Synergistic antibacterial activity of baicalin and EDTA in combination with colistin against colistin-resistant *Salmonella*

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ABSTRACT The emergence and rapid spread of multidrug resistant (**MDR**) Gram-negative bacteria have posed a serious threat to global health and security. Because of the time-consuming, high cost and high risk of developing new antibiotics, a significant method is to use antibiotic adjuvants to revitalize the existing antibiotics. The purpose of the study is to research the traditional Chinese medicine baicalin with the function of inhibiting the efflux pump and EDTA whether their single or combination can increase the activity of colistin against colistin-resistant *Salmonella in vitro* and *in vivo*, and to explore its molecular mechanisms. *In vitro* antibacterial experiments, we have observed that baicalin and EDTA alone could enhance the antibacterial activity of colistin. At the same time, the combination of baicalin and EDTA also showed a stronger synergistic effect on colistin, reversing the colistin resistance of all *Salmonella* strains. Molecular docking and RT-PCR results showed that the combination of baicalin and EDTA not only affected the expression of *mcr-1*, but also was an effective inhibitor of MCR-1. In-depth synergistic mechanism analysis revealed that baicalin and EDTA enhanced colistin activity through multiple pathways, including accelerating the tricarboxylic acid cycle (**TCA cycle**), inhibiting the bacterial antioxidant system and lipopolysaccharide (**LPS**) modification, depriving multidrug efflux pump functions and attenuating bacterial virulence. In addition, the combinational therapy of colistin, baicalin and EDTA displayed an obvious reduction in bacterial loads cfus of liver and spleen compared with monotherapy and 2-drug combination therapy. In conclusion, our study indicates that the combination of baicalin and EDTA as a novel colistin adjuvant can provide a reliable basis for formulating the therapeutic regimen for colistin resistant bacterial infection.

Key words: colistin, *mcr-1*, resistance, combination therapy, *Salmonella*

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INTRODUCTION

The emergence and spread of multidrug-resistant (**MDR**) gram-negative bacteria have almost made medical and veterinary clinics reach the point where no antimicrobials are available, leading to renewed interest in the use of colistin. Colistin is a class of cationic polypeptide antibiotics, which kill gram-negative pathogens through disruption of membrane permeability via polar and hydrophobic interactions. Despite having serious adverse effects, colistin has been considered the last-resort antimicrobial for the treatment of multidrug-resistant gram-negative bacterial infections (Andrade et al., 2020). The resistance of Gram-negative bacteria to colistin is primarily due to the modification of lipid A of outer membrane lipopolysaccharide (**LPS**) (Jeannot et al., 2017). Moreover, a transferable plasmid-mediated colistin resistance gene *mcr-1* was recently found in China, which encodes a pEtN transferase that modifies lipid A, triggering concerns about the rapid spread of colistin resistance in the world (Liu et al., 2016; Wang et al., 2018). Nowadays, the development of antibiotics is facing many challenges, and new antibiotics are difficult to introduce into clinics (Brown and Wright, 2016). Therefore, a variety of antibiotic adjuvants have been widely used to improve the efficacy of antibiotics or delay the emergence of drug resistance. More than 60 adjuvants have been reported to reverse colistin resistance, such as natural compounds

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such as resveratrol and Osthol (Cannatelli et al., 2018; Zhou et al., 2019).

Baicalin is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi. It is one of the most commonly and versatile herbal medicines used to antibacterial, antiviral, anti-inflammatory, liver and nerve protection in China (Chen et al., 2018; Sass et al., 2019). It has been reported that baicalin has a synergistic effect with β-lactam antibiotics, tetracycline and oxytetracycline to enhance their antibacterial activity against *Staphylococcus aureus* (Guz et al., 2001; Novy et al., 2011). Baicalin is considered to be an effective and promising curative strategy against *S. saprophyticus* biofilm formation and the accessory gene regulator system through inhibiting efflux pump (Wang et al., 2019). However, there is currently no study on the synergistic effect of baicalin on colistin.

As a metal ion chelating agent, EDTA can increase the permeability of the outer membrane (**OM**), displacing LPS with phospho-lipids in the OM (Ellison et al., 1988; Nikaido, 1996). Both exposure to EDTA and reduced LPS may result in misfolded envelope proteins and activate the CpxAR system (Nikaido, 1996). In addition, chelators agents such as EDTA can sensitize the outer membrane of *Escherichia coli* and other Gram-negative bacteria, increasing their susceptibility to colistin (Vaara, 1992).

Our previous study indicated that TCS CpxAR and the AcrAB-TolC efflux pump have reciprocal effects on colistin resistance-related gene transcription (Zhai et al., 2018, 2020). The deletion of the multidrug efflux pump gene *acrB* and the overexpression of *cpxR* (JSΔ*acrB*Δ*cpxR*/p*cpxR*) can cause the supercritical expression of CpxR and increase significantly the susceptibility of *Salmonella* to colistin. Considering these aspects, this work evaluated whether or baicalin, EDTA single or their combination could effectively reverse colistin resistance. Interestingly, we found that baicalin and EDTA effectively reverses colistin resistance of *Salmonella* isolates *in vivo* and *in vitro* by multiple means. The discovery of baicalin combined with EDTA as a novel and secure colistin adjuvant provides a potential treatment for the infections of colistin resistant gram-negative bacteria.

MATERIALS AND METHODS

Salmonella *Isolates, Chemicals and Reagent*

Table 1 lists the *Salmonella* isolates used in this study. Twenty *Salmonella* isolates including 16 colistin-resistant strains and 4 colistin-sensitive strains from multifarious chicken and human samples were included in this study. Of the tested strains, seven *Salmonella* strains including 5 *mcr-1*-negative colistin-resistant strains and 2 *mcr-1*-positive colistin-resistant strains have previously conducted mutation analysis of the TCSs PmrAB and PhoPQ related genes through whole-genome sequencing to study the resistance mechanism to colistin (Table S1 for reviewers' information only). *Salmonella enterica serovar* Typhimurium CVCC 541 (JS) was used as the control. Colistin (COL, C4461, ≥19,000 IU/mg) and baicalin (BAI, 572667, 95 %) were purchased from Sigma-Aldrich (Darmstadt, Germany). EDTA was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Antibacterial Tests

Exploration of the effect of the efflux pump inhibitor baicalin and CpxAR activator EDTA on the MIC of colistin: First, the MIC of colistin, baicalin and EDTA for *Salmonella* strains was determined by the broth microdilution method (Clinical and Laboratory Standards Institute, 2021). Briefly, drugs were 2-fold diluted in Mueller-Hinton broth (**MHB**) and mixed with an equal volume of bacterial suspensions containing approximately 5×10^5 colony-forming units (cfus) /mL in a clear UV-sterilized 96-well microliter plate. After 18 h incubation at 37°C, the MIC values were defined as the lowest concentrations of antibiotics with no visible growth of bacteria.

The next 3 colistin susceptibility tests were performed simultaneously: One added baicalin, one added EDTA, and another added baicalin and EDTA to MHB. The MIC of colistin produced after the addition of baicalin and EDTA was compared with the critical value of colistin resistance (2 mg/L) (Clinical and Laboratory Standards Institute, 2021), and the effect was considered to be reversed if the strain was again susceptible to colistin. The MIC fold changes of colistin produced after the addition of baicalin and EDTA was calculated as the ratio of the MIC level of colistin to the MIC level of colistin after the addition of baicalin and EDTA. All experiments were performed with three biological replicates.

Checkerboard Assays

Synergistic activity of colistin and EDTA, baicalin was evaluated by checkerboard assays. Each well is inoculated with 50 μL of 5×10^5 cfu/mL test strain suspension, and the final volume is 100 μL. The inoculum was prepared by suspending bacterial cultures grown overnight in LB broth into MHB. When the 3 drugs were combined, baicalin and EDTA were diluted in a reaction well at the same time. The concentration ratio of baicalin and EDTA was the optimal concentration ratio selected in the above-mentioned combined drug susceptibility test. The FIC index (**FICI**).

The results were read after incubation at 37°C for 16 to 20 h. The FICI was calculated according to the formula as follows: FIC index = $MIC_{ab}/MIC_a + MIC_{ba}/MIC_b = FIC_a + FIC_b$. MIC_a is the MIC of compound A alone, MIC_{ab} the MIC of compound A in combination with compound B, MIC_b the MIC of compound B alone, MIC_{ba} the MIC of compound B in combination with compound A, and FIC_a is the FIC of compound A and FIC_b the FIC of compound B. The FICI $\leq$ 0.5, synergistic effect; 0.5 < FICI $\leq$ 1, additive action; FICI > 2, antagonism; FICI 1 to

Table 1. Summary of strains used in this study.

				BAI Microdilution assay (mg/L)				EDTA Microdilution assay (mg/L)				BAI and EDTA Microdilution assay (mg/L)		
Name	Source	Mechanism of resistance to COL	MIC of COL	MIC of BAI	MIC of 1/2 BAI + COL	MIC fold change	Conclusion effect	MIC of EDTA	MIC of 1/2 EDTA + COL	MIC fold change	Conclusion effect	MIC of 1/2 BAI + 1/2 EDTA	MIC fold change	Conclusion effect
JS	–	–	1/2	2500	1/2	1	–	125	1/32	16	–	1/256	128	–
SR01	chicken	Table S1	32	2500	4	8	None	125	2	16	Reverse	1/8	256	Reverse
SR02	chicken	Table S1	4	2500	1/4	16	Reverse	250	1/8	32	Reverse	1/256	1024	Reverse
SR03	chicken	Table S1	32	2500	8	4	None	250	1	32	Reverse	1/16	512	Reverse
SR04	chicken	Table S1	32	2500	2	16	Reverse	125	1/2	64	Reverse	1/32	1024	Reverse
SR05	chicken	Table S1	4	2500	1	4	Reverse	125	1/4	16	Reverse	1/32	128	Reverse
SR06	chicken	Unknown	16	2500	1/2	32	Reverse	125	4	4	None	1/32	512	Reverse
SR07	chicken	Unknown	16	2500	2	8	Reverse	125	2	8	Reverse	1/8	128	Reverse
SR08	chicken	Unknown	8	2500	1	8	Reverse	125	1	8	Reverse	1/32	256	Reverse
SR09	chicken	Table S1	32	2500	2	16	Reverse	125	2	16	Reverse	1/128	4096	Reverse
SR10	chicken	Table S1	32	2500	1	32	Reverse	125	1	32	Reverse	1/512	16384	Reverse
SR11	chicken	Unknown	32	2500	8	4	None	125	4	8	None	1/4	128	Reverse
SR12	chicken	Unknown	64	2500	4	16	None	125	4	16	None	1/8	512	Reverse
SR13	chicken	*mcr-1*	16	2500	2	8	Reverse	125	1	16	Reverse	1/16	256	Reverse
SR14	chicken	*mcr-1*	32	2500	4	8	None	125	2	16	Reverse	1/8	256	Reverse
SR15	human	*mcr-1*	64	2500	1	64	Reverse	125	4	16	None	1/4	256	Reverse
SR16	human	Unknown	32	2500	4	8	None	125	1/2	64	Reverse	1/64	2048	Reverse
SF01	human	–	1	2500	1/4	4	–	500	1/128	128	–	1/1024	1024	–
SF02	human	–	1/2	2500	1/4	2	–	250	1/2	1	–	1/8192	4096	–
SF03	chicken	–	1/8	2500	1/32	4	–	125	1/128	16	–	1/2048	256	–
SF04	chicken	–	1	2500	1/4	4	–	250	1/4	4	–	1/256	256	–

Note: The "–" means that the strain is sensitive to colistin; BAI, baicalin. COL, colistin.

2 had no interaction. The data was obtained in at least 3 independent experiments.

Time-kill Study

The time-kill curve study was performed on four strains, namely SR03, SR04, SR14 and the standard strain JS. Bacterial cultures were inoculated under the following 7 conditions: MHB, MHB + 1/2 MIC colistin, MHB + 1/2 MIC baicalin, MHB + 1/2 MIC EDTA, MHB + 1/2 MIC colistin + 1/2 MIC baicalin, MHB + 1/2 MIC colistin + 1/2 MIC EDTA, MHB + 1/2 MIC colistin + 1/2 MIC baicalin + 1/2 MIC EDTA and incubated with shaking at 37°C for 24 h. Dilutions of each culture were spread on LB nutrient agar at 0, 2, 4, 8, 16, and 24 h and colony counts were performed after 16 to 18 h of incubation. Take the common Log_{10} cfus of colony count at each time point as the vertical axis, and take incubation time (h) as the horizontal axis to establish the time sterilization curve.

Transcriptomic Analysis

Salmonella SR14 grown to the early exponential stage in LB and was treated with PBS (control group), baicalin (1/2 MIC 1250 mg/L), EDTA (1/2 MIC 62.5 mg/L) or a combination of baicalin and EDTA for 4 h. After centrifugation, liquid nitrogen was used for quick-frozen bacterial and sent to Tsingke Biotechnology Co, Ltd. for prokaryotic transcriptome sequencing. Raw sequencing reads were filtrated and mapped according to the reference genome of JS. Differentially expressed genes were identified using the FPKM (transcript fragments per million mapped reads) method with P-value ≤ 0.05 and fold change (**FC**) value ≥ 2 ($\log_2 FC \geq 1$ or $\log_2 FC \leq -1$). Differentially expressed genes were identified between pairwise comparisons: PBS treated versus baicalin (1,250 mg/L) treated, PBS treated vs. EDTA (62.5 mg/L) treated, PBS treated vs. baicalin (1250 mg/L) + EDTA (62.5 mg/L) treated.

RT-PCR Analysis

In order to verify the transcriptome results, we selected *Salmonella* strain SR28 for fluorescence quantitative PCR of some related genes, and the culture conditions of the strain were the same as those of the transcriptome. In addition, in order to evaluate the effect of baicalin combined with EDTA on *mcr-1* gene transcription, we quantified the expression of *mcr-1* gene in the recombinant plasmid PUC18: *mcr-1* (the obtained plasmid was verified by PCR and direct DNA sequencing). We inoculated colonies into 7 different LB broth media, including 2 mg/L colistin, 625 mg/L baicalin (1/4 MIC), 31.25 mg/L EDTA (1/4 MIC), 2 mg/L colistin + 625 mg/L baicalin or 31.25 mg/L EDTA, 2 mg/L colistin + 625 mg/L baicalin + 31.25 mg/L EDTA.

These cultures were shaken at 37°C for 4 h. Extract 10 mL of this culture corrected to OD600 0.5 using the TRIzolV RMaxTMB bacterial RNA isolation method. Total RNA was extracted by the phenol-chloroform method, and the OD260/OD280 value was evaluated by Trace Nucleic Acid Protein Analyzer Spectrophotometer. cDNA samples were synthesized and then RT-PCR was performed according to the method of Zhai (Zhai et al., 2020). Each quantification was performed in triplicate. The 16S rRNA gene was used as a calibrator, and the primers of RT-PCR are listed in Table S2.

Molecular Docking

Molecular simulations of the interaction between MCR-1, baicalin and EDTA were performed using the AutoDock software (Trott and Olson, 2010). In this research, the initial structure of MCR-1 was obtained from the 3D structure of X-ray(PDB ID: 5GRR) (Ma et al., 2016). The structure of baicalin and EDTA comes from The Pubchem Project (PubChem CID: 64982 and 6049). Weighting parameters for the scoring function include steric interactions, hydrophobic interactions, hydrogen bonding energy, and the number of rotatable bonds in the ligand. The lower the parameter, the more likely the ligand-active pocket will bind. The Discovery Studio molecular graphics system was used to analyze their interaction patterns with binding site residues.

Mouse Abdominal Cavity Infection Model

Mice were maintained in strict accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11-14-1988). The mouse experiments were approved by the Henan Science and Technology Department (protocol number SCXK 2019-0002). Female KM mice (Taconic Biosciences) aged 7 to 9 d were infected intraperitoneally for about $\sim 2 \times 10^6$ cfus *mcr-1* negative colistin resistant *Salmonella* SR04, or the *mcr-1* positive colistin resistant *Salmonella* SR14. Infections persisted for 2 h and were treated with intraperitoneal injections as described above.

Each group of mice were injected with PBS, colistin (20 mg/kg), colistin + baicalin (20 + 50 mg/kg), colistin + EDTA (20 +10 mg/kg) or a combination of the three drugs (20 + 50 + 10 mg/kg). 48 hours after infection, the mice were euthanized through cervical dislocation. The spleen and liver were aseptically removed, homogenized, continuously diluted, inoculated on SS plate, cultured at 37°C for 14 to 16 h, and the numbers of bacteria were counted.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 7 and SPSS software. All data was presented as mean ± SD. Statistical assessments were performed using

unpaired 2-tailed t-tests, one-way ANOVA, 2-way ANOVA, or log-rank tests. Differences at P-value <0.05 were considered significant. Significance levels are indicated with asterisks: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS

Synergistic Activity of Colistin in Combination With Baicalin and EDTA

MICs of baicalin and EDTA were 2,500 mg/L and ≥ 125 mg/L for all tested strains, respectively, showing the lack of any intrinsic antimicrobial activity of baicalin and EDTA alone against these *Salmonella* isolates. When 1/2 MIC baicalin and 1/2 MIC EDTA were separately used with colistin, the MICs of colistin for *Salmonella* isolates were significantly decreased, reversing colistin resistance of some *Salmonella* isolates, and the reversal rate of colistin resistance were 62.5% and 75%, respectively (Figure 1). To further evaluate the potentiation, the potency of baicalin in combination with EDTA on colistin was tested in diverse *Salmonella* isolates. The combination of 1/2 MIC baicalin and 1/2 MIC EDTA fully reversed the colistin resistance of all colistin resistant*Salmonella* isolates with the MICs of colistin being decreased from 4 to 64 mg/L to 1/4 to 1/512 mg/L, and the mean fold change of colistin MIC was 1,736 (Table 1). In addition, we have observed significant synergistic effects in colistin-resistant *Salmonella* with different resistance mechanisms, suggesting that the combination of baicalin and EDTA is an antibiotic adjuvant to reverse colistin-resistant Gram-negative pathogens. Meanwhile, the combination of baicalin and EDTA also enhanced the activity of colistin against the sensitive *Salmonella* isolates with a decrease of MICs, ranging from 1 to 1/8 mg/L to 1/256 to 1/8,192 mg/L.

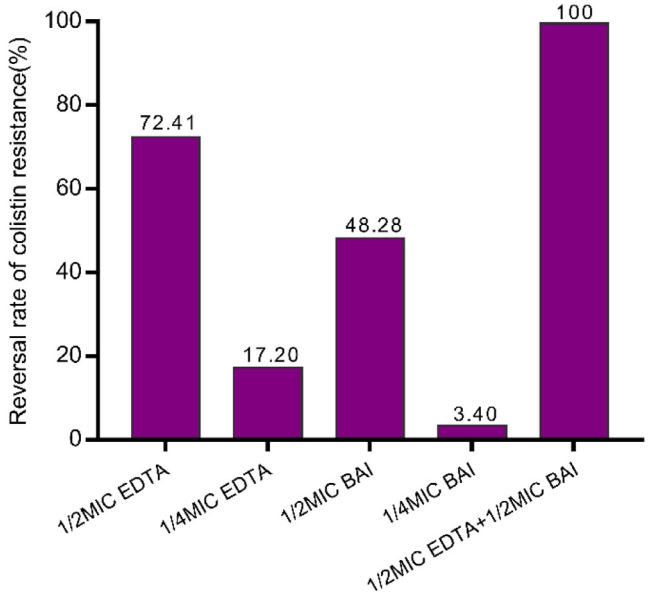

Figure 1. Resistance reversal rate (%) of colistin when baicalin and EDTA are used alone or in combination. BAI, baicalin. The reversal rate of colistin resistance was calculated as follows: the number of strains whose MICs of colistin reversed to sensitive after the combination was divided to the number of all strains that were colistin-resistant in this study.

Table 2. FICI of two-drug and three-drug combinations against *Salmonella* isolates.

Salmonella isolates	FICI		
	Colistin + Baicalin	Colistin + EDTA	Colistin + Baicalin + EDTA
JS	1	0.75	0.5
SR02	0.75	0.75	0.375
SR05	1	0.75	0.5
SR08	0.75	0.375	0.25
SR10	0.75	1.5	0.5
SR14	1	0.75	0.5
SR15	0.75	0.625	0.5

To further explore this enhanced effect, we evaluated the potentiation effect of baicalin and EDTA in combination with colistin against 7 *Salmonella* strains by the checkerboard test. Table 2 presents the combined FICI values for all studied strains. Standard strains JS and clinical isolates of *Salmonella* exhibited strong synergistic interaction in 2-drug and 3-drug combinations (FICI = 0.25−1). There was no difference in the synergistic activity of the 2 drug combinations: colistin + baicalin and colistin + EDTA (fractional inhibitory concentration index FICI = 0.375−1). However, the combination of colistin + baicalin + EDTA showed a synergistic effect on almost all *Salmonella* isolates (FICI = 0.25−0.5) (Table 2).

Although the combined antibacterial tests and checkerboard assays showed that baicalin and EDTA have an enhanced effect on colistin, the direct synergistic bactericidal activity test may directly prove these findings. Therefore, we subsequently conducted the time-kill curve experiments on *Salmonella* isolates in the exponential growth stage. We found that neither baicalin nor EDTA nor colistin alone could kill all *Salmonella* strains. In contrast, the combination of colistin with baicalin or EDTA can reduce the bacterial load by about 2-$\log_{10}$ (Figure 2). While the 3-drug combination of colistin + baicalin + EDTA showed stronger bactericidal activity against all strains, resulting in a ~3-$\log_{10}$ reduction in bacterial load (Figure 2).

Baicalin and EDTA Directly Inhibited MCR-1 Activity

In order to systematically investigate the molecular mechanism of baicalin combined with EDTA on colistin sensitivity of *Salmonella* isolates, we used quantitative RT-PCR to further research the mRNA expression level of colistin resistance gene *mcr-1*. The *mcr-1* gene of *Salmonella* strain SR14 was introduced into *E. coli* DH5α through clone and transformation leading to resistance to colistin. Then, the effect of both baicalin and EDTA on *mcr-1* gene expression was determined on *E. coli* DH5α (pUC18-*mcr-1*). The addition of 2 mg/L colistin, 2 mg/L colistin + 625 mg/L baicalin and 2 mg/L colistin + 31.25 mg/L EDTA led to a decrease of 1.67-

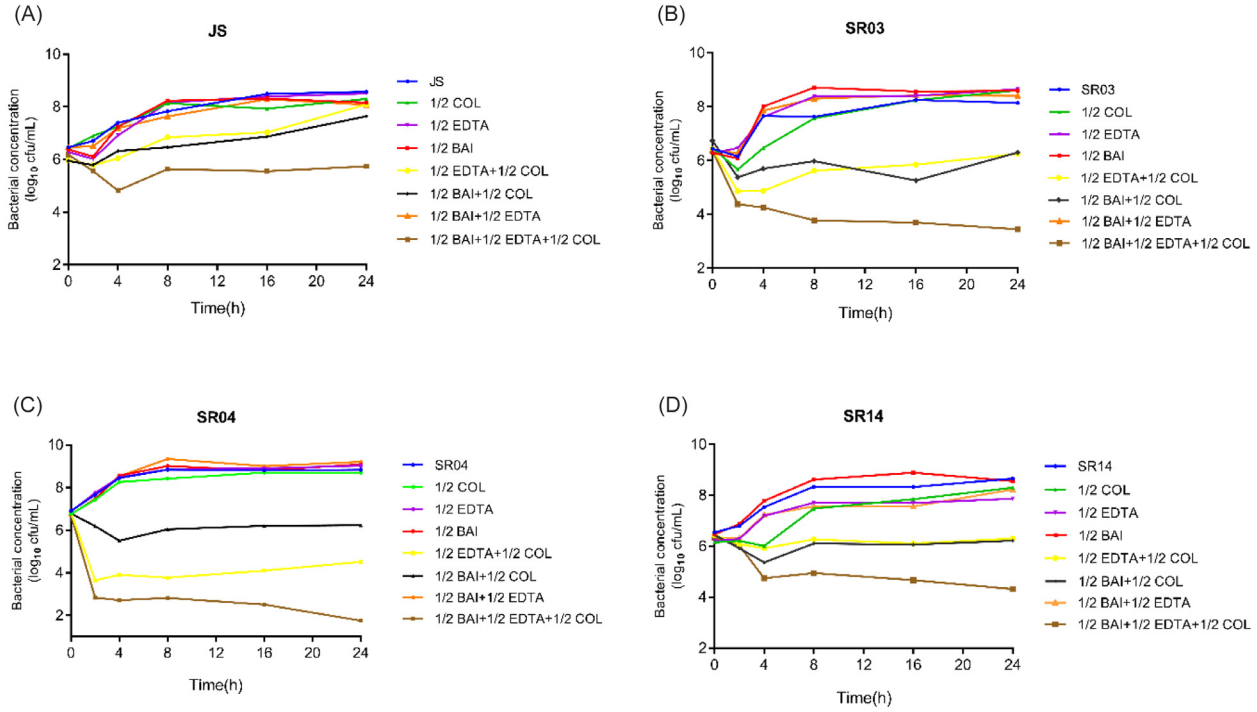

Figure 2. Baicalin and EDTA potentiates colistin activity against *Salmonella in vitro*. BAI, baicalin. COL, colistin. (A) Time-kill curves of the *Salmonella* JS in the presence of baicalin (1,250 mg/L), EDTA (62.5 mg/L) or colistin (sub-MIC) alone or in combination for 24 h. (B) (C) (D) Time-kill curves of the clinical strain SR03, SR04 and SR14 (*mcr-1*) in the presence of baicalin (1,250 mg/L), EDTA (125 mg/L or 62.5 mg/L) or colistin (sub-MIC) alone or in combination for 24 h. Data are representative of 3 independent experiments and shown as mean ± SD.

fold, 1.143-fold (not statistically significant) and an increase of 1.46-fold for *E. coli* DH5α (pUC18-*mcr-1*), respectively. However, the combination of 2 mg/L colistin + 625 mg/L baicalin, 2 mg/L colistin + 31.25 mg/L EDTA, 625 mg/L baicalin + 31.25 mg/L EDTA and 2 mg/L colistin + 625 mg/L baicalin + 31.25 mg/L EDTA decreased *mcr-1* expression 1.27-fold, 2.17-fold, 1.8-fold, and 26.9-fold (Figure 3).

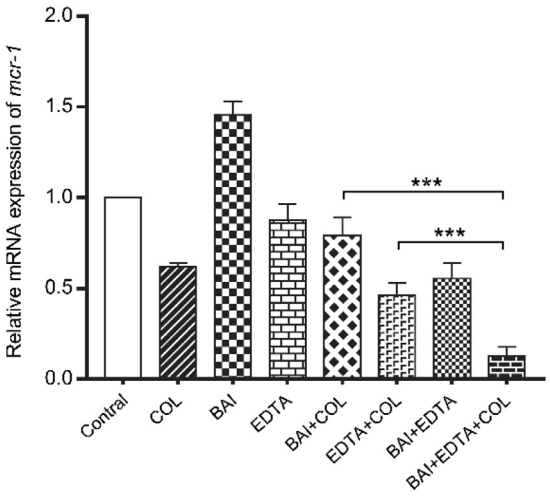

Figure 3. *mcr-1* gene expression in *E.coli* DH5α (pUC18-*mcr-1*) in the absence of antibiotic and in the presence of baicalin (625 mg/L), EDTA (31.25 mg/L) or colistin (2 mg/L) alone or in combination. BAI, baicalin. COL, colistin. Significant differences were evaluated by one-way ANOVA analysis and shown with $***P < 0.001$.

The spatial conformation of MCR-1 protein after docking with bioactive groups of baicalin and EDTA was elucidated by standard molecular docking and molecular modeling. The docking results showed that the binding free energy between MCR-1 and baicalin was −6.54 kcal/mol. As shown in Figure 4, it is obvious that baicalin can bind to MCR-1 through hydrogen bond and hydrophobic actions. Specifically, the binding model between baicalin and *mcr-1* shows that baicalin can form a strong interaction with lys307, lys333, asp331, asp337, gly334, and tyr308, indicating that baicalin can be combined with MCR-1.

In addition, the binding free energy between MCR-1 and EDTA is −1.07 kcal/mol, which is mainly connected by hydrogen bonds (Lys307, ASP337, Tyr308, and THP219), which indicated that the bonding stability of EDTA to MCR-1 is poor. Previous studies have confirmed that MCR-1 is a zinc metalloprotein. EDTA is a strong metal chelating agent, which can chelate the zinc ion in the active center of MCR-1, so as to make MCR-1 lose its activity (Son et al., 2019).

Baicalin Combined With EDTA can Promote Oxidative Damage, Reduce LPS Modification, Inhibit Efflux Pump and Attenuate Bacterial Virulence

To identify specific molecular mechanisms of baicalin combined with EDTA enhancing the antibacterial activity of colistin, SR14 (carrying *mcr-1*) was analyzed by

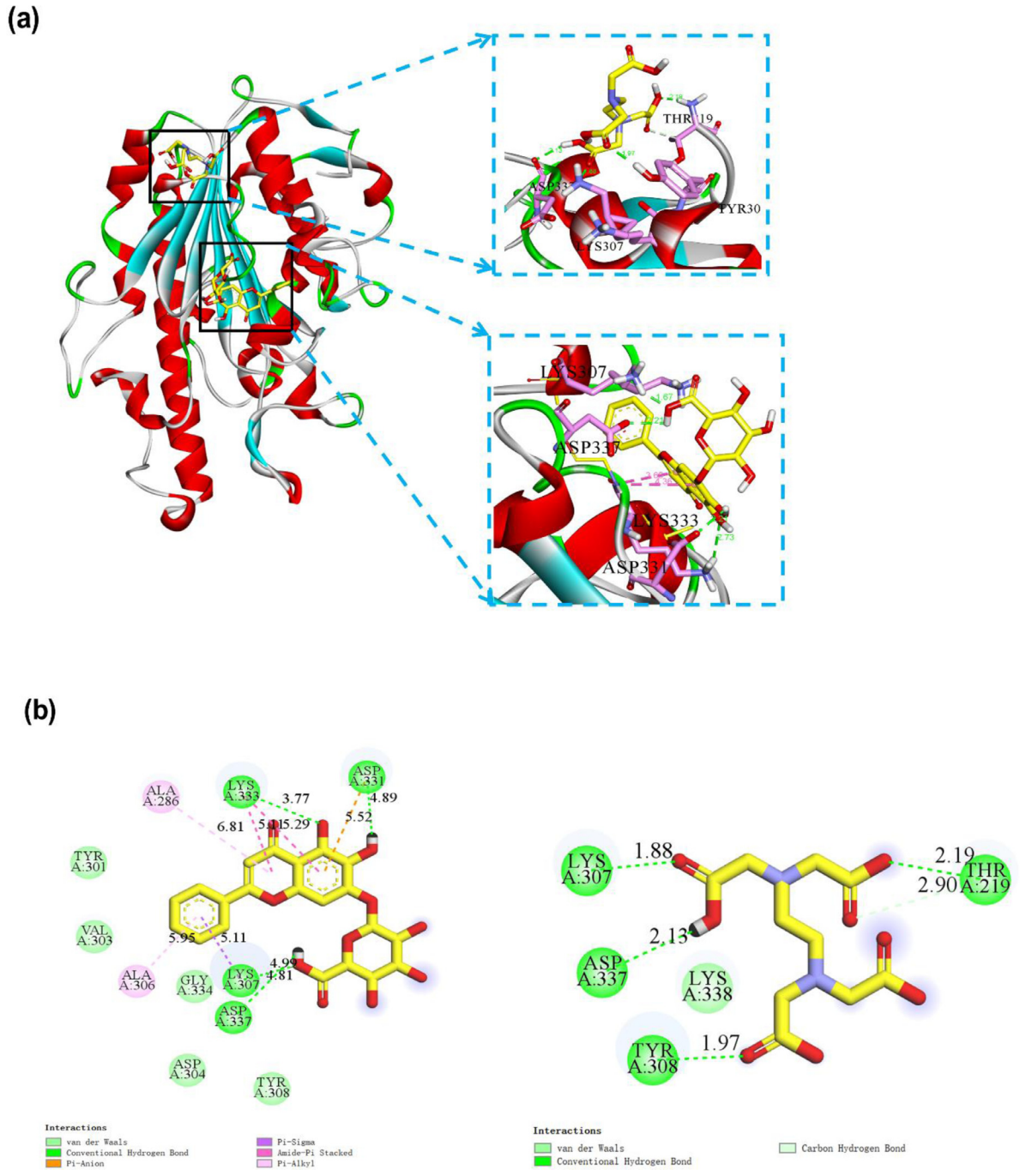

Figure 4. Putative pattern of interaction among baicalin, EDTA and MCR-1 protein. (A) The interactions formed between the amino acid residues (stick) and the docked baicalin and EDTA molecule (ball and stick) in the MCR-1 binding site. (B) Interaction of planar amino acids among baicalin, EDTA and MCR-1 molecule.

transcriptome after treatment with baicalin, EDTA or baicalin + EDTA for 4 h. Compared with the control group, the expression of 111 genes and 196 genes were up-regulated in the baicalin and EDTA alone treatment groups, respectively, and the expressions of 89 and 66 genes were down-regulated (>2-fold). In the baicalin combined with EDTA group, 182 up-regulated genes and 209 down-regulated genes (>2-fold) were found. According to KEGG enrichment analysis, it is clear that differentially expressed genes (**DEG**) are mainly involved in pathways such as ABC transporters, TCS, bacterial antioxidant system and bacterial metabolism (Figure 5a). Concretely, we found that the genes with increased expression were related to the tricarboxylic acid cycle (**TCA cycle**) (*citX, fumC* and *acnB*), and the decreased expression genes were related to antioxidant function (*soxR, sufB* and *sodC*), LPS modification (*pmrB, arnB, phoP* and *phoQ*), ABC transporters (*cheY, rbsD, phnS* and *cheB*), Multidrug efflux pumps (*marA, ramA, tolC* and *norM*) and *Salmonella* infection and virulence (*fliC, fliB, sipB, sipC, sipA, yscJ, yscC* and *yscR*) (Figure 5b).

To verify the transcriptome results, we performed RT-PCR experiments on the LPS modification and

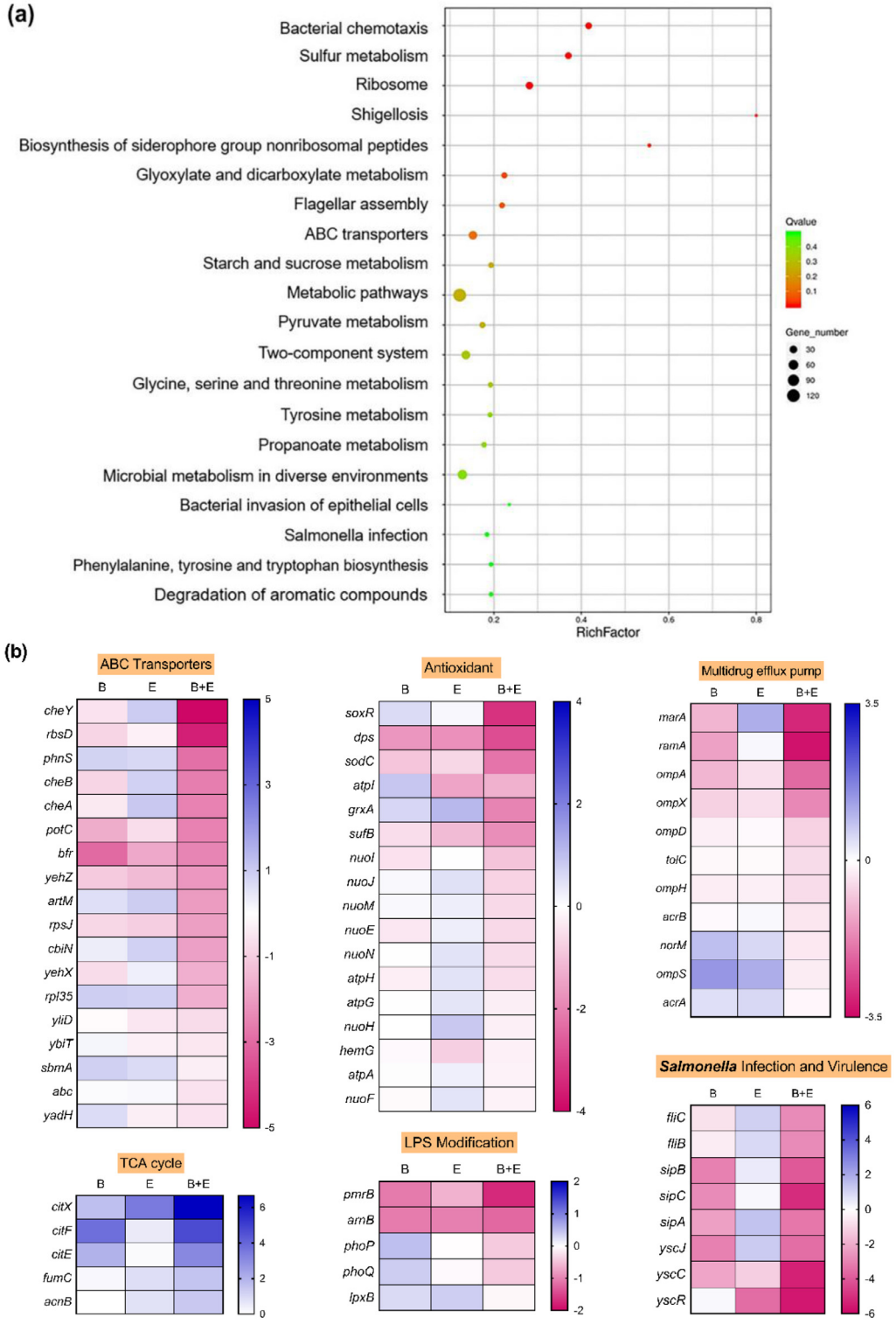

Figure 5. Transcriptomic analysis of SR14 treated by baicalin, EDTA, and baicalin plus EDTA. KEGG enrichment analysis (A) of the differential expression genes (DEGs) in SR14 after exposure to baicalin plus EDTA. The x and y axis in A represent the expression changes and corresponding statistically significant degree, respectively. (B) Selected DEGs involved in TCA cycle, antioxidant response, LPS modification, ABC transporters, multidrug efflux pump and *Salmonella* infection and virulence. B, baicalin alone; E, EDTA alone; B+E, baicalin in combination with EDTA.

multidrug efflux pumps pathways using a *Salmonella* strain SR14. RT-qPCR results showed that baicalin and EDTA inhibited the expression of *acrD, tolC, acrB, marA, ramA* and *robA* genes in the multidrug efflux pumps system; increased the expression of the colistin resistance-related gene *mgrB* gene, and significantly reduced *pmrA, pmrB, phoQ, pmrH, cptA* and *mcr-1* gene expression (Figure 6). The expression of representative genes analyzed by RT-PCR was consistent with the results of the transcriptome.

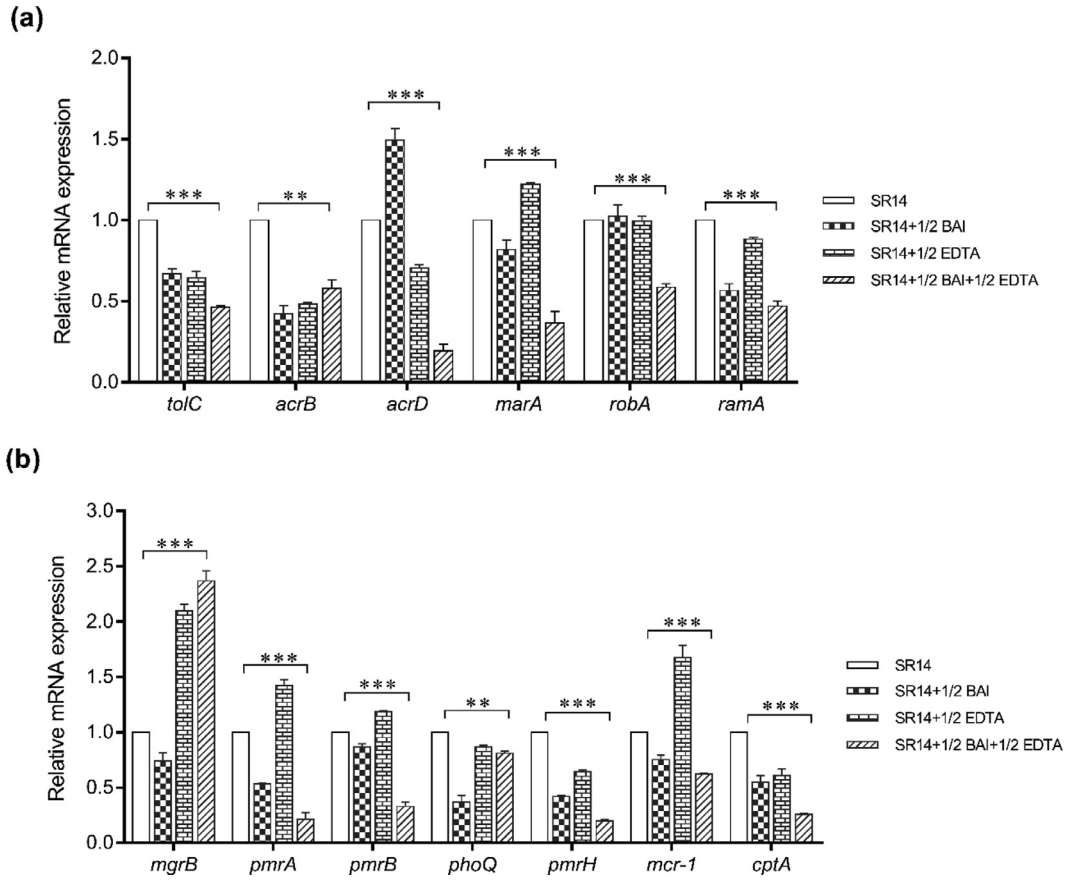

Figure 6. Relative expression of LPS modification and multidrug efflux pumps pathways related genes. Data were presented as mean ± SD from three biological replicates. Significant differences were evaluated by one-way ANOVA analysis and shown with $^{**}P < 0.01$, $^{***}P < 0.001$.

Baicalin in Combination With EDTA Restores Colistin Activity in Vivo

In view of the excellent synergistic bactericidal activity of colistin, baicalin and EDTA against *Salmonella in vitro*, its therapeutic potential was further evaluated *in vivo*. The combinational efficacy was tested in a mouse abdominal cavity infection model infected with *mcr-1* positive and *mcr-1* negative *Salmonella* strains (Figure 7). Colistin in combination with baicalin or EDTA both displayed≈1-$\log_{10}$ reductions in bacterial loads cfus of liver and spleen, compared with PBS control group in *mcr-1* positive and *mcr-1* negative *Salmonella* strains (except SR14 spleen bacterial loads cfus). Encouragingly, the combinational therapy of colistin, baicalin, and EDTA (20 + 50 + 10 mg/kg) displayed an obvious reduction in bacterial loads cfus of the liver and spleen compared with monotherapy and 2-drug combination therapy. *In vivo* efficacious results demonstrated that the combination of baicalin and EDTA is a potential adjuvant for the treatment of bacterial infections caused by colistin-resistant pathogens.

DISCUSSION

Colistin is recognized worldwide as the "last resort" of defense for the treatment of MDR Gram-negative bacterial infection. However, the emergence of more and more colistin resistant strains has seriously reduced its clinical efficacy. Consequently, there is an urgent need for some efficient and more cost-effective strategies to solve the crisis of colistin resistance. For example, melatonin was found to enhance bacterial OM permeability, promote oxidative damage, to restore colistin susceptibility to MCR-positive Gram-negative pathogens (Liu et al., 2020b); The antidiabetic drug metformin potentiates tetracycline antibiotic activity against Gram-negative bacteria by disrupting bacterial OM (Liu et al., 2020a). These existing examples inspire us to search for potential non antibacterial agents as potential synergists of colistin.

In this study, despite antibacterial activity against the tested strains was weak, baicalin or EDTA exhibited a potentiation (1 to 128-fold) on colistin against resistant *Salmonella* isolates, while the combination of baicalin and EDTA showed the highest enhancement effect (16 −32,768-fold) on colistin. Additionally, this activity is independent of the resistance gene of colistin. In addition, this activity was independent of the colistin resistance gene. As far as we know, it is the first time to apply the co-application of baicalin and EDTA to rescue colistin susceptibility in colistin-resistant *Salmonella* strains.

The horizontal transfer of plasmid carrying *mcr-1* between various bacterial species may accelerate the spread of polymyxin resistance. Therefore, more MCR-1 inhibitors, such as 1-Phenyl-2-(phenylamino) ethanone derivatives and osthole, were chosen for research

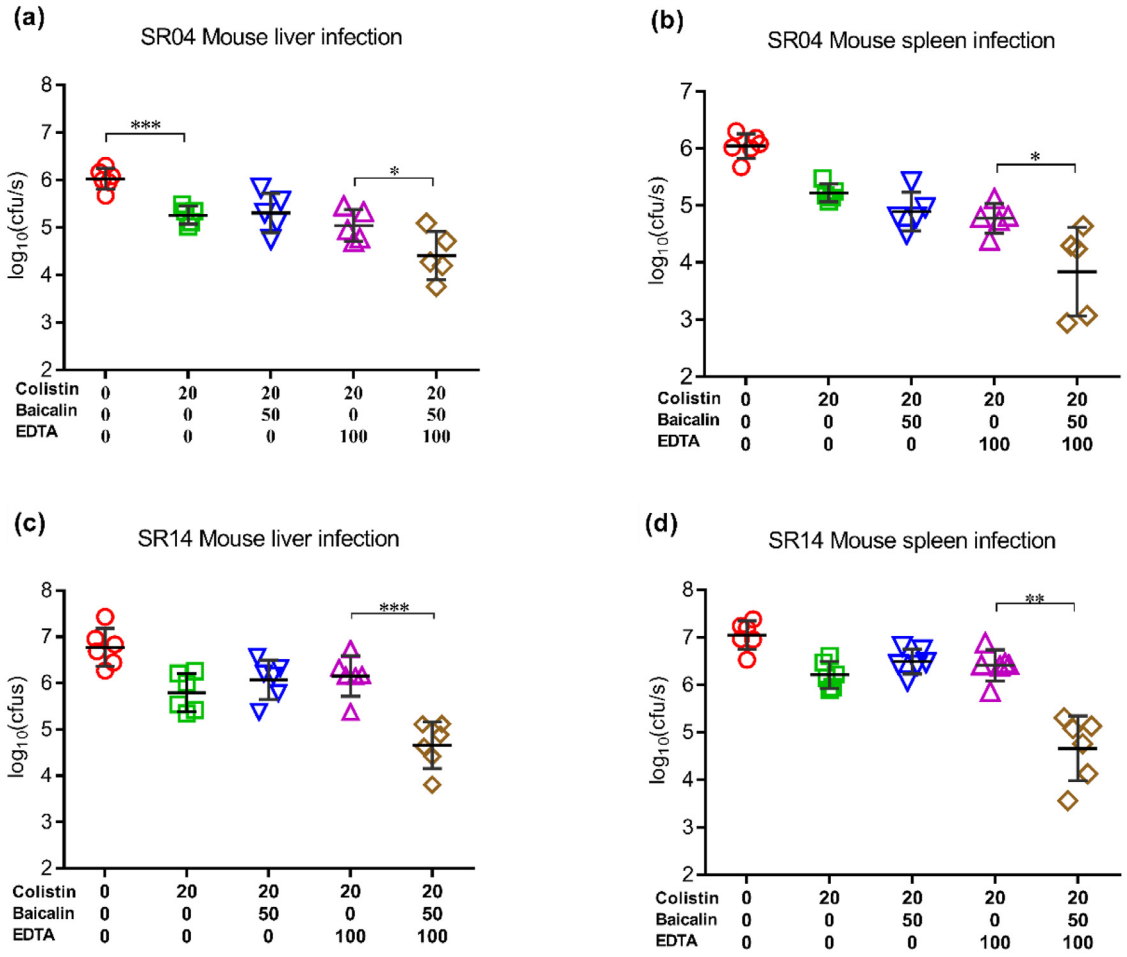

Figure 7. Baicalin and EDTA rescues colistin activity in three animal infection models. Decreased bacterial load in the mouse abdominal cavity infection model by combination therapy. Female KM mice (n=6 per group) were intraperitoneally infected given a non-lethal dose of *Salmonella* SR04 and SR14 (*mcr-1*) (1.0×10^5 cfus), and treated with a single dose of colistin(20 mg/kg), colistin + baicalin (20 + 50 mg/kg), colistin + EDTA (20 + 10 mg/kg) or a combination of the three drugs (20 + 50 + 10 mg/kg), or PBS by intraperitoneal injection. *P* values were determined by Mann-Whitney *U* test. *P* values ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) are reported. Data are presented as mean ± SD.

(Lan et al., 2019; Zhou et al., 2019). Among them, osthole could localize to the binding pocket of MCR-1 (residue 330−350). Due to the binding of osthole with MCR-1, the binding of substrate with MCR-1 was blocked, leading to inactivated biological activity of MCR-1. Additionally, 1-Phenyl-2-(phenylamino) ethanone derivatives could interact with MCR-1 protein (Glu246 and Thr285) through hydrogen bonds and occupy the cavity of MCR-1 protein, thereby inhibiting the enzyme activity of MCR-1. Our research showed that the combination of baicalin and EDTA can significantly down-regulate the mRNA expression of the *mcr-1* recombinant strain. The interaction mechanism between baicalin and MCR-1 was explored by molecular dynamics simulation. Our investigation showed that baicalin could bind to the active pocket of MCR-1 (Lys307, Lys333, ASP331, ASP337, Gly334, and Tyr308). baicalin binds to MCR-1 and thus hinders the ability of MCR-1 to bind its substrate, reducing the biological activity of MCR-1. Previous studies have confirmed that EDTA can chelate the zinc ion in the active center of MCR-1 and make MCR-1 inactive (Son et al., 2019). These results further indicate that baicalin and EDTA act directly on MCR-1 and affect its activity.

In depth synergistic mechanism analysis demonstrated that baicalin and EDTA enhanced colistin activity by multiple ways, including accelerating the TCA cycle, inhibiting bacterial antioxidant system and LPS modification, depriving multidrug efflux pump function and attenuating bacterial virulence (Figure 8). It is generally believed that colistin acts against Gram-negative bacteria only by inducing membrane lysis (Yu et al., 2015). Colistin can electrostatically interact with negatively charged LPS and displace divalent cations on the OM (Velkov et al., 2013), which penetrates OM through a self-promoting uptake mechanism leading to inner membrane (**IM**) leakage and eventually cell death. Recent reports presented an alternative mechanism of colistin against Gram-negative bacteria, which colistin stimulates the TCA cycle and respiratory chain leading to the enhancement of NADH metabolism, stimulating the production of highly harmful hydroxyl radicals (•OH), and causing oxidative damage to Gram-negative pathogens (Sampson et al., 2012).

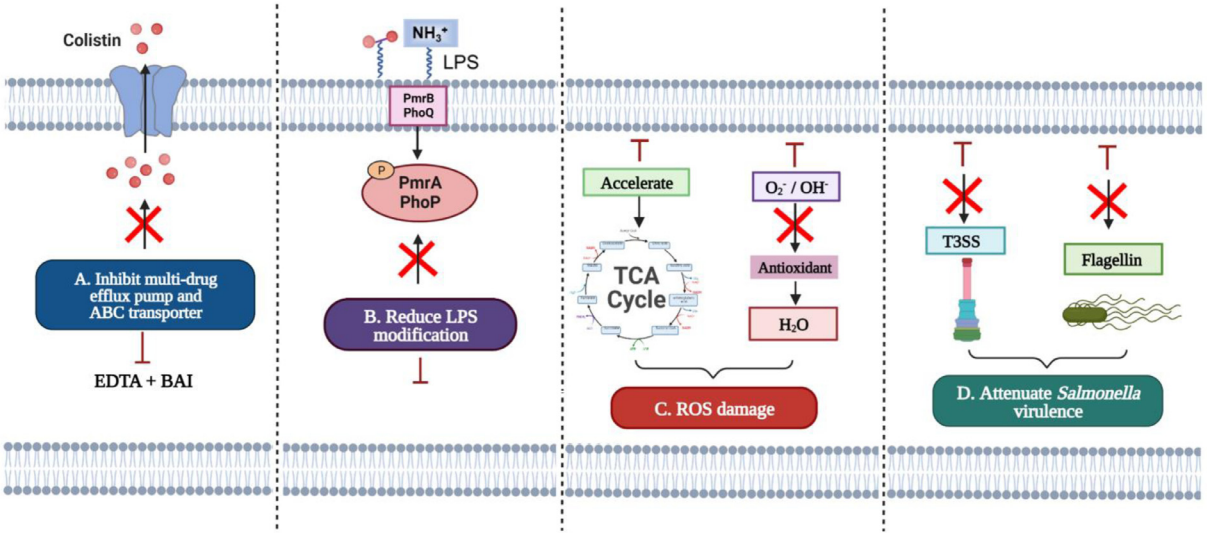

Figure 8. Scheme summarizing the mechanisms of rescuing colistin susceptibility of baicalin in combination with EDTA. BAI, baicalin. (A) Promoting intracellular accumulation of colistin in drug-resistant bacteria by inhibiting the multidrug efflux pumps and ABC transporters. (B) Inhibit the LPS modification by reducing the expression of TCSs PmrAB and PhoPQ systems. (C) Enhances bacterial oxidative damage by accelerating the TCA and inhibiting the oxidation-reduction system. (D) attenuates *Salmonella* virulence by inhibiting the expression of T3S system and flagellin in *Salmonella*.

In bacteria, the acceleration of the TCA cycle is always accompanied by the enhancement of bacterial respiration and the production of reactive oxygen species. It has been reported that TCA cycle metabolites, such as fumarate can potentiate tobramycin lethality in stationary phase cells with metabolic dormancy and phenotypic tolerance by enhancing TCA cycle activity, increasing downstream cellular respiration and proton motive force (Meylan et al., 2017). In this study, we found that the combination of baicalin and EDTA increased the oxidative damage by accelerating the TCA cycle and inhibiting the bacterial antioxidant system, which restored colistin susceptibility of *Salmonella* isolates. Furthermore, baicalin combined with EDTA can reduce expression of the genes related to LPS modification and multidrug efflux pump. For example, the combination of baicalin and EDTA inhibits the expression of multidrug-efflux transporter NorM, a member of the MATE family, which has been described to confer tolerance toward colistin in *Burkholderia vietnamien* (Fehlner-Gardiner and Valvano, 2002).

A recent study confirmed that the nitroisoaniline natural product des-Hostatin inhibits intracellular virulence of *Salmonella* by interfering with the 2-component system SsrA-SsrB and sensitizes *Salmonella* to the last-resort antibiotics colistin and polymyxin B (Tsai et al., 2020). Here, we uncover the combinations of baicalin and EDTA disrupting T3S system (**T3SS**) regulatory signaling (*yscJ, yscC* and *yscR*) and attenuating bacterial virulence. Although the secretory systems of different pathogens may have different effects on bacterial virulence and motility, an important role in pathogenicity and virulence is usually considered to be the T3SS (Rosqvist et al., 1995). In the era of antibiotic resistance, the combination of baicalin and EDTA is a promising treatment strategy. More research is needed to determine this specific mechanism of attenuating bacterial virulence.

CONCLUSIONS

In conclusion, our study indicates that the combination of baicalin and EDTA can increase the activity of colistin against *Salmonella* both *in vitro* and *in vivo*. The combination of baicalin and EDTA as a novel colistin adjuvant highlights the great potential of non-antibiotic agents against bacterial infectious diseases. Moreover, more prospective clinical trials are still required to verify the potentiation activity of the combination of baicalin and EDTA with colistin *in vivo*. Our findings provide a potential treatment strategy of using existing antibacterial agents in combination with adjuvants, to suppress the infections caused by MDR Gram-negative bacteria.

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Ethical statement: This study was carried out in accordance with the guidelines of Henan Agricultural University Animal Ethics Committee.

DISCLOSURES

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102346.

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