Medicinal Plant *Iris Japonica* as Histidine Kinase Inhibitor Candidate Can Enhance Antibiotic Susceptibility of *Escherichia Coli* Resistant to β -Lactam Antibiotics

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Research Article

Keywords: Histidine kinase inhibitor, TCSs, *I. japonica*, *Escherichia coli*, Multidrug resistance

Posted Date: February 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-149416/v1>

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Abstract

Background

Bacteria can respond to antibiotics in the environment by histidine kinase (HK) sensor then induce drug-resistance. In this article,antibiotic susceptibility and ESBLs genes of β -lactam-resistant *E. coli* interfered by *I. japonica* were investigated to reveal *I. japonica* can be HK inhibitor candidate[1].

Methods

E-test were applied to measure the MIC of *E. coli* to penicillin G and cefotaxime sodium according to 2017CLSI. Microplate dilution methods were applied to explore sensitivity variation of penicillin G and cefotaxime sodium of *Escherichia coli*, which contain only one single β -lactamase gene. Real-time fluorescent quantitative PCR were performed to determine the inducing effects of penicillin G and cefotaxime sodium to *E. coli* *TEM*, *SHV*, *CTX-M* and *KPC* single gene expression, and the interrupting effect of *I. japonica* extracts. Immobilized metal ion affinity chromatography and bacterial protein phosphorylation detection kit were performed to determine the change of bacterial protein phosphorylation concentration of *Escherichia coli*, after the induction of 1/4 penicillin G and cefotaxime sodium, and the interrupting effect of *I. japonica* extracts,HK inhibitor closantel was used as the positive control.

Results

1/4 MIC penicillin G or cefotaxime sodium could induce more than 10 times elevation of *TEM*, *SHV* and *CTX-M* mRNAs. While, *I. japonica* aqueous extracts (250 mg/mL) and ethanol extracts (100 μ g/mL or 50 μ g/mL) could decreased more than 40% of gene expression induced by antibiotics. *I. japonica* extracts could significantly enhance the sensitivity of β -lactam antibiotic-resistant *E. coli* strains to penicillin G or cefotaxime sodium, and could block the *TEM*, *CTX-M* and *SHV* mRNA induced by 1/4 MIC concentration antibiotics. Penicillin G and cefotaxime sodium at 1/4 MIC induced the protein phosphorylation of *TEM*, *SHV* and *CTX-M* promoted. Closantel (132.5 μ g/mL), *I. japonica* aqueous extracts (250 mg/mL) and ethanol extracts (100 μ g/mL) could inhibit the protein phosphorylation induced by above mentioned antibiotics.

Conclusion

I. japonica extracts can enhance the sensitivity of β -lactam antibiotic-resistant *Escherichia coli*. The mechanism may be that *I. japonica* extracts can be used as the inhibitor of HK phosphorylation, inhibit the transmission of antibiotic signal in TCSS, and reduce the expression of ESBLs gene.

Introduction

In the last decade, we have witnessed a dramatic increase in the proportion and absolute number of bacteria resistant to multiple antibacterial agents. Multidrug-resistant bacteria infection is currently considered as an emergent global disease and a major public health problem [2–4]. The emergence of resistant microorganisms, depending on mutations or the acquisition of mobile genetic elements carrying resistance genes, and this may take place irrespective of the presence of antibacterial agents. But these drugs provide the necessary selective pressure for the rise and spread of resistant pathogens. Therefore, the driving force behind the increasing rates of resistance can ultimately be attributed to the abuse and misuse of antibacterial agents, whether used in patients and livestock or released into the environment [5]. Multidrug-resistant bacteria infection is no longer a medical issue, It has become a global health threat that will require the coordinated action of many different stakeholders to tackle antibiotic resistance at its very root.

Bacterial resistance will be expressed, when they perceive the presence of antibiotics in the environment. Bacterial capability of responding to external stimuli is conferred by two-component signaling systems (TCSs). The TCSs comprises a histidine kinase (HK) sensor protein and its corresponding response regulator protein (RRP)[6]. Aimee et al have reported that TCSs are a major mechanism by which bacteria sense and respond to changes in their environment. And TCSs may play a critical role in the formation of bacteria antibiotic resistance[7]. Gotoh et al. (2010) have reported that HK in TCSs is capable of autophosphorylation in response to antibiotics, and the RRP interacts with the phosphorylated HK. RRP binds to the upstream-regulatory region of bacterial drug resistance genes and control their expression, such as β -lactamases genes *TEM*, *SHV*, *KPC* and *CTX-M*[8]. Therefore, HK is the core protein in the TCSs, and does not exist in mammals. If HK is inhibited, TCCs will be blocked. Then bacterial resistance gene may not be expressed, which will be sensitive to antibiotic again. The features of the HK and its essential role in TCCS signal transduction make it an attractive target for structure-based of biochemical inhibitors[9, 10]. There are few studies on HK inhibitors, most of which are limited to their toxicity, such as Closantel and some imidazole compounds, could inhibit the HK autophosphorylation of TCSs. But these inhibitors are rarely used in clinical practice, because their highly hydrophobic properties, and they cause serious diseases such as hemolysis[11, 12].

Traditional Chinese medicine has a long history and high medicinal value. If some herbs can be used as HK inhibitors, there may be a new way to treat drug-resistant bacteria. Medicinal plant *I. japonica* is native to temperate and tropical regions of Asia and has been used as a folk medicine for treating bronchitis, internal injuries, rheumatism and swellings. Laboratory studies indicates that *I. japonica* can prevent excessive oxidation of biomolecules based on various antioxidant mechanisms and the other biological activities proven by a lot of studies on italic included: anti-mutagenic, anti-angiogenic, anti-inflammatory, hypoglycemic. The main ingredients of *I. japonica* are tectoridin, tectorigenin and irigenin [13, 14].

In this study, antibiotic susceptibility and ESBLs genes of β -lactam-resistant *E. coli* interfered by *I. japonica* extracts were investigated in order to reveal *I. japonica* can be HK inhibitor candidate.

Methods

Preparation and analysis of *I. japonica* extracts:

I. japonica was collected from Huanglong cave in Hangzhou, and was identified by Professor Yao Zhensheng, college of pharmaceutical science, Zhejiang Chinese Medical University. *I. japonica* aqueous extracts were obtained by decocting method. Crush 200 g of dried *I. japonica* rhizome, and soak in 4000 mL distilled water for 30 min. Then boiled for 30 min on high fire, filtration under reduced pressure while hot. Add 2000 mL distilled water to the filter residue, decocting and filtration are the same as above. Combine the two filtrates, concentrate under reduced pressure, and dry for later use. *I. japonica* ethanol extracts were obtained by ethanol refluxing process. Crush 200 g of dried *I. japonica* rhizome. Use 70% ethanol with six times the volume of the rhizome to heat and reflux for three times, once every 2 hours. Filtration under reduced pressure, the three filtrates were combined and concentrate under reduced pressure. [15-17]. Macroporous resin (Kelong Chemical Reagent Factory, Chengdu, Lot: 20070118) technology was applied to purify crude *I. japonica* extracts [18, 19]. The color reaction under ultraviolet light, ammonia reaction, aluminium trichloride colorimetric determination and hydrochloric acid-magnesium reaction colorimetric method were used by *I. japonica* extracts to qualitative flavonoids. Treating the tectoridin standard (Phytopurify Technology Development Company, Chengdu, Lot: 13060409) as a control, UV spectrophotometry methods were used to determine the content of tectoridin [20, 21].

Bacterial isolates

A total of 183 isolates were donated by the Second Affiliated Hospital of Zhejiang University School of Medicine, Zhejiang Provincial People's Hospital and Ningbo Women and Children's Hospital, so the ethical approval was not required. The strains were isolated from January 2011 to June 2013, of which 60 were isolated from sputum specimens of patients with lower respiratory tract infection, 38 were isolated from urinary tract infections in patients with urine samples, 29 strains were isolated from vaginitis vaginal secretions, 23 strains of pleural effusion specimens from patients with infectious pleurisy, 21 strains from cerebrospinal fluid samples of neonatal purulent meningitis, 5 strains from peritoneal effusion secondary to intestinal perforation, and 7 strains from peripheral blood from patients with sepsis.

MIC of *E. coli* to penicillin G and cefotaxime sodium

Gradient strip methods according to Clinical and Laboratory Standards Institute (CLSI) 2017 were applied to measure the MIC of 183 *E. coli* to penicillin G and cefotaxime sodium. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms [22].

Detection of ESBLs

Randomly picked single colonies were inoculated into liquid Luria Bertani broth and allowed to grow overnight at 37°C in shaking table. The bacterial DNA from these isolates was extracted using the Gene Extraction Kit. DNA content and purity were measured using a UV/Vis spectrophotometer. The extracted

DNA was used for subsequent PCRs. Based on the primer sequences of the amplified ESBLs fragments reported in the literature, each primer was synthesized by Shanghai Invitrogen Corporation [23, 24]. Four sets of specific primers were used to screen for b-lactamase encoding genes belonging to the *bla CTX-M*, *bla SHV*, *bla TEM* and *bla KPC* families (Table 1). The above-mentioned target gene fragment was amplified using a high-fidelity PCR kit (TaKaRa). According to manufacturer's guidelines, the total PCR reaction volume is 100 mL, contains 2.5 mol/L dNTP, 200 nmol/L primers, 20 mol/L MgCl₂, 2.5 U EX-Taq DNA polymerase, 100 ng DNA template and 1×PCR buffer solution (pH 8.3). PCR assay was used and the amplification conditions were applied as follows: 94°C for 5 min; repeated 30 times with a cycle of 94°C for 30 s, annealing temperature 45°C for 30 s, 72°C for 1 min, and finally 72°C for 10 min. The PCR results were detected by 1.5% agarose gel electrophoresis pre-stained with ethidium bromide.

MIC and MBC of *I. japonica* extracts:

Microtube dilution methods according to CLSI 2017 were applied to measure MIC and MBC of *I. japonica* extracts to 183 *E. coli*. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control organisms [22].

TABLE1 The primers of *TEM*, *CTX-M*, *SHV*, *KPC* genes and reference used in PCR

Primers	Sequence5'-3'	Application	Product sizebp
<i>TEM</i>	FTCGGGCAAATGTGCG	<i>TEM</i> gene detection	972
	RTGCTTAATCAGTGAGGCACC		
<i>CTX-M</i>	F GTTACAGCCCTTCGGCGATGATTC	<i>CTX-M</i> gene detection	881
	R GCGCATGGTGACAAAGAGAGTGCA		
<i>SHV</i>	F GCCGGGTTATTTTATTTGTCGC	<i>SHV</i> gene detection	1017
	RTCTTTCCGATGCCGCCGCCAGTCA		
<i>KPC</i>	F GCTACACCTAGCTCCACCTCC	<i>KPC</i> gene detection	1050
	RTCAGTGCTCTACAGAAAACC		
16S RNA	F AGAGCAAGCGGACCTCATAAA	PCR internal reference	113
	RAACGTATTACCGTGACATTCTG		

FForward primer; RReverse primer

Effect of *I. japonica* extracts to *E. coil* antibiotic sensitive variation

Microtube dilution methods according to CLSI 2017 were applied to explore sensitive variation to penicillin G and cefotaxime sodium of *E. coli* E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺), E004 (*SHV*⁺), E005 (*TEM*⁺), E006 (*CTX-M*⁺), E007 (*CTX-M*⁺), E008 (*TEM*⁺), E009 (*SHV*⁺) and E010 (*TEM*⁺). These strains

were resistant to β -lactam antibiotics and containing only one single β -lactamase gene. After the action 0.5 h of *I. japonica* aqueous extracts (working concentration were 250 mg/mL, 125 mg/mL and 62.5 mg/mL) and ethanol extracts (working concentration were 100 mg/mL, 50 mg/mL and 25 mg/mL), these strains were in subinhibitory concentrations of *I. japonica* extracts. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (National Institutes for Food and Drug Control, China) were used as quality control organisms.

Effect of *I. japonica* extracts to the gene expression of ESBLs

Real-time fluorescent quantitation PCR (RT-PCR) were performed to determine the inducing effects of 1/4 MIC penicillin G and cefotaxime sodium to *E. coli* *TEM*, *SHV*, *CTX-M* and *KPC* single gene expression, and the interrupting effect of *I. japonica* aqueous extracts (working concentration were 250 mg/mL, 125 mg/mL and 62.5 mg/mL) and ethanol extracts (working concentration were 100 mg/mL, 50 mg/mL and 25 mg/mL). After the action 0.5 h of *I. japonica* aqueous extracts and ethanol extracts, then the expression of ESBLs was detected by extracting RNA from *E. coli*. According to manufacturer's guidelines, the total PCR reaction volume is 100 μ L, contains 2.5 mol/L dNTP, 200 nmol/L primers, 20 mol/L $MgCl_2$, 2.5 U EX-Taq DNA polymerase, 100 ng DNA template and 1 $\times$ PCR buffer solution (pH 8.3). RT-PCR assay was used and the amplification conditions were applied as follows: at 95°C for 5 min; repeated 40 times with a cycle of 94°C for 5 min; repeated 30 times with a cycle of 94°C for 30 s, annealing temperature at 45°C for 30 s, at 72°C for 1min, and finally at 72°C for 10 min.

Effect of *I. japonica* extracts to the HK of TCSs

Escherichia coli E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺) and E004 (*SHV*⁺) which contain only one single β -lactamase gene, were pre-incubated with *I. japonica* aqueous (250 mg/mL, 125 mg/mL and 62.5 mg/mL) and ethanol extracts (100 μ g/mL, 50 μ g /mL and 25 μ g /mL) at 37°C for 30 min, respectively. 1/4 MIC penicillin G or cefotaxime sodium was added and incubated at 37°C for 1 h, then centrifugation at 5000 rpm for 15 min. The resulting bacteria precipitation was washed with normal saline for remove the interference of extracts and antibiotic. According to the instructions of the Bacterial Protein Phosphorylation Enrichment Kit (BestBio), adding the lysate into the bacteria precipitation, followed by ice bath for 30 min, then disrupted by ultrasonication (300w, 3 s each with 3 s interval, 15 min), and centrifugation at 12000 rpm, 4°C, for 5 min to collect the supernatant. The protein concentration of the supernatant was determined by the BCA Kit (Generay). The protein samples were adjusted to 0.5 mg/mL with the above bacteria lysate, and the protein phosphorylation was enriched with the immobilized metal ion affinity chromatography (Bestbio). The bacterial protein phosphorylation level was detected by Protein Phosphorylation Detection Kit (Sangon) and spectrometer (Bio Rad). The concentration of protein phosphorylation in each sample was obtained according to the standard curve of protein phosphorylation standard concentration and O.D. 600 nm value. Compared with the results of E001, E002, E003 and E004 without the action of *I. japonica* extracts, and analyzed the effect of *I. japonica* extracts on HK. HK inhibitor closantel (132.5 μ g/mL) was used as the positive control.

Statistical methods

The ESBLs genes detection rate, protein phosphorylation concentration and ESBLs-mRNAs levels were statistically analyzed using SPSS13.0 statistical software χ^2 test and t test, respectively, $P < 0.05$ was statistically significant significance.

Results

Qualitative and quantitative analysis of tectoridin in *I. japonica* extract

The standard tectoridin as a control, *I. japonica* aqueous extracts and ethanol extract under UV light color reaction were: gray under visible light, brown and fluorescent spots under UV light. Ammonia reaction were: ammonia smoked yellow or brownish yellow fluorescent spots. Aluminium trichloride colorimetric determination phenomena were: Yellowish green fluorescent spots under UV light. Hydrochloric acid - magnesium reaction was: foam was purple. The reaction results were positive, indicating that *I. japonica* aqueous extracts and ethanol extract contains flavonoids.

MIC of penicillin G and cefotaxime sodium to *E. coli*

E-test results showed that the MIC of penicillin G and cefotaxime sodium to 183 clinical isolates of *E. coli* resistant to β -lactam antibiotics respectively were ≥ 32 mg/mL and ≥ 16 mg/mL. These results concord with the mensuration of hospitals which used by microtube dilution methods. Therefore, the isolates were resistant to penicillin G and cefotaxime sodium.

Detection of ESBLs

PCR results showed that *TEM*, *CTX-M*, *KPC* and/or *SHV* genes all can be detected in 183 *E. coli* resistant to β -lactam antibiotics (Table 2). *TEM* (83.1%, 152/183) gene had the highest detection rate, then *CTX-M* (77.1%, 141/183), *SHV* (8.2%, 15/183), *KPC* had the lowest detection rate (2.2%, 4/183). As a result, *TEM* and *CTX-M* gene detection rate were significantly higher than *SHV* and *KPC* gene. 31.7% (58/183) strains carried *KPC*, *TEM*, *CTX-M* or *SHV* single gene, 68.3% (125/183) strains carried two or more ESBLs genes among the total. Therefore, the detection rate of strains who carried two or more ESBLs genes was significantly higher than the detection rate of strains who carried only one gene. 92.0% (115/125) strains carried *TEM* and *CTX-M* genes (*TEM*+*CTX-M*) among the total, and these strains were significantly higher than the number of strains who carried *TEM*+*SHV* (4.8%, 6/125) or *TEM*+*CTX-M*+*SHV* (3.2%, 4/125).

Owing to the gene expression mechanism of single gene strains relative to polygenic strains is relatively simple and clear, we selected E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺), E004 (*SHV*⁺), E005 (*TEM*⁺), E006 (*CTX-M*⁺), E007 (*CTX-M*⁺), E008 (*TEM*⁺), E009 (*SHV*⁺), E010 (*TEM*⁺) to investigate the variation of β -lactam antibiotic sensitivity of these 10 strains, after the pre-incubated at 37°C for 30 min of *I. japonica* aqueous extracts (250 mg/mL, 125 mg/mL and 62.5 mg/mL) and ethanol extracts (100 μ g/mL, 50 μ g/mL and 25 μ g/mL). At the same time, we selected E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺) and E004

(*SHV*⁺), four strains to detect the effect of *I. japonica* extracts to expression of ESBLs single resistance gene.

MIC and MBC of *I. japonica* extracts

The bactericidal results of *I. japonica* aqueous extracts, ethanol extracts to 183 *Escherichia coli*, respectively in Microtube dilution methods were MIC $\geq$ 500 mg/mL and $\geq$ 200 μ g/mL, MBC $\geq$ 1000 mg/mL and $\geq$ 400 μ g/mL. To provide an experimental basis for determining the working concentration of *I. japonica* aqueous extracts (250 mg/mL, 125 mg/mL and 62.5 mg/mL) and ethanol extracts (100 mg/mL, 50 mg/mL and 25 mg/mL), as well as exploring the variation of β -lactam antibiotic sensitivity for *I. japonica* extracts to *E. coli*, and the drug resistance gene expression (Table 3).

TABLE 2 ESBLs gene types of 183 β -lactam antibiotics-resistant isolates

Gene type	strains (n)	(%)
<i>TEM</i>	27	14.8
<i>CTX-M</i>	22	12.0
<i>SHV</i>	5	2.7
<i>KPC</i>	4	2.2
<i>TEM+CTX-M</i>	115	62.8
<i>TEM+SHV</i>	6	3.3
<i>TEM+CTX-M+SHV</i>	4	2.2

TABLE 3 MIC and MBC of *I. japonica* to 183 *E. coli*

	aqueous extracts (mg/mL)	ethanol extracts (mg/mL)
MIC	$\geq$ 500	$\geq$ 200
MBC	$\geq$ 1000	$\geq$ 400

Effect of *I. japonica* extracts to penicillin G and cefotaxime sodium

After the incubation of 250 mg/mL, 125 mg/mL, and 62.5 mg/mL *I. japonica* aqueous extracts for 0.5 h, we knew that only E003 (*KPC*⁺) invariant resistant to penicillin G at 250 mg/mL *I. japonica* aqueous extracts, and the remaining 9 isolates were resistant to sensitive. Such as the MIC of penicillin G to E001, E004, E006 and E009 from the original 32 μ g/mL to 8 μ g/mL, to E002 and E005 from the original 32 μ g/mL to 4 μ g/mL, to E007 and E010 from the original 64 μ g/mL to 16 μ g/mL, while to E008, the MIC

from the original 128 µg/mL changed to 16 µg/mL. While, *I. japonica* aqueous extracts (125 mg/mL) could only enhance the susceptibility of E002 from the original 32 µg/mL to 8 µg/mL, *I. japonica* aqueous extracts (62.5 mg/mL) had hardly any effect on resistant *E. coli* to penicillin G (Table 4).

TABLE 4 Effect of penicillin G on MIC of *E. coli* after *I. japonica* aqueous extracts

Strain	ESBLs gene	Effect of <i>I. japonica</i> aqueous extracts on the MIC of <i>E. coli</i> isolates resistant to penicillin G (mg/mL)			
		0	62.5	125	250
E001	<i>TEM</i>	32	16	16	8*
E002	<i>CTX-M</i>	32	32	8*	4*
E003	<i>KPC</i>	64	64	64	32
E004	<i>SHV</i>	32	32	32	8*
E005	<i>TEM</i>	32	32	16	4*
E006	<i>CTX-M</i>	32	32	16	8*
E007	<i>CTX-M</i>	64	32	32	16*
E008	<i>TEM</i>	128	128	64	16*
E009	<i>SHV</i>	32	16	16	8*
E010	<i>TEM</i>	64	64	32	16*

penicillin G ≤ 8 µg/mL was sensitive, > 8 µg/mL was resistant; *compared with before the inhibitor, P < 0.05

After the incubation of 100 mg/mL, 50 mg/mL, and 25 mg/mL of the *I. japonica* ethanol extracts for 0.5 h, we knew that only E003 (*KPC*⁺) invariant resistant to penicillin G at 100 mg/mL *I. japonica* ethanol extracts, the remaining 9 isolates were resistant to sensitive. Such as the MIC of penicillin G to E001 and E002 from the original 32 µg/mL to 4 µg/mL, to E004, E005, E006 and E009 from the original 32 µg/mL to 8 µg/mL; to E007 and E010 from the original 64 µg/mL to 8 µg/mL or 16 mg/mL, to E008, the MIC from the original 128 µg/mL changed to 16 µg/mL, to E003, the MIC still is 64 µg/mL. While, *I. japonica* ethanol extracts (50 mg/mL) could only enhance the susceptibility of E001 from the original 32 µg/mL to 8 µg/mL, *I. japonica* ethanol extracts (25 mg/mL) had hardly any effect on resistant *E. coli* to penicillin G (Table 5).

TABLE 5 Effect of Penicillin G on MIC of *E. coli* after *I. japonica* ethanol extracts

strain	ESBLs gene	Effect of <i>I. japonica</i> ethanol extracts on the MIC of <i>E. coli</i> isolates resistant to penicillin G (mg/mL)			
		0	25	50	100
E001	<i>TEM</i>	32	16	8*	4*
E002	<i>CTX-M</i>	32	32	32	4*
E003	<i>KPC</i>	64	64	64	64
E004	<i>SHV</i>	32	32	16	8*
E005	<i>TEM</i>	32	32	32	8*
E006	<i>CTX-M</i>	32	16	16	8*
E007	<i>CTX-M</i>	64	64	32	8*
E008	<i>TEM</i>	128	128	128	16*
E009	<i>SHV</i>	32	32	32	8*
E010	<i>TEM</i>	64	64	16	16*

cefotaxime sodium $\leq 8 \mu\text{g/mL}$ are sensitive, $> 8 \mu\text{g/mL}$ are resistant; *compared with before the inhibitor, $P < 0.05$

After the incubation of *I. japonica* aqueous extracts for 0.5 h, we knew that only E003 (*KPC*⁺) invariant resistant to cefotaxime sodium at 250 mg/mL *I. japonica* aqueous extracts, the remaining 9 isolates were resistant to sensitive. Such as the MIC of cefotaxime sodium to E001 and E002 from the original 16 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$, to E004, E006, E007 and E009 from the original 32 $\mu\text{g/mL}$ to 8 $\mu\text{g/mL}$. *I. japonica* aqueous extracts (125 mg/mL) could enhance the susceptibility of E001, E002, E005, E007 and E010 to cefotaxime sodium. While *I. japonica* aqueous extracts (62.5 mg/mL) had hardly any effect on resistant *E. coli* to cefotaxime sodium (Table 6).

After the incubation of *I. japonica* ethanol extracts for 0.5 h, we knew that only E003 (*KPC*⁺) invariant resistant to cefotaxime sodium at 100 mg/mL *I. japonica* ethanol extracts, the remaining 9 isolates are resistant to sensitive. *I. japonica* ethanol extracts (50 mg/mL) can enhance the susceptibility of E002, E004, E007 and E008 to cefotaxime sodium. *I. japonica* ethanol extracts (25 mg/mL) had hardly any effect on resistant *E. coli* to cefotaxime sodium (Table 7).

TABLE 6 Effect of cefotaxime sodium on MIC of *E. coli* after *I. japonica* aqueous extracts

strain	ESBLs gene	Effect of <i>I. japonica</i> aqueous extracts on the MIC of <i>E. coli</i> isolates resistant to cefotaxime sodium (mg/mL)			
		0	62.5	125	250
E001	<i>TEM</i>	16	16	4*	4*
E002	<i>CTX-M</i>	16	16	4*	4*
E003	<i>KPC</i>	32	32	16	32
E004	<i>SHV</i>	32	32	16	8*
E005	<i>TEM</i>	32	16	8*	4*
E006	<i>CTX-M</i>	32	16	16	8*
E007	<i>CTX-M</i>	32	32	8*	8*
E008	<i>TEM</i>	64	64	64	8*
E009	<i>SHV</i>	32	32	32	8*
E010	<i>TEM</i>	64	64	16*	16*

penicillin G ≤ 8 $\mu\text{g/mL}$ are sensitive, > 8 $\mu\text{g/mL}$ are resistant; *compared with before the inhibitor, $P < 0.05$

Effect of *I. japonica* extracts to the gene expression of ESBLs:

with 16S RNA as a reference, compared with the blank control group, 1/4 MIC penicillin G or cefotaxime sodium could induce more than 10 times elevation of *TEM*, *SHV* and *CTX-M*, while the gene expression of *KPC* was almost unchanged. We could find that 1/4 MIC antibiotics could induce elevation of the gene expression (Figure 1).

TABLE 7 Effect of cefotaxime sodium on MIC of *E. coli* after *I. japonica* ethanol extracts

strain	ESBLs gene	Effect of <i>I. japonica</i> ethanol extracts on the MIC of <i>E. coli</i> isolates resistant to cefotaxime sodium (mg/mL)			
		0	25	50	100
E001	<i>TEM</i>	16	16	8	4*
E002	<i>CTX-M</i>	16	16	4*	4*
E003	<i>KPC</i>	32	32	32	32
E004	<i>SHV</i>	32	16	8	4*
E005	<i>TEM</i>	32	16	16	4*
E006	<i>CTX-M</i>	32	32	8	4*
E007	<i>CTX-M</i>	32	32	8	8*
E008	<i>TEM</i>	64	64	16	8*
E009	<i>SHV</i>	32	32	16	8*
E010	<i>TEM</i>	64	64	32	16*

cefotaxime sodium $\leq 8 \mu\text{g/mL}$ are sensitive, $> 8 \mu\text{g/mL}$ are resistant; *compared with before the inhibitor, $P < 0.05$

When *I. japonica* aqueous extracts (250 mg/mL, 125 mg/mL or 62.5 mg/mL) and ethanol extracts (100 mg/mL, 50 mg/mL or 25 mg/mL) worked separately on E001 (*TEM*⁺), 250 mg/mL *I. japonica* aqueous extracts and 100 mg/mL, 50 mg/mL ethanol extracts could decreased more than 50% of gene expression, demonstrating that *I. japonica* extracts in these concentrations could interrupt the antibiotics-induced elevation of ESBLs-mRNAs (Figure 2).

When *I. japonica* aqueous extracts (250 mg/mL, 125 mg/mL or 62.5 mg/mL) and ethanol extracts (100 mg/mL, 50 mg/mL or 25 mg/mL) worked separately on E002 (*CTX-M*⁺), 250 mg/mL *I. japonica* aqueous extracts and 100 mg/mL, 50 mg/mL ethanol extracts could decreased more than 40% of gene expression, demonstrating that *I. japonica* extracts in these concentrations could interrupt the antibiotics-induced elevation of ESBLs-mRNAs (Figure 3).

When *I. japonica* aqueous extracts (250 mg/mL, 125 mg/mL or 62.5 mg/mL) and ethanol extracts (100 mg/mL, 50 mg/mL or 25 mg/mL) worked separately on E004 (*SHV*⁺), 250 mg/mL *I. japonica* aqueous extracts and 100 mg/mL, 50 mg/mL ethanol extracts could decreased more than 50% of gene expression, demonstrating that *I. japonica* extracts in these concentrations could interrupt the antibiotics-induced elevation of ESBLs-mRNAs (Figure 4).

Effect of *I. japonica* extracts to the HK in TCSS:

The protein phosphorylation of *E. coli* E001 (*TEM*⁺), E002 (*CTX-M*⁺) and E004 (*SHV*⁺) significantly promoted, because of the induction of 1/4 MIC penicillin G and cefotaxime sodium, while the E003 (*KPC*⁺) was almost unchanged. (Figure 5) ($P < 0.05$)

250 mg/mL *I. japonica* aqueous extracts and 100 mg/mL ethanol extracts pretreated E001 (*TEM*⁺), E002 (*CTX-M*⁺) and E004 (*SHV*⁺), respectively. The phenomenon of protein phosphorylation promoted induced by 1/4 MIC penicillin G and cefotaxime sodium was inhibited. ($P < 0.05$). While 150 mg/mL *I. japonica* aqueous extracts or 50 µg/mL ethanol extracts pretreated *E. coli*, only the phenomenon of protein phosphorylation promoted induced by 1/4 MIC cefotaxime sodium was inhibited. ($P < 0.05$), yet little influence on the protein phosphorylation induced by 1/4 MIC penicillin G (Figure 6). HK inhibitor Closantel (132.5 µg/mL) was used as the positive control.

Discussion

The emergence and spread of bacterial resistance have been considered the result of a process of natural selection, directed influenced by the pressure of status of antibiotics use [25]. Faced the antibiotic challenge, bacteria had evolved to acquire resistance. In this strong selective pressure, only the fittest survive, and it leading to the spread of resistance mechanisms and resistant clones [26]. Tierney et al.'s study (2019) showed that bacteria have evolved the TCSs to respond appropriately to different environmental changes, and TCSs is capable of autophosphorylation in response to an environmental signal [7]. Gotoh et al. (2010) and Tiwari et al. (2017) have reported that TCSs is capable of autophosphorylation in response to antibiotic pressure, and the response regulator protein interacts with the phosphorylated HK [6, 27]. Phosphorylated response regulator protein bind to the upstream-regulatory region of bacterial drug resistance genes, and it can control their expression [6, 27, 28]. Therefore, TCSs may play a critical role in the formation of bacterial antibiotic resistance.

How to overcome bacterial resistance is always a difficult problem in clinical treatment of bacterial infections. TCSs is an important signaling system, related to bacterial resistance, its normal transmission is the key of resistance expression. HK is the core protein in the TCSs, which has the activity of kinase, phosphotransferase and phosphatase. The features of the HK and its essential role in TCSs signal transduction make it an attractive target for structure-based of biochemical inhibitors.

Since the first reported of HK inhibitors, which were isothiazolone and imidazolium, in 1993 by Roychoudhury et al, lots of HK inhibitor compounds had been found or synthesized [29]. Keith et al. (2000) have reported that closantel (Fig. 7a), ofloxacin (Fig. 7b), and some compounds which were synthesized by themselves, could inhibit the HK autophosphorylation of TCSs in *E. Coli* [12]. Yamamoto et al. (2001) have verified that HK inhibitors, such as synthetic imidazole, zerumbone derivatives and aranorosinol B were effective antibacterial agents against *Bacillus subtilis* [11]. However, none of the identified inhibitors have put into clinical use, even nor are any of these compounds even in clinical trials. Because many of inhibitors suffer from poor bioavailability stemming from their highly hydrophobic properties, and some inhibitors lead to hemolysis [16]. And for most of these inhibitors, it's unclear

whether the ability to inhibit HK as identified in italic assays is the causation for antimicrobial activities or whether killing of bacteria is independent of in italic HK inhibitory activity [12, 29]. Previous studies with HK inhibitors showed these compounds to have the hydrophobic planar aromatic molecules where minor substituent groups play a major role in specificity [30].

In the treatment of multidrug-resistant bacteria, apart from the combined use of different antibiotics antibiotic combining with traditional Chinese medicines have been demonstrated in various studies. And traditional Chinese medicines have lower toxicity [31]. We found that tectoridin (Fig. 7c), the main active ingredient of *I. Japonica* extracts has the similar structure as hydrophobic planar aromatic molecules [12]. And we speculate that *I. japonica* extracts may as HK inhibitor, and it can combine with antibiotics to against bacterial resistance.

We selected the clinical isolated *E. coli* which had β -lactamases, in order to explore the relationship between TCSs and β -lactamase genes, and study the effect of antibiotics on the expression of a single β -lactamase genes, understand whether *I. Japonica* extracts can inhibit the HK phosphorylation in TCSs and enhance antibiotic susceptibility against multidrug-resistant *E. Coli*. ESBLs are defined as enzymes mediated by plasmid, and ESBLs are able to hydrolyze β -lactam antibiotics which containing oxo imine, and can be inhibited by β -lactamases inhibitors [12, 32]. It has been reported that *E. coli* is resistant to β -lactam antibiotics due to carrying a variety of ESBLs genes. There are many types of ESBLs genes, and the important and prevalent β -lactamases are *bla CTX-M*, *bla SHV*, *bla TEM* and *bla KPC* in clinical. We selected E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺), E004 (*SHV*⁺), which contain only a single β -lactamase gene, as the research objects.

Phosphorylation is the main mode of HK activation, and closantel is the inhibitor of HK as positive control. There is no commercial kit to detect HK phosphorylation. Therefore, in order to explore the effect of antibiotics and *I. Japonica* extracts on protein phosphorylation levels in bacteria, we used Protein Phosphorylation Enrichment Kit and Protein Phosphorylation Level Detection Kit, and HK Specific Inhibitor Inhibition Test. By detecting the variety of protein phosphorylation levels in bacteria, we analyzed the activation of HK and its response regulatory protein situation. The results showed that compared with the results of E001 (*TEM*⁺), E002 (*CTX-M*⁺), E003 (*KPC*⁺) and E004 (*SHV*⁺) without the action of antibiotics, the protein phosphorylation of E001 (*TEM*⁺), E002 (*CTX-M*⁺) and E004 (*SHV*⁺) induced by 1/4 MIC penicillin G and cefotaxime sodium significantly increased ($P < 0.05$). But the phenomenon of protein phosphorylation promoted can be inhibited by the 250 mg/mL *I. japonica* aqueous extracts, 100 μ g/mL *I. japonica* ethanol extracts and Closantel (132.5 μ g/mL), ($P < 0.05$). While 150 mg/mL *I. japonica* aqueous extracts or 50 g/mL *I. japonica* ethanol extracts pretreated *E. coli* respectively, only the protein phosphorylation induced by 1/4 MIC cefotaxime sodium was inhibited ($P < 0.05$). These results suggest that sublethal penicillin or cefotaxime can be used as signal molecules to activate TCSs, and the phenomenon could be inhibited by *I. japonica* extracts and closantel.

In addition, we quantitatively detected the variations of *TEM*, *CTX-M*, *KPC* or *SHV* mRNA levels in *Escherichia coli* (E001 ~ E004 strains) before and after the incubation of 1/4 MIC penicillin G or

cefotaxime sodium. The results showed that except *KPC* mRNA, the levels of *TEM* mRNA, *CTX-M* mRNA and *SHV* mRNA were significantly increased ($P < 0.05$). More importantly, 132.5 µg/mL closantel, 250 mg/mL *I. japonica* aqueous extracts or 100 µg/mL *I. japonica* ethanol extracts pretreated *Escherichia coli* (E001 ~ E004 strains), the phenomenon of mRNA levels promoted eliminated ($P < 0.05$). Different from eukaryotic cell gene expression with dual regulatory mechanisms of transcription and post transcription, prokaryotic cells such as bacteria only regulate gene expression at the transcription level, so the level of prokaryotic cell mRNAs can truly reflect its protein expression levels. These results suggest that sublethal penicillin G or cefotaxime sodium can induce the up-regulated of ESBLs genes (*TEM*, *CTX-M* and *SHV*) expression through the HK signaling system (TCSs) of *E. coli*. But *I. japonica* extracts can inhibit the genes expression by inhibiting the HK of TCSs. Surprisingly, the expression of *KPC* gene in *E. coli* wasn't induced up-regulated by sublethal penicillin G or cefotaxime sodium, the reason is worth to further study.

At present, the anti β -lactamase drugs, such as sulbactam, tazobactam and clavulanic acid, can bind to the β -lactamase and make it inactive, but have no function of inhibiting the expression of ESBLs gene [3, 33, 34]. Closantel is a veterinary drug for the treatment of liver fluke disease in animals, and it has great toxic and side effects. It was found that Closantel can specifically inhibit the phosphorylation of HK.

In this study, we selected the closantel as the positive control, and *I. japonica* aqueous extracts and ethanol extracts as the candidate molecules of HK inhibitor. The study found that 250 mg/mL *I. japonica* aqueous extracts or 100 g/mL *I. japonica* ethanol extracts could significantly reduce the phosphorylation of HK and the level of *TEM* mRNA, *CTX-M* mRNA, *SHV* mRNA, which induced by 1/4 MIC penicillin G and cefotaxime sodium. While 150 mg/mL *I. japonica* aqueous extracts or 50 g/mL *I. japonica* ethanol extracts only could significantly reduce the phosphorylation of HK and the level of *TEM* mRNA, *CTX-M* mRNA, *SHV* mRNA, which induced by 1/4 MIC cefotaxime sodium. The results showed that *I. japonica* extracts can inhibit the expression of β -lactamase at the gene level, and enhancement of antibiotic susceptibility against multidrug-resistant *E. Coli*. Therefore, the *I. japonica* aqueous and alcohol extracts have the potential to be used as a new HK inhibitor based on drug resistance signal blocking. We will further research the mechanism of *I. japonica* extracts inhibit HK activity.

Conclusion

The present study showed that *I. japonica* extracts can be used as HK inhibitor to solve the problem of bacterial resistance. And we found the 250 mg/mL *I. japonica* aqueous extracts and 100 or 50 µg/mL ethanol extracts could significantly enhance the sensitivity of β -lactam antibiotic-resistant *E. coli* strains to penicillin G or cefotaxime sodium, and could block the *TEM*, *CTX-M* and *SHV* mRNA induced by 1/4 MIC concentration antibiotics increased.

Abbreviations

I. japonica

Iris japonica; *E. Coli*: *Escherichia coli*; HK: histidine kinase; CLSI: Clinical and Laboratory Standards Institute; PCR: Polymerase Chain Reaction; TCSs: two-component signaling systems; RRP: response regulator protein; ESBLs: extended-spectrum β -Lactamase; MIC: minimum inhibitory concentration; TEDA: *I. japonica* aqueous extracts; TEDE: *I. japonica* ethanol extracts;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All primer sequences *TEM*, *CTX-M*, *SHV*, *KPC* genes and reference are listed in Table 1 in manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

The work was supported by the Xinmiao Talent program of Zhejiang Province (Grant no. 2018R410044).

Authors' contributions

ZKX, YHW, and DQC conceived and designed the works. YHW performed the experiments. ZKX and DQC completed the writing and translation of the manuscript. JC and ZKX completed the data analysis and interpretation of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We are grateful to Professor Jie Yan, who is from Department of Pathogenic Biology, College of Medicine, Zhejiang University, for the critical reading and valuable comments to the work and manuscript.

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Figures

Figure 1

Influence of antibiotics on ESBLS-mRNA levels *Comparison of ESBLS-mRNAs with non-acting strains of antibiotics, $P < 0.05$

Figure 2

Inhibition of *I. japonica* extracts on β -lactam antibiotics-induced E001(TEM+) bacterial ESBLs-mRNAs
 TEDA: *I. japonica* aqueous extracts; TEDE: *I. japonica* ethanol extracts *Comparison of ESBLs-mRNAs with antibiotics and non-acting strains, ESBLs-mRNAs levels were statistically analyzed using t test, $P < 0.05$

Figure 3

Inhibition of *I. japonica* extracts on β -lactam antibiotics-induced E002 (CTX-M+) bacterial ESBLs-mRNAs
 TEDA: *I. japonica* aqueous extracts; TEDE: *I. japonica* ethanol extracts *Comparison of ESBLs-mRNAs
 with antibiotics and non-acting strains, ESBLs-mRNAs levels were statistically analyzed using t test, $P < 0.05$

Figure 4

Inhibition of *I. japonica* extracts on β -lactam antibiotics-induced E004(SHV+) bacterial ESBLs-mRNAs
 TEDA: *I. japonica* aqueous extracts; TEDE: *I. japonica* ethanol extracts *Comparison of ESBLs-mRNAs with antibiotics and non-acting strains, ESBLs-mRNAs levels were statistically analyzed using t test, $P < 0.05$

Figure 5

Effect of antibiotics on induction of bacterial protein phosphorylation

Figure 6

Inhibition of *I. japonica* extracts on β -lactam antibiotics-induced E001(TEM+) $\square$ E002(CTX-M+) and E004 (SHV+) bacterial protein phosphorylation TEDA: *I. japonica* aqueous extracts; TEDE: *I. japonica* ethanol extracts *Comparison of protein phosphorylation with antibiotics and non-acting strains, ESBLs-mRNAs levels were statistically analyzed using t test, $P < 0.05$

Figure 7

(a) closantel, (b) ofloxacin, (c) tectoridin of *I. Japonica* extracts

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