Journal of Microbiology, Immunology and Infection 55 (2022) 946–955

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

Role of *Coptis chinensis* in antibiotic susceptibility of carbapenem-resistant *Klebsiella pneumoniae*

Cheng-Yin Tseng [a,b], Mao-Feng Sun [c,d], Tzu-Chien Kao [e], Tsai-Chung Li [f], Ching-Ting Lin [g,*]

[a] *Graduate Institute of Chinese Medicine, School of Chinese Medicine, China Medical University, 91 Hsueh-Shih Rd, Taichung City, 40402, Taiwan*
[b] *Section of Infectious Disease, Hsinchu Mackay Memorial Hospital, 690, Sec. 2, Guang-fu Rd., East Dist., Hsinchu City, 30071, Taiwan*
[c] *Department of Chinese Medicine, China Medical University Hospital, Taichung, Taiwan*
[d] *Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan*
[e] *Graduate Institute of Chinese Medicine, School of Chinese Medicine, China Medical University, Taichung, Taiwan*
[f] *Department of Public Health, College of Public Health, China Medical University, Taichung, Taiwan*
[g] *School of Chinese Medicine, China Medical University, 91 Hsueh-Shih Rd, Taichung City, 40402, Taiwan*

Received 2 February 2021; received in revised form 18 May 2021; accepted 5 July 2021
Available online 22 July 2021

KEYWORDS
Antibiotic susceptibility;
Carbapenem-resistant *Klebsiella pneumoniae*;
Colistin;
Coptis chinensis;
Ferulic acid

Abstract *Background:* The incidence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has rapidly increased. This study aimed to assess the effect of *Coptis chinensis* and its compounds on the minimal inhibitory concentrations (MICs) of eight antibiotics against CRKP.
Methods: Cell cultures were used to investigate the effects of *C. chinensis* and its compounds on the MICs of eight antibiotics against CRKP. The MICs for antibiotics alone and antibiotics with *C. chinensis* or compounds were measured and compared. Furthermore, the effects of *C. chinensis* on cell membrane injury and intracellular adenosine triphosphate (ATP) CRKP concentration were also measured. The Mann–Whitney rank-sum test was used to analyze the differences between means.
Results: C. chinensis exhibits a notable MIC bacteriostatic effect at 5 mg/mL on CRKP. A significant MIC reduction against CRKP exists when *C. chinensis* was added to colistin and colistin-containing two-antibiotic combinations. Moreover, *C. chinensis* could damage cell membrane integrity and decrease intracellular ATP concentration in CRKP. Thus, *C. chinensis* exhibits antimicrobial activity superiority with colistin against CRKP. Furthermore, the effects of identified compounds in *C. chinensis* on the MICs of colistin, four-to eight-, two-to four-, and

* Corresponding author. Fax: +886 4 22032295.
E-mail addresses: chengyin.tseng@gmail.com (C.-Y. Tseng), maofeng@mail.cmuh.org.tw (M.-F. Sun), applekid.tw@gmail.com (T.-C. Kao), tcli@mail.cmu.edu.tw (T.-C. Li), gingting@mail.cmu.edu.tw (C.-T. Lin).

https://doi.org/10.1016/j.jmii.2021.07.003

one-to two-fold reductions were found in ferulic acid, magnoflorine, and jatrorrhizine hydrochloride, respectively. Among these compounds, ferulic acid destroys membrane integrity and decreases intracellular ATP concentration.
Conclusion: C. chinensis and ferulic acid can potentiate the antimicrobial activity of colistin and may represent a promising component of combination therapy against CRKP infections in a clinical setting.

Introduction

Klebsiella pneumoniae is a gram-negative facultative anaerobe that causes diseases, such as pneumonia, bacteremia, and urinary tract infections. Hypervirulent *K. pneumoniae* could cause severe diseases in healthy people.[1] Moreover, carbapenem antibiotics resistance emerged and disseminated in *K. pneumoniae* and currently represents a serious threat to public health; carbapenem-resistant *K. pneumoniae* (CRKP) was first reported in 1997, with high mortality rates attributed to CRKP infections.[2] Furthermore, hypervirulent *K. pneumoniae* strains with carbapenem resistance were recently found in China, with increased prevalence worldwide. These organisms can cause complicated infections that are difficult to treat and present a serious global public health threat.[3,4] Carbapenems, tigecycline, colistin, and aminoglycosides are used against some CRKPs. Moreover, colistin combined with an aminoglycoside, tigecycline, or carbapenem is a promising treatment option.[5] Furthermore, a combination therapy with two active drugs, one of which was carbapenem, can lower the mortality rate.[2] Currently, options for antimicrobial combination therapies are limited, and an ever-increasing threat of antibiotic resistance exists against these last-resort antibiotics. Consequently, deeper solving of antibiotic resistances in CRKP is a promising future development of therapeutic targets.[6,7]

Traditional Chinese medicine (TCM) has been used for the treatment of infectious diseases since ancient times. Many treatments and their underlying mechanisms of action have been proposed, and many reports have been published on TCM antimicrobial therapy in the scientific literature, such as the classic Chinese medical monograph "Treatise on Febrile Diseases (*shāng hán lùn*)." Liu et al. reported that 44.8 % of Chinese herbal medicines used in Taiwan exhibit antibacterial activity against antibiotic-resistant *Pseudomonas aeruginosa*, and one of the herbal medicines, *Ramulus Cinnamomi*, demonstrates a synergistic effect with antibiotics.[8] Furthermore, heat-clearing Chinese herbs (HCCHs) may exhibit anti-inflammatory and antimicrobial effects.[9—11] HCCHs and TCM formulations are frequently used as components of combined therapy or as monotherapy to treat common infectious diseases (e.g., pulmonary tuberculosis, methicillin-resistant *Staphylococcus aureus*, and sepsis).[12—14] TCM formulations may be useful for treating CRKP infections because they reduce fever, detoxify, *disperse wind* (anti-inflammation), and reduce swelling.

Coptis chinensis could demonstrate significant antimicrobial activity against many microorganisms including *S. aureus*, *P. aeruginosa*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Vibrio cholerae*.[9,15,16] Over 120 chemical components have been identified from *C. chinensis*, including alkaloids, organic acids, coumarins, phenylpropanoids, and quinones.[17] Berberine, ferulic acid (FA), magnoflorine, and jatrorrhizine hydrochloride (JH) are commonly identified compounds in *C. chinensis* and reported to kill bacteria and fungi.[18—21] However, the antibacterial role of *C. chinensis* for CRKP remains unknown. Therefore, this paper first reported the effects of *C. chinensis* on the minimal inhibitory concentrations (MICs) of different antibiotics against CRKP. *C. chinensis* was found to decrease the MIC of colistin against CRKP. Moreover, the effects of *C. chinensis* on cell membrane injury and intracellular adenosine triphosphate (ATP) concentration were also measured.

Methods

Strains and culture media

CRKP-P13 was recovered from the isolates, which were remnants from patients, at China Medical University Hospital. In addition to strain confirmation according to the MIC criteria of the Clinical and Laboratory Standards Institute (CLSI), CRKP-P13 was further identified as a *K. pneumoniae* carbapenemase (KPC)-producing strain using polymerase chain reaction (PCR) with the primer set KPC-Fm/KPC-Rm.[22] In addition, the mobilized colistin resistance (*mcr*-1) gene in this study was not detected in CRKP-P13 using PCR with the two primer sets,[23] CLR5-F/CLR5-R and MCR-LF/MCR-LR. Regarding the validity of this study, *E. coli* ATCC 25922 was used as a quality control strain following the CLSI guideline. The Luria—Bertani (LB) broth was used as culture media in this study. Bacteria were routinely cultured at 37 °C in LB broth or agar plate.

Preparation of *C. chinensis* and compounds

The concentrated *C. chinensis* paste (product: 8010) was obtained from Kaiser Pharmaceutical Co., Ltd (Tainan, Taiwan), which was manufactured under the good manufacturing practice and supervised by the Ministry of Health and Welfare of Taiwan. The pure compounds of *C. chinensis* were purchased from ChemFaces (Hubei, China), which has an ISO9001:2015 certification. All the indicated

concentrations of *C. chinensis* paste and experimental compounds were prepared in LB broth and filtered using a 0.45-μm filter, respectively.

C. chinensis antibacterial activity for CRKP-P13

Serial concentrations with equal differences between 10 and 100 mg/mL of *C. chinensis* were sequentially added to CRKP-P13 suspension using the broth microdilution method, followed by incubation to determine the inhibitory concentration of *C. chinensis*. CRKP-P13 growth was stopped at a *C. chinensis* concentration of 20 mg/mL. Then, the *C. chinensis* paste was serially diluted two-fold from 10 mg/mL for testing concentration (0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 mg/mL). Moreover, a concentration with 80 % bactericidal activity was defined as MIC of *C. chinensis* for CRKP-P13.

Determination of MICs of antibiotics and mixtures of antibiotics with TCM

CRKP-P13 was treated with different antibiotics, without or with *C. chinensis*, and then cultured in the LB culture media at 37 °C for 16—18 h. Consequently, the average MICs of antibiotics alone and antibiotics with *C. chinensis* were measured and compared. The results listed are representative of three independent experiments.

MICs of antibiotics alone and antibiotic combinations for CRKP-P13

This study selected eight clinically applicable antibiotics for the treatment of Enterobacteriaceae infections, including ertapenem, imipenem, ceftriaxone, cefepime, amikacin, colistin, tigecycline, and cefoperazone/sulbactam. According to the drug instructions, two-fold serial dilutions of antibiotics were used to determine the MIC values for each test drug. Serial concentrations of each of the eight antibiotics were added to the preset media of CRKP-P13 suspension to test its susceptibility. The detected MICs of each antibiotic were recorded. The MICs of various combinations of two antibiotics, which were prepared with a 1:1 mixture of 2 × detected-MIC of each two selected antibiotics, were also determined. Six regimens of a two-antibiotic combination (various combinations of colistin, tigecycline, imipenem, and amikacin) were used to measure any changes in the MICs. Moreover, >3 independent experiments were conducted for each antibiotic. The average MICs of each antibiotic alone and the two-antibiotic combination were then calculated.

MICs of mixtures of antibiotics and TCM for CRKP-P13

Three fixed concentrations of *C. chinensis* (MIC, 1/2 × MIC, and 1/4 × MIC) were separately added to the media of each of the eight antibiotics alone and to the six regimens of two-antibiotic combinations to evaluate the potential enhancing activity of *C. chinensis* on the inhibition of CRKP-P13 growth by antibiotics. The average MICs of the antibiotics alone and the antibiotic *C. chinensis* combinations were measured and compared.

Assessing CRKP-P13 cell membrane integrity

Cell membrane integrity was determined according to a previously modified method.[41] Fluorescence was measured using SpectraMax GEMINI XPS microplate reader (Molecular Devices, San Jose, CA, USA). The excitation/emission maxima for the dyes are 485/542 nm for SYTO 9 (bacteria) and 485/610 nm for propidium iodide (disrupted cell membrane). To determine the effect of *C. chinensis* and experimental compounds (FA, magnoflorine, and JH) on cell membrane integrity, bacterial suspensions were treated with the indicated concentrations of *C. chinensis* paste and experimental compounds at 37 °C for 15 min. The results were expressed as a relative percentage of viable CRKP-P13 present after treatment with the indicated concentrations of *C. chinensis* or experimental compounds. The percentage of untreated viable CRKP-P13 was set as 100.

Time-kill kinetics of the indicated concentration of *C. chinensis* and FA on the CRKP-P13 cell membrane integrity were further conducted, respectively. The results of viable CRKP-P13 were presented at 0 (control), 5, 10, 15, 30, 60, and 120 min after treatments with the indicated concentrations of *C. chinensis* and FA. The procedures were performed as described above.

Measurement of intracellular ATP concentrations of CRKP-P13

The cell suspension of CRKP-P13 (OD600 = 0.6) was used and treated with *C. chinensis* in a 2.0-mL eppendorf tube. *C. chinensis* was added to each tube resulting in final concentrations of 0 (control), 0.313, 0.625, 1.25, 2.5, 5, and 10 mg/mL, respectively. Likewise, the final concentrations of tested compounds were 0 (control), 0.063, 0.125, 0.25, 0.5, and 1 mg/mL. All the samples were maintained at 37 °C for 30 min. For intracellular ATP determination, 400 μL of cell culture were quickly transferred into boiling water and incubated for 10 min at 100 °C with constant shaking at 600 rpm. Next, cell debris was removed by centrifugation for 10 min at 13,000 rpm. The ATP concentration in the supernatant was analyzed using the CellTiter-Glo 2.0 Assay (Promega, Madison, WI, USA), according to the manufacturer's instruction.

Statistical analysis

The mean colony-forming units (CFU) of CRKP-P13 were compared between the control and the various concentrations of *C. chinensis*. The relative percentage of live CRKP-P13 cells and fluorescence units were also calculated similarly: untreated cells compared with indicated concentrations of *C. chinensis* and three identified compounds. The results were expressed as mean percentage ±standard deviation. The Mann—Whitney rank-sum test was used to analyze the differences between means. All reported p values were two-tailed, and statistical significance was set to $^{*}p < 0.05$ and $**p < 0.001$.

Results

MIC determination of *C. chinensis* for CRKP-P13

The inhibitory effect of *C. chinensis* against CRKP was not investigated. To understand the role of *C. chinensis* against CRKP-P13, the CFU of CRKP-P13 formed in the LB medium incubated with different concentrations of *C. chinensis* were calculated and compared with CRKP-P13 formed in the LB medium alone (the control group). The MIC value of *C. chinensis* was determined at 5 mg/mL for CRKP-P13. The relative survival percentages of CRKP-P13 were 95.18 % ± 2.77 %, 93.46 % ± 32.81 %, 95.04 % ± 37.03 %, 52.99 % ± 13.25 %, 28.81 % ± 10.57 %, 17.71 % ± 6.77 %, and 1.04 % ± 0.37 % at the concentrations of 0.156 mg/mL (1/32 × MIC), 0.313 mg/mL (1/16 × MIC), 0.625 mg/mL (1/8 × MIC), 1.25 mg/mL (1/4 × MIC), 2.5 mg/mL (1/2 × MIC), 5 mg/mL (MIC), and 10 mg/mL (2 × MIC), respectively (Fig. 1). Thus, *C. chinensis* exhibited a significant inhibitory effect on the growth.

Effect of *C. chinensis* on the MICs of antibiotics against CRKP-P13

MIC of single antibiotic plus *C. chinensis* against CRKP-P13

The MICs of different antibiotics against CRKP-P13 cotreated with MIC, 1/2 × MIC, and 1/4 × MIC *C. chinensis* were conducted to determine whether *C. chinensis* would affect the antibacterial activity of the indicated antibiotics against CRKP-P13. Table 1 shows that CRKP-P13 cotreated with MIC and 1/2 × MIC of *C. chinensis* could decrease the MIC of colistin by two-to four-fold, with dose-dependent effects. The addition of *C. chinensis* also provided reductions in the MICs of cefepime and cefoperazone/sulbactam. Therefore, *C. chinensis* may have a strengthening effect on colistin against CRKP infection.

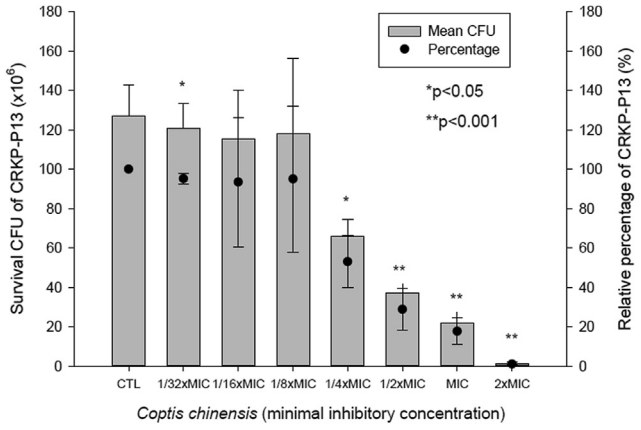

Figure 1. CRKP-P13 susceptibility to *Coptis chinensis*. The CFU formed from CRKP-P13 in the LB medium alone (the control group) and after treatment with the indicated concentrations of *C. chinensis* were counted by plating. Survival CFU and the relative percentage were expressed. The Mann–Whitney rank-sum test determined the statistically significant reductions (*asterisk*) in relative percentage of CRKP-P13 survival CFU observed for 1/32 × MIC, 1/4 × MIC, 1/2 × MIC, MIC, and 2 × MIC following the addition of *C. chinensis*. *CFU* colony-forming unit, *CTL* control, *MIC* minimal inhibitory concentration. *Error bars* indicate standard deviations. *$p < 0.05$ and **$p < 0.001$.

Table 1 The minimal inhibitory concentration of antibiotics alone and combination of antibiotics plus *C. chinensis* for CRKP-P13.

Antibiotic	Minimal inhibitory concentration (MIC, μg/mL)			
	Antibiotic alone	Antibiotic plus *C. chinensis* (mg/mL)		
		5 (MIC)	2.5 (1/2 × MIC)	1.25 (1/4 × MIC)
ETP	128	**64**–128[a]	ND	ND
IPM	32	32	ND	ND
CRO	8192	8192	ND	ND
FEP	4096	2048	1024–4096	**1024**–4096
AMI	32	16–**32**	ND	ND
CL	4	0.5–**1**	2	4
TGC	2–4	4–8	ND	ND
CPZ/Sulb	1024	512	1024	ND

[a] Range of MIC measurements. *Bold font* denotes the main MIC for each measurement.
ETP ertapenem, *IPM* imipenem, *CRO* ceftriaxone, *FEP* cefepime, *AMI* amikacin, *CL* colistin, *TGC* tigecycline, *CPZ/Sulb* cefoperazone/sulbactam, *ND* no detection.

MIC of antibiotic combination plus *C. chinensis* against CRKP-P13

The combination of colistin with a carbapenem or aminoglycoside (e.g., meropenem or amikacin) was suggested for CRKP infections.[24,25] The MICs of two-antibiotic combinations against CRKP-P13 are listed in Table 2. All combination groups, except for tigecycline and amikacin, led to reductions in the MICs of each two antibiotics against CRKP-P13. The combinations of colistin–imipenem, colistin–amikacin, tigecycline–colistin, imipenem–tigecycline, and imipenem–amikacin reduced the MICs of each antibiotic two-to four-fold compared with the use of single antibiotics. The fractional inhibitory concentration (FIC) indexes of individual combinations were colistin–imipenem (0.5), colistin–amikacin (0.5–1), tigecycline–colistin (1), tigecycline–amikacin (2), imipenem–tigecycline (0.5–1), and imipenem–amikacin (0.5). This indicated that all six combinations resulted in additive or indifference effect (FIC = 0.5–2) against CRKP-P13. The indicated concentrations of *C. chinensis* (MIC, 1/2 × MIC, and 1/4 × MIC) were applied to further evaluate the synergistic effect on MIC reductions of two-antibiotic combinations. Table 2 shows that the addition of MIC *C. chinensis* to the three colistin-containing groups, which were colistin–imipenem, colistin–amikacin, and tigecycline–colistin provided reductions in their MICs. Only the combination of colistin–amikacin showed a similar enhancing effect after the additions of 1/2 × MIC and 1/4 × MIC *C. chinensis*.

Table 2 Minimal inhibitory concentration for two-antibiotic combinations and additions of *C. chinensis* for CRKP-P13.

Antibiotic regimen[a]	Minimal inhibitory concentration (MIC, μg/mL)				
	Before mixing of two antibiotics	Mixing of two antibiotics[b]	Addition of *C. chinensis* (mg/mL)		
			5 (MIC)	2.5 (1/2 × MIC)	1.25 (1/4 × MIC)
CL/IPM	4/64	1/16	0.5/8	1/16	1/16
CL/AMI	4/16	1/4—**2/8**	0.5/2—**1/4**	0.5/2—**1/4**	1/4
TGC/CL	2/4	1/2	0.5/1	2/4	ND
TGC/AMI	2/16	2/16	4/32	ND	ND
IPM/TGC	64/2	16/0.5—**32/1**[c]	32/1	ND	ND
IPM/AMI	64/16	16/4	16/4	ND	ND

[a] Clinically selected combination of antibiotics for the treatment of *K. pneumoniae* infections.
[b] Measured MIC for every two antibiotics after 16—18 h.
[c] Range of MIC measurements. *Bold font* denotes the main measurement for experiments.
CL colistin, *IPM* imipenem, *TGC* tigecycline, and *AMI* amikacin, *ND* no detection.

Effects of different identified compounds on the MIC of colistin against CRKP-P13

Synergistic treatment effects were found in FA, magnoflorine, and JH when comparing the MICs of colistin-alone treatment (Table 3). The reductions on the MIC of colistin against CRKP-P13 were four-to eight-, two-to four-, and one-to two-fold for FA, magnoflorine, and JH, respectively. The FA provided a more significant synergistic effect on colistin antibacterial activity. Thus, the possible evidence of the amplifying activity of *C. chinensis* on colistin for CRKP-P13 was shown.

Effect of *C. chinensis* on cell membrane injury

The cell membrane was the primary colistin target. Membrane depolarization was induced, and cell wall synthesis was inhibited by inserting itself into the cell membrane of gram-negative bacteria such as *K. pneumoniae*.[26] The current experiment showed that the additions of MIC and 1/2 × MIC *C. chinensis* could lower four- and two-fold the MIC of colistin, respectively. Therefore, the *C. chinensis* activity in destroying the cell membrane integrity of CRKP-P13 was determined. The results showed that *C. chinensis* provided reductions in the relative percentage of viable CRKP-P13 of 94.734 ± 3.321, 79.343 ± 2.114, 49.458 ± 1.848, and 39.387 ± 1.363 at *C. chinensis* concentrations of 1/4 × MIC, 1/2 × MIC, MIC, and 2 × MIC, respectively (Fig. 2). *C. chinensis* showed significant effects on cell membrane injury at 1/2 × MIC, MIC, and 2 × MIC. Thus, 1/2 × MIC, MIC and 2 × MIC *C. chinensis* could damage the CRKP-P13 cell membrane integrity.

Effect of the compounds on cell membrane injury

To further observe whether FA, magnoflorine, and JH could destroy the cell membrane integrity, the fluorescence units of viable CRKP-P13 cells in the treatment were evaluated. Fig. 3a showed that FA caused significant cell membrane injury in CRKP-P13. The results were 70.502 ± 1.635,

Table 3 Effect on the minimal inhibitory concentration of colistin against CRKP-P13 for identified compounds of *C. chinensis*.

No.	Identified compound	Concentration (mg/mL)[a]	Minimal inhibitory concentration (MIC, μg/mL)	
			Colistin	Colistin plus compound
1	Palmatine hydrochloride	0.625	4	4
2	Coptisine chloride	0.83	4	4
3	Coptisine sulfate	0.83	4	4
4	Ferulic acid	0.5	4	**0.5**—1[b]
5	Magnoflorine	0.5	4	1—2
6	Jatrorrhizine hydrochloride	0.5	4	2—**4**
7	(R)-(+)-Corypalmine	0.83	4	4
8	Demethyleneberberine	0.83	4	8
9	Columbamine	0.83	4	4
10	Worenine	0.83	4	4
11	Berberine hydrogen sulfate	0.83	4	4
12	Berberrubine	0.83	4	4
13	Oxyepiberberine	0.5	4	4
14	Berberine	0.625	4	4

[a] The working concentration was determined according to the solubility of each *C. chinensis*-identified compound.
[b] Range of MIC measurements. *Bold font* denotes the main experiment measurements.

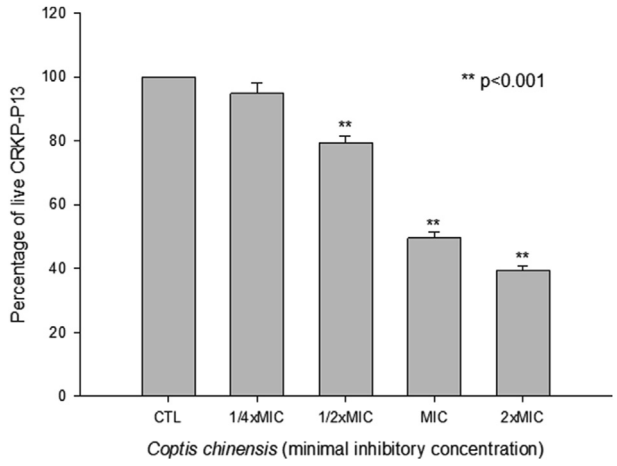

Figure 2. Percentage of viable CRKP-P13 cells after treatment with various concentrations of *Coptis chinensis*. The activity of *C. chinensis* on the injury of CRKP-P13 cell membrane integrity was determined by fluorescence units of viable cells. The fluorescence units of each bacterial suspension in LB broth alone (the control group) and after treatment with the indicated concentrations of *C. chinensis* were determined. *CTL* control, *MIC* minimal inhibitory concentration. $^{**}p < 0.001$.

24.131 ± 2.993, 17.040 ± 1.559, and 6.691 ± 0.370 at FA concentrations of 0.125, 0.25, 0.5, and 1 mg/mL, respectively. However, cell membrane injury was not measured for magnoflorine and JH (Fig. 3b and c). This finding might indicate the role of FA in *C. chinensis*-mediated antibacterial activity of colistin against CRKP-P13.

Time-kill kinetics of *C. chinensis* and ferulic acid on cell membrane integrity

The time-kill kinetics of *C. chinensis* and FA in destroying the CRKP-P13 membrane integrity were further studied. Fig. 4a shows that *C. chinensis* showed rapid killing during the first 5 min of the assay for concentrations above the MIC. Slow declines were observed after 5 min of exposure. In contrast, during exposures of 1/4 × MIC and 1/2 × MIC *C. chinensis*, slow reductions in the percentage of live CRKP-P13 were observed during 2 h of incubation. Regarding the time-kill curves of ferulic acid for CRKP-P13 (Fig. 4b), a rapid decline in the live CRKP-P13 percentage during the first 5 min for exposures of 1.0 and 0.5 mg/mL FA was disclosed. The lowest percentage of live CRKP-P13 within 1 and 2 h was revealed as well. However, a slow decline of the percentage of live CRKP-P13 was observed during exposures of ≦0.25 mg/mL FA.

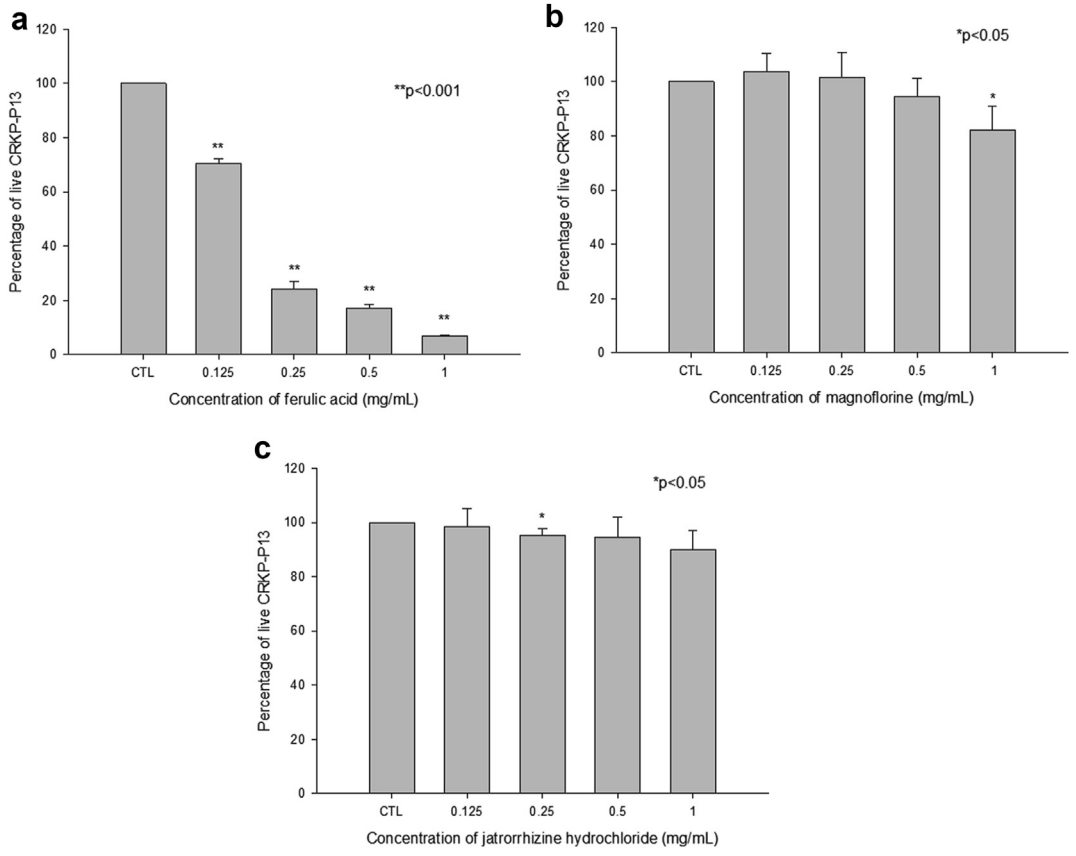

Figure 3. Percentage of viable CRKP-P13 cells after treatment with indicated compound concentrations. The activities of three identified compounds for destroying CRKP-P13 cell membrane integrity were determined by viable cell fluorescence units. The reduction levels of CRKP-P13 fluorescence with ferulic acid (**a**), magnoflorine (**b**), and jatrorrhizine hydrochloride (**c**) at 0.125, 0.25, 0.5, and 1 mg/mL were measured. The results were expressed as mean percentages ± standard deviation and analyzed by the Mann–Whitney rank-sum test. $^{*}p < 0.05$ and $^{**}p < 0.001$.

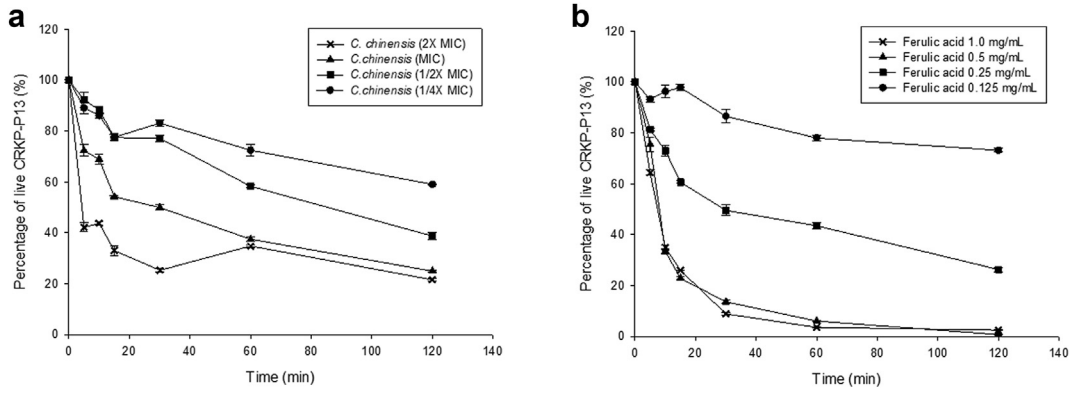

Figure 4. Time-kill curves for CRKP-P13 using four different concentrations of *C. chinensis* and ferulic acid. Concentrations of 1/4 × MIC, 1/2 × MIC, MIC, and 2 × MIC for *C. chinensis* (**a**) and 0.125, 0.25, 0.5, and 1.0 mg/mL for ferulic acid (**b**) were added to LB suspensions, respectively. The percentage of viable CRKP-P13 for all groups was determined at 0, 5, 10, 15, 30, 60, and 120 min. The percentage of untreated viable CRKP-P13 at per timepoint was set as 100.

Effects of *C. chinensis* on intracellular ATP concentration

The injury of the cell membrane integrity may lead to the decrease of intracellular ATP concentration in bacteria.[27] The effect of *C. chinensis* on intracellular ATP concentration in CRKP-P13 was further observed. Fig. 5 shows reductions in intracellular ATP concentrations of CRKP-P13 after treatment with indicated concentrations of *C. chinensis* in a dose-dependent manner. Furthermore, the intracellular ATP concentration trend in response to *C. chinensis* was consistent with that of cell membrane injury. Thus, *C. chinensis* could reduce the CRKP-P13 intracellular ATP concentration via cell membrane injury.

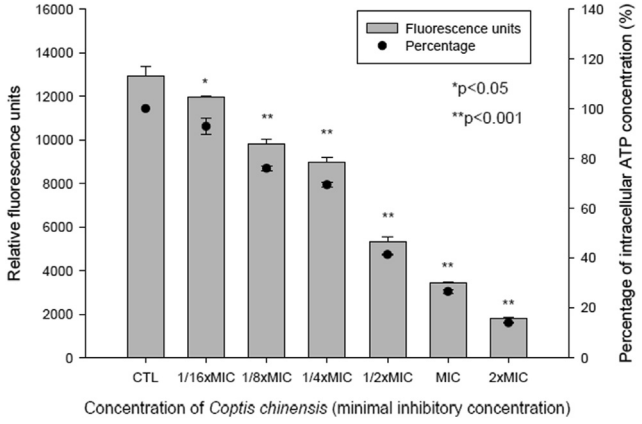

Figure 5. Effects of *C. chinensis* on intracellular ATP CRKP-P13 concentration. Intracellular ATP was measured by the CellTiter-Glo 2.0 Assay (Promega). Statistically significant reductions of intracellular ATP production by CRKP-P13 were noted when comparing control cells with cells treated with *C. chinensis* at all indicated concentrations. *CTL* control, *MIC* minimal inhibitory concentration. *Error bars* indicate standard deviations. *$p < 0.05$ and **$p < 0.001$.

Effect on the intracellular ATP concentrations for compounds

Intracellular ATP concentrations were measured to further validate the antibacterial activity against CRKP-P13 after the treatment of FA, magnoflorine, and JH. Significant reductions of intracellular ATP productions of CRKP-P13 were revealed for all three identified compounds after treatment (Fig. 6).

Discussion

Limited therapeutic options for CRKP infections are a global consensus. The combination of last-resort antibiotics, such as colistin and carbapenem, is recommended in the literature. Combination therapy does not necessarily provide a successful outcome for all CRKP infections currently, especially for CRKP bacteremia, which is associated with a mortality rate of 12.5%—68 %.[28,29] Next, the combination of Western and Chinese medicines may provide promising results for the overwhelming threat of resistance against these last-resort antibiotics. Previous studies indicate that *C. chinensis* can inhibit gram-negative bacteria.[16,30] In this study, *C. chinensis* showed an antimicrobial effect on the CRKP-P13 strain.

Synergistic antibacterial activity was measured following the addition of 5 mg/mL C. *chinensis* with colistin, ertapenem, cefepime, and cefoperazone/sulbactam. Moreover, the MIC reduction for colistin was the most prominent. Furthermore, three colistin-containing antibiotic combinations demonstrated an improved synergistic effect following the addition of *C. chinensis*. The antibacterial mechanisms for these four antibiotics are diversely distributed. Ertapenem, cefepime, and cefoperazone/sulbactam interfere with cell wall synthesis in bacteria, whereas colistin is categorized as a cell membrane disruptor. Colistin is a cyclic polypeptide antibiotic used for the treatment of gram-negative infections since the early 1970s. It can bind to lipopolysaccharide (LPS) and disrupt the bacterial cell

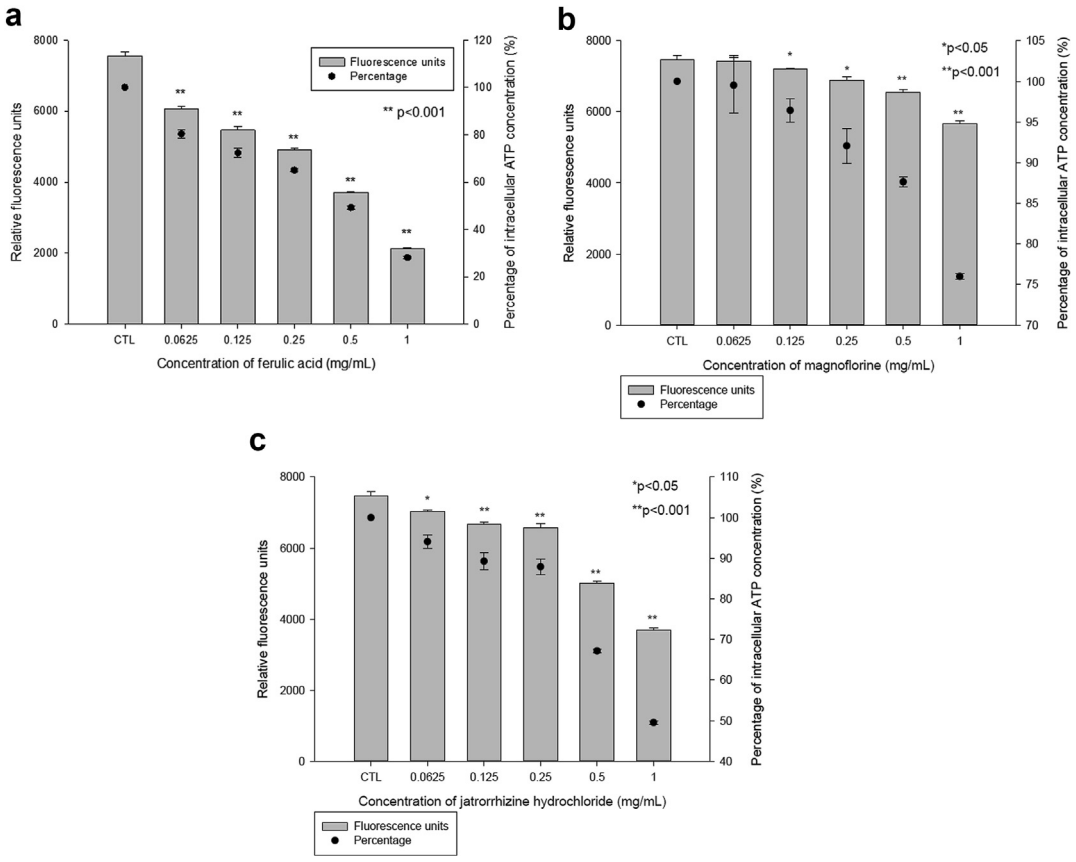

Figure 6. Effects of ferulic acid, magnoflorine, and jatrorrhizine hydrochloride on CRKP-P13 intracellular ATP concentration. Intracellular ATP was measured by CellTiter-Glo 2.0 Assay (Promega). The reductions of intracellular ATP productions by CRKP-P13 with ferulic acid (**a**), magnoflorine (**b**), and jatrorrhizine hydrochloride (**c**) at 0 (control) and indicated concentrations were measured. Statistically significant reductions of intracellular ATP concentrations for all three identified compounds were found with the analysis of the Mann–Whitney rank-sum test. Moreover, the ferulic acid would be more potent on it. *CTL* control. *Error bars* indicate standard deviations. $*p < 0.05$ and $**p < 0.001$.

membranes through competitive replacement of divalent cations in the membrane lipids, leading to bacterial death.[31] Recently, colistin was also reported to bind to LPS within the cytoplasmic membrane. *Mobilized colistin resistance-1* genes may help the bacteria to modify LPS in the cytoplasmic membrane of the bacteria, leading to colistin resistance.[32] Novel antibiotic research and development are relatively slow, and it may take many years before new antibiotics are ready for clinical use. One earlier study reported that *C. chinensis* can decrease LPS-stimulated cytokine secretion and change cell permeabilities.[33] This study also demonstrated that *C. chinensis* could destroy membrane integrity and decrease intercellular ATP concentration. Therefore, this ability of *C. chinensis* to disrupt bacterial cell membranes does not only inhibit CRKP growth but also improve the MIC of colistin against CRKP (Table 1). Thus, *C. chinensis* could be a potential novel drug for the treatment of colistin-resistant CRKP infections.

C. chinensis contains several alkaloids with antibacterial activity.[30] The alkaloids including berberine, the most representative component with 5%–7% of the ingredients,[34] exhibit stronger antibacterial activity against gram-positive bacteria than against gram-negative bacteria. Furthermore, berberine can effectively kill bacteria at higher concentrations.[18] Moreover, magnoflorine and JH are also classified as alkaloids; magnoflorine exhibited inhibitory activity against *Candida* strains and reduced the biofilm formation of *Candida albicans*.[20] Likewise, antimicrobial activities of JH were reported against coagulase-negative staphylococci and *C. albicans*.[21] The identified compounds, magnoflorine and JH, in this study provided mild-to-moderate synergistic effects on colistin against CRKP-P13 (Table 3). Next, magnoflorine and JH provided significant decreases in intracellular ATP concentration although the effects on the injury of cell membrane integrity were negligible. Studies demonstrate that cytoplasmic ATP can be affected by the cell wall structure,[27,35] cell membrane hyperpolarization, and cytoplasmic pH reduction.[19,36] Thus, we assumed that magnoflorine and JH reduce intracellular ATP in CRKP-P13 by similar mechanisms and lead to cell death.

FA is the most common phenylpropanoids in herbal medicine including *C. chinensis*.[17] The intracellular ATP of bacteria leaks through defective cell membranes under irreversible impairment in membrane permeability. The ATP concentration can be used to determine and quantify the amount of viable microbial cells. FA shows antibacterial activity against a foodborne opportunistic pathogen, *Cronobacter sakazakii*, and can lower the intracellular ATP of

C. sakazakii by destroying the cell membrane integrity.[19] Cell membrane dysfunction was further confirmed as the bactericidal mechanism.[37] This study showed that the antimicrobial effect of FA against CRKP-P13 was consistent with similar reports in the literature. In addition, FA provided significantly linear correlations between tested concentrations and both viable cells and decreases in CRKP-P13 intracellular ATP concentration. Thus, based on our finding, FA could play a role in the *C. chinensis* activity by increasing the antibacterial activity of colistin for CRKP. Presumably attributable to FA, *C. chinensis* could amplify the effect of colistin in destroying the integrity of the CRKP cell membrane. Clinically, the combination of FA with antibiotics, especially colistin, may provide potential in treating multidrug-resistant bacterial infections.

Toxicity studies have shown that the extract of *C. chinensis* has cytotoxic effects on several cell lines.[17,38] In contrast, no observed treatment-related toxicity exists to extract *C. chinensis* in the *in vivo* experiment for 90 days. Furthermore, Yi et al. demonstrated no abnormalities in the highest dose of *C. chinensis* alkaloids in the subchronic toxicity assay.[38] Several *in vivo* studies recently exist to provide evidence that the use of *C. chinensis* is relatively safe.[39,40] However, the clinical efficacy, safety, and metabolism of *C. chinensis* in the treatment of infectious diseases remain largely unknown. Therefore, more clinical experiments are warranted to verify the adjuvant role of *C. chinensis* in antibacterial therapy against CRKP infections. In the current era of multidrug resistance against majority of antibiotics, it is worth investigating the *in vivo* validity of *C. chinensis* and its compounds. Developing new antimicrobial agents that can treat these strains is crucial, and investigating traditional ethnobotanical remedies may provide a possible solution. Thus, *C. chinensis* and FA may be promising components as a combination therapy with colistin against CRKP infections in a clinical setting.

Funding

This study was supported by the grants from Ministry of Science and Technology (MOST 109-2320-B-039-049-MY3), China Medical University (CMU109-S-31), and section of medical research of Hsinchu Mackay Memorial Hospital (MMH-HB-10603).

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank Lih-Tsu Li of the Medical Laboratory of Hsinchu Mackay Memorial Hospital for assistance in preparing the strains and Pei-Ju Tsai of the Graduate Institute of Chinese Medicine at China Medical University for helping with the experiments and data collection. The authors would also like to thank Kaiser Pharmaceutical Co., Ltd. for providing the TCM formulations for the experiments.

References

1. Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: going on the offense with a strong defense. *Microbiol Mol Biol Rev* 2016 80; **3**:629—61.
2. Carmeli Y, Akova M, Cornaglia G, Daikos GL, Garau J, Harbarth S, et al. Controlling the spread of carbapenemase-producing Gram-negatives: therapeutic approach and infection control. *Clin Microbiol Infect* 2010 16;**2**:102—11.
3. Anna Maria Spagnolo PO, Donatella Panatto, Fernanda Perdelli, Maria Luisa Cristina. An overview of carbapenem-resistant Klebsiella pneumoniae: epidemiology and control measures. *Rev Med Microbiol* 2014;**25**:7—14.
4. Du P, Zhang Y, Chen C. Emergence of carbapenem-resistant hypervirulent Klebsiella pneumoniae. *Lancet Infect Dis* 2018 18;**1**:23—4.
5. Souli M, Galani I, Antoniadou A, Papadomichelakis E, Poulakou G, Panagea T, et al. An outbreak of infection due to beta-Lactamase Klebsiella pneumoniae Carbapenemase 2-producing K. pneumoniae in a Greek University Hospital: molecular characterization, epidemiology, and outcomes. *Clin Infect Dis* 2010 50;**3**:364—73.
6. Yang TY, Hsieh YJ, Kao LT, Liu GH, Lian SH, Wang LC, et al. Activities of imipenem-relebactam combination against carbapenem-nonsusceptible Enterobacteriaceae in Taiwan. *J Microbiol Immunol Infect* 2022;**55**:86—94.
7. Jean SS, Lee WS, Ko WC, Hsueh PR. In vitro susceptibility of ceftaroline against clinically important Gram-positive cocci, *Haemophilus* species and *Klebsiella pneumoniae* in Taiwan: results from the Antimicrobial Testing Leadership and Surveillance (ATLAS) in 2012-2018. *J Microbiol Immunol Infect* 2021; **54**(4):627—31.
8. Liu CS, Cham TM, Yang CH, Chang HW, Chen CH, Chuang LY. Antibacterial properties of Chinese herbal medicines against nosocomial antibiotic resistant strains of Pseudomonas aeruginosa in Taiwan. *Am J Chin Med* 2007;**35**(6):1047—60.
9. Muluye RA, Bian Y, Alemu PN. Anti-inflammatory and antimicrobial effects of heat-clearing Chinese herbs: a current review. *J Tradit Complement Med* 2014 4;**2**:93—8.
10. Wojtyczka RD, Dziedzic A, Kepa M, Kubina R, Kabala-Dzik A, Mularz T, et al. Berberine enhances the antibacterial activity of selected antibiotics against coagulase-negative Staphylococcus strains in vitro. *Molecules* 2014 19;**5**:6583—96.
11. Zhao Q, Chen XY, Martin C. Scutellaria baicalensis, the golden herb from the garden of Chinese medicinal plants. *Sci Bull* 2016 61;**18**:1391—8.
12. Lai F, Zhang Y, Xie DP, Mai ST, Weng YN, Du JD, et al. A systematic review of rhubarb (a traditional Chinese medicine) used for the treatment of experimental sepsis. *Evid Based Complement Alternat Med* 2015:131283. 2015.
13. Yu YY, Wang H, Zhang SW, Wang BE. Inhibition of methicillin-resistant Staphylococcus aureus by the compound Qingre granules. *Chin Med J* 2010 123;**8**:1017—20.
14. Shi G, Zhang L. Effects on type 2 diabetes complicated with pulmonary tuberculosis: regiment of insulin, isoniazid, rifampicin, pyrazinamide and ethambutol versus the regiment plus Qi-boosting and Yin-nourishing decoction of Traditional Chinese Medicine. *J Tradit Chin Med* 2015;**35**(3): 260—5.
15. Leach FS. Anti-microbial properties of Scutellaria baicalensis and Coptis chinensis, two traditional Chinese medicines. *Bioscience Horizons* 2011 4;**2**:119—27.
16. Sun D, Abraham SN, Beachey EH. Influence of berberine sulfate on synthesis and expression of Pap fimbrial adhesin in uropathogenic Escherichia coli. *Antimicrob Agents Chemother* 1988 32;**8**:1274—7.

17. Wang J, Wang L, Lou GH, Zeng HR, Hu J, Huang QW, et al. Coptidis Rhizoma: a comprehensive review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *Pharm Biol* 2019 57;**1**:193—225.
18. Peng L, Kang S, Yin Z, Jia R, Song X, Li L, et al. Antibacterial activity and mechanism of berberine against Streptococcus agalactiae. *Int J Clin Exp Pathol* 2015 8;**5**:5217—23.
19. Shi C, Zhang X, Sun Y, Yang M, Song K, Zheng Z, et al. Antimicrobial activity of ferulic acid against cronobacter sakazakii and possible mechanism of action. *Foodb Pathog Dis* 2016 13;**4**: 196—204.
20. Kim J, Ha Quang Bao T, Shin Y-K, Kim K-Y. Antifungal activity of magnoflorine against Candida strains. *World J Microbiol Biotechnol* 2018;**34**(11):167.
21. Slobodnikova L, Kost'alova D, Labudova D, Kotulova D, Kettmann V. Antimicrobial activity of Mahonia aquifolium crude extract and its major isolated alkaloids. *Phytother Res* 2004 18;**8**:674—6.
22. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis* 2011 70;**1**:119—23.
23. Li B, Yin F, Zhao X, Guo Y, Wang W, Wang P, et al. Colistin resistance gene mcr-1 mediates cell permeability and resistance to hydrophobic antibiotics. *Front Microbiol* 2019;**10**:3015.
24. Karaiskos I, Lagou S, Pontikis K, Rapti V, Poulakou G. The "old" and the "new" antibiotics for MDR gram-negative pathogens: for whom, when, and how. *Front Public Health* 2019;**7**:151.
25. Yu L, Zhang J, Fu Y, Zhao Y, Wang Y, Zhao J, et al. Synergetic effects of combined treatment of colistin with meropenem or amikacin on carbapenem-resistant Klebsiella pneumoniae in vitro. *Front Cell Infect Microbiol* 2019;**9**:422.
26. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* 2010 8;**6**:423—35.
27. Caillet S, Ursachi L, Shareck F, Lacroix M. Effect of gamma radiation and oregano essential oil on murein and ATP concentration of Staphylococcus aureus. *J Food Sci* 2009 74;**9**: M499—508.
28. Veeraraghavan B, Shankar C, Karunasree S, Kumari S, Ravi R, Ralph R. Carbapenem resistant Klebsiella pneumoniae isolated from bloodstream infection: Indian experience. *Pathog Glob Health* 2017 111;**5**:240—6.
29. Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, et al. Treatment outcome of bacteremia due to KPC-producing Klebsiella pneumoniae: superiority of combination antimicrobial regimens. *Antimicrob Agents Chemother* 2012 56;**4**:2108—13.
30. Hu JP, Takahashi N, Yamada T. Coptidis rhizoma inhibits growth and proteases of oral bacteria. *Oral Dis* 2000 6;**5**:297—302.
31. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther* 2012 10;**8**:917—34.
32. Akshay Sabnis AK, Becce Michele, Katheryn L, Hagart H, Evans Lindsay E, Christopher R, et al. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. *January* 2020;**29**:1—32.
33. Yang Z, Grinchuk V, Ip SP, Che CT, Fong HH, Lao L, et al. Anti-inflammatory activities of a Chinese herbal formula IBS-20 in vitro and in vivo. *Evid Based Complement Alternat Med* 2012:491496. 2012.
34. Organization WH. *WHO monographs on selected medicinal plants***1**; 1999. p. 105—14.
35. Caillet S, Shareck F, Lacroix M. Effect of gamma radiation and oregano essential oil on murein and ATP concentration of Escherichia coli O157:H7. *J Food Protect* 2005 68;**12**: 2571—9.
36. Sanchez E, Garcia S, Heredia N. Extracts of edible and medicinal plants damage membranes of Vibrio cholerae. *Appl Environ Microbiol* 2010 76;**20**:6888—94.
37. Dasagrandhi C, Park S, Jung WK, Kim YM. Antibacterial and biofilm modulating potential of ferulic acid-grafted chitosan against human pathogenic bacteria. *Int J Mol Sci* 2018;**19**:8.
38. Yi J, Ye X, Wang D, He K, Yang Y, Liu X, et al. Safety evaluation of main alkaloids from Rhizoma Coptidis. *J Ethnopharmacol* 2013 145;**1**:303—10.
39. Ho CE, Goh YL, Zhang C. From prejudice to evidence: the case of rhizoma coptidis in Singapore. *Evid Based Complement Alternat Med* 2014:871720. 2014.
40. Linn YC, Lu J, Lim LC, Sun H, Sun J, Zhou Y, et al. Berberine-induced haemolysis revisited: safety of Rhizoma coptidis and Cortex phellodendri in chronic haematological diseases. *Phytother Res* 2012 26;**5**:682—6.
41. Alakomi HL, Matto J, Virkajarvi I, Saarela M. Application of a microplate scale fluorochrome staining assay for the assessment of viability of probiotic preparations. *J Microbiol Methods* 2005;**62**(1):25—35. https://doi.org/10.1016/j.mimet.2005.01.005.