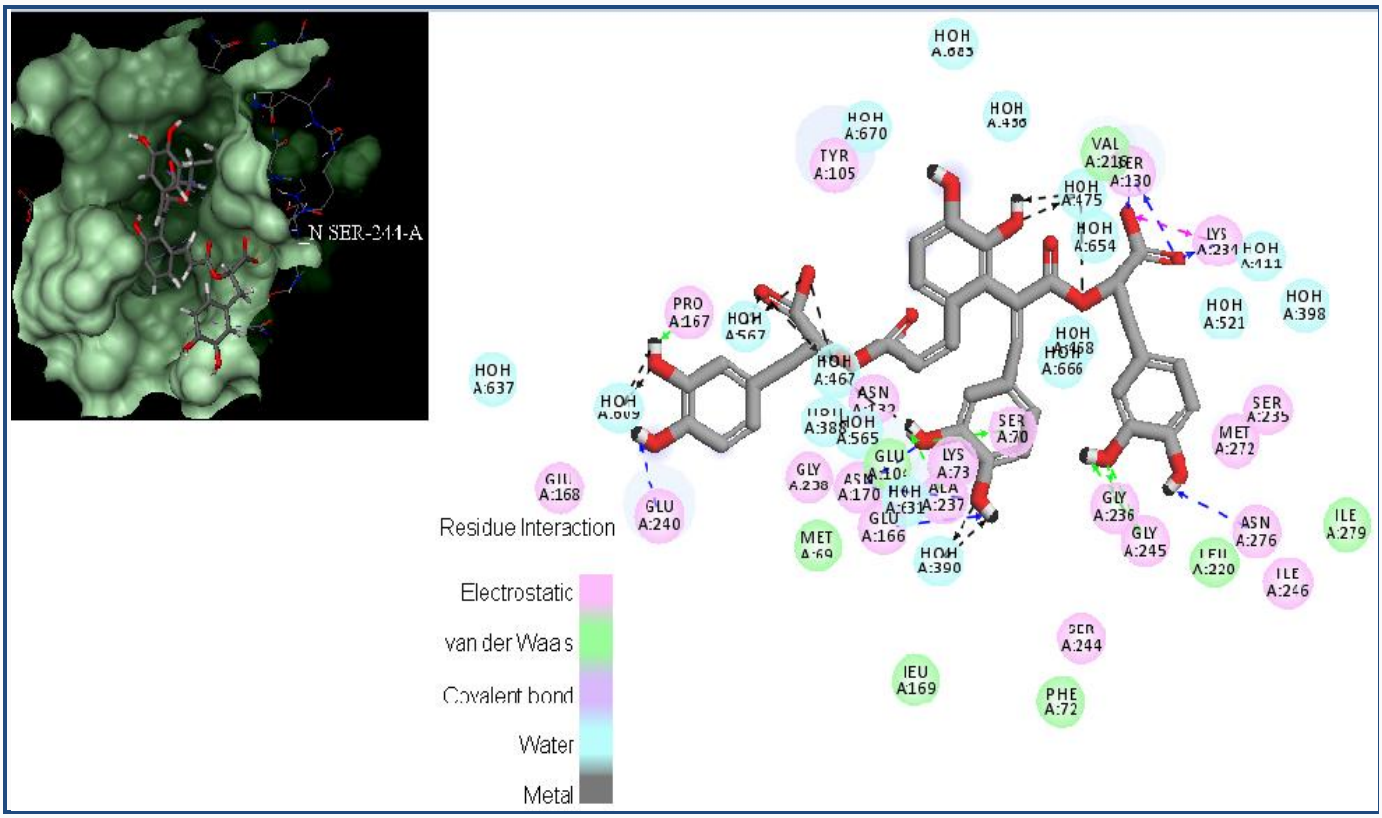

Figure 3: Binding of salvianolic acid E within the active site of R244S. (Please cite figure 3 in main text)

Discussion:

Inhibitors-resistant mutants of TEM β-lactamase

In this study, three mutant strains which resistant enzyme inhibitors were constructed by site direct mutagenesis**.** Based on the experiment of mutant strains resistanted to enzyme inhibitors, the results showed that the sulbactam could effectively inhibit the activity of wild-type β-lactamase, leading to cefoperazone could inhibit the growth of bacteria with the concentration of 1ug/ml . It also showed that R244S mutant, S130G mutant and M69I mutant could certain resistant the inhibitor, sulbactam. In the experiment, R244S mutant had the best result of inhibitors-resistance, the MIC could reach 32ug/ml. The result of S130G mutant is worst, but its MIC could still reach 4ug/ml. And the MIC of M69I mutant is 8ug/ml. The results was consistent with previously reported **[7, 8, 9]**, showed that mutation in these three positions could lead to resistance to β-lactamase inhibitors, turning the wild type β-lactamases into IRTs.

Molecular docking

Virtual screening of chemical databases is an efficient method of computer aided drug design **[20].** In this study, we used virtual screening in order to find out new compounds with high affinity against three IRTs from traditional Chinese medicine ingredients. According to the results of molecular docking, caffeic acid, curcumin, salvianolic acid E, ferulic acid and p-coumaric acid have high binding score with the mutants. Hence, these five Chinese medicine ingredients were chosen as inhibitors for in vitro study.

Combination of antibiotic and Chinese herbal medicinal ingredients

The invitro studies of five Chinese medicine ingredients aimed to verify the accuracy of molecular docking. The results showed that using the Chinese medicinal ingredients alone could not inhibit the growth of mutated *E. coli* in vitro. But the combination of cefperazone-sulbactam and Salvianolic acid E could inhibit the growth of R244S mutated strain effectively **(Figure 1).** The MIC of cefperazone-sulbactam reduced from 32ug/ml to 1ug/ml by using salvianolic acid E.

Experiments show that salvianolic acid E did not have antibacterial activity, but it could reduce the MIC of cefperazone-sulbactam when it was used in conjunction with cefperazone-sulbactam. This illustrates that the mechanism of salvianolic acid E was binding with β-lactamase as enzyme inhibitor, protecting cefperazone from hydrolysis, to achieve the purpose of inhibiting bacterial growth. This is consistent with the results of molecular docking. The other Chinese herbal medicinal ingredients such as caffeic acid, curcumin and so on, also had high affinity for these β-lactamase mutants, but they did not show this feature, the reason of that remained to be further in-depth study.

Conclusion:

Computer aided modeling followed by *invitro* validation show that the ingredient of traditional Chinese medicine salvia, salvianolic acid E inhibits the r244s β-lactamase mutant. The MIC of cefoperazone significantly reduced, and drug-resistant mutant strains of Escherichia coli growth has been effectively suppressed by combination of cefperazone-sulbactam and salvianolic acid E. This finds application in the development of compounds to combat inhibitor resistant strains.

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Supplementary material:

Table 1: The top 10 docking results of ligands docked with M69I.

Ligand id	Score	match	lipo	ambig	clash	rot
3461	-38.0729	-43.8594	-5.9867	-9.0790	2.8521	12.6000
6013	-33.2017	-45.1958	-12.1111	-10.3221	3.8272	25.2000
2216	-29.4123	-34.4233	-2.1872	-4.5544	2.1527	4.2000
1879	-27.0653	-35.6529	-3.8365	-7.0511	2.8752	11.2000
3421	-26.0189	-37.6213	-2.6193	-6.1883	1.0100	14.0000
2695	-25.1269	-24.8539	-9.0500	-7.3946	3.7716	7.0000
1903	-23.9318	-30.5255	-4.4616	-5.0443	2.2996	8.4000
6489	-23.6351	-36.0236	-1.6046	-5.2103	1.2034	12.6000
1941	-21.9863	-35.4600	-2.9998	-5.4638	1.1373	15.4000
1680	-21.1125	-23.1296	-2.7826	-2.6377	0.6374	1.4000

Table 2: The top 10 docking results of ligands docked with R244S.

Ligand id	Score	match	lipo	ambig	clash	rot
6013	-33.0889	-41.8441	-15.1835	-19.4142	12.7530	25.2000
3467	-26.3783	-36.5709	-4.4271	-5.9692	3.9888	11.2000
3460	-26.0524	-32.5309	-5.0422	-7.4187	3.7395	9.8000
3461	-24.3798	-36.9619	-5.9988	-7.3360	7.9169	12.6000
2695	-21.7085	-21.9024	-6.4866	-7.9611	2.2415	7.0000
6489	-21.2277	-31.8608	-3.4952	-5.9134	2.0417	12.6000
1879	-20.2515	-30.0084	-4.0625	-4.1898	1.4092	11.2000
1066	-19.8870	-21.2996	-3.2711	-6.1131	2.5968	2.8000
3070	-19.2058	-21.8566	-5.4698	-5.4406	5.3612	2.8000
1028	-19.0708	-25.7814	-2.8802	-4.0446	1.2355	7.0000

Table 3: The top 10 docking results of ligands docked with S130G.

Ligand id	Score	match	lipo	ambig	clash	rot
2216	-30.5969	-37.5431	-2.5496	-4.8227	4.7185	4.2000
3461	-28.9950	-44.2409	-2.9714	-6.7375	6.9548	12.6000
6013	-28.5353	-42.3716	-10.9649	-9.6868	3.8880	25.2000
1879	-27.5260	-38.6640	-2.7796	-5.4456	2.7631	11.2000
1680	-25.7674	-26.9908	-3.1570	-3.2357	0.8161	1.4000
3070	-25.7660	-28.9473	-4.5676	-3.6264	3.1752	2.8000
1066	-25.4115	-27.7853	-2.9621	-3.6165	0.7523	2.8000
3467	-24.6374	-32.3822	-4.5707	-6.8795	2.5950	11.2000
3460	-23.2770	-32.7962	-4.2271	-5.9397	4.4861	9.8000
7001	-22.1122	-28.7722	-2.9580	-5.0786	0.8966	8.4000

Table 4: Doubling dilution method to determine the MIC. The parent strain has no resistance to Cefperazone-Sulbactam, but all the mutant strains showed high resistance to it.

Strains	Cefperazone-Sulbactam ug/mL											
	2048	1024	512	256	128	64	32	16	8	4	2	1
primordial strain	-	-	-	-	-	-	-	-	-	-	-	-
R244S	-	-	-	-	-	-	+	+	+	+	+	+
S130G	-	-	-	-	-	-	-	-	-	+	+	+
M69I	-	-	-	-	-	-	-	-	+	+	+	+
blank control	-	-	-	-	-	-	-	-	-	-	-	-

Table 5: Combination of antibiotic and Chinese herbal medicinal ingredients against the mutant strains, the MIC was determined by the doubling dilution method. The holes which the bacterial have grown were marked as "+", and the holes which the bacteria which the bacterial could not grow were marked as "-".

Drugs against	concentration ug/mL											
M69I strain	2048	1024	512	256	128	64	32	16	8	4	2	1
Cefperazone-Sulbactam	-	-	-	-	-	-	-	-	+	+	+	+
combination of Cefperazone-Sulbactam and caffeic acid	-	-	-	-	-	-	-	+	+	+	+	+
combination of Cefperazone-Sulbactam and curcumin	-	-	-	-	-	-	-	-	+	+	+	+
combination of Cefperazone-Sulbactam and Salvianolic acid E	-	-	-	-	-	-	-	+	+	+	+	+
combination of Cefperazone-Sulbactam and p-coumaric	-	-	-	-	-	-	-	-	+	+	+	+
combination of Cefperazone-Sulbactam and ferulic acid	-	-	-	-	-	-	-	-	+	+	+	+
blank control	-	-	-	-	-	-	-	-	-	-	-	-
	Cefperazone-Sulbactam ug/mL											
Drugs against S130G strain	2048	1024	512	256	128	64	32	16	8	4	2	1
Cefperazone-Sulbactam	-	-	-	-	-	-	-	-	-	+	+	+
combination of Cefperazone-Sulbactam and caffeic acid	-	-	-	-	-	-	-	-	-	+	+	+
combination of Cefperazone-Sulbactam and curcumin	-	-	-	-	-	-	-	-	-	+	+	+
combination of Cefperazone-Sulbactam and Salvianolic acid E	-	-	-	-	-	-	-	-	-	+	+	+
combination of Cefperazone-Sulbactam and p-coumaric	-	-	-	-	-	-	-	-	-	+	+	+
combination of Cefperazone-Sulbactam and ferulic acid	-	-	-	-	-	-	-	-	-	+	+	+
blank control	-	-	-	-	-	-	-	-	-	-	-	-
Drugs against	**concentration ug/mL**											
R244S strain	2048	1024	512	256	128	64	32	16	8	4	2	1
Cefperazone-Sulbactam	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and caffeic acid	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and curcumin	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and Salvianolic acid E	-	-	-	-	-	-	-	-	-	-	-	-
combination of Cefperazone-Sulbactam and p-coumaric	-	-	-	-	-	+	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and ferulic acid	-	-	-	-	-	-	+	+	+	+	+	+
blank control	-	-	-	-	-	-	-	-	-	-	-	-
Drugs against	**concentration ug/mL**											
R244S strain	2048	1024	512	256	128	64	32	16	8	4	2	1
Cefperazone-Sulbactam	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and caffeic acid	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and curcumin	-	-	-	-	-	-	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and Salvianolic acid E	-	-	-	-	-	-	-	-	-	-	-	-
combination of Cefperazone-Sulbactam and p-coumaric	-	-	-	-	-	+	+	+	+	+	+	+
combination of Cefperazone-Sulbactam and ferulic	-	-	-	-	-	-	+	+	+	+	+	+

acid												
blank control	-	-	-	-	-	-	-	-	-	-	-	-
		concentration		**ug/mL**								
Drugs	2048	1024	512	256	128	64	32	16	8	4	2	1
caffeic acid	+	+	+	+	+	+	+	+	+	+	+	+
curcumin	+	+	+	+	+	+	+	+	+	+	+	+
Salvianolic acid E	+	+	+	+	+	+	+	+	+	+	+	+
p-coumaric	+	+	+	+	+	+	+	+	+	+	+	+
ferulic acid	+	+	+	+	+	+	+	+	+	+	+	+
blank control	-	-	-	-	-	-	-	-	-	-	-	-