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ORIGINAL ARTICLE

Basic Study

Inhibitory effects of emodin, baicalin, schizandrin and berberine on *hefA* gene: Treatment of *Helicobacter pylori*-induced multidrug resistance

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Abstract

AIM: To investigate the inhibitory effects of emodin, baicalin, *etc*. on the *hefA* gene of multidrug resistance (MDR) in *Helicobacter pylori* (*H. pylori*).

METHODS: The double dilution method was used to screen MDR *H. pylori* strains and determine the minimum inhibitory concentrations (MICs) of emodin, baicalin, schizandrin, berberine, clarithromycin, metronidazole, tetracycline, amoxicillin and levofloxacin against *H. pylori* strains. After the screened MDR stains were treated with emodin, baicalin, schizandrin or berberine at a 1/2 MIC concentration for 48 h, changes in MICs of amoxicillin, tetracycline, levofloxacin, metronidazole and clarithromycin were determined.

MDR strains with reduced MICs of amoxicillin were selected to detect the *hefA* mRNA expression by real-time quantitative PCR.

RESULTS: A total of four MDR *H. pylori* strains were screened. Treatment with emodin, baicalin, schizandrin and berberine significantly decreased the MICs of amoxicillin and tetracycline against some strains, decreased by 1 to 2 times, but did not significantly change the MICs of clarithromycin, levofloxacin, and metronidazole against MDR strains. In the majority of strains with reduced MICs of amoxicillin, *hefA* mRNA expression was decreased; one-way ANOVA (SPSS 12.0) used for comparative analysis, $P < 0.05$.

CONCLUSION: Emodin, baicalin, schizandrin and berberine significantly decreased the MICs of amoxicillin and tetracycline against some *H. pylori* strains, possibly by mechanisms associated with decreasing *hefA* mRNA expression.

Key words: Traditional Chinese medicine; Multidrug resistance; *Helicobacter pylori*; Efflux pump; hefA

Core tip: Clinical signs of *Helicobacter pylori* (*H. pylori*)-induced drug resistance have become more and more prevalent, thus resulting in reduced cure rates. In this study, we used herbal extracts, such as berberine, to inhibit multidrug resistance in *H. pylori*. The results indicated that the minimum inhibitory concentration of amoxicillin and tetracycline was lowered after the intervention; the regulatory mechanism was related to down-regulation of efflux pump *hefA* mRNA expression. This suggests a novel strategy for prophylaxis and treatment of *H. pylori*-induced resistance.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the pathogen of multiple digestive system diseases including chronic active gastritis and peptic ulcer, and has been categorized as a class Ⅰ carcinogen by the World Health Organization[1,2]. Globally, the rate of *H. pylori* infection is as high as 50%[3-5], and it is even higher in developing countries. In China, the rate of *H. pylori* infection is 60%-90%[6,7]. Studies have found that 15% of clinical isolates of *H. pylori* develop multiple drug resistance (MDR; resistance to three or more antibiotics)[8-11], and this causes a decline in *H. pylori* eradication rate, posing a serious threat to human health. Therefore, the mechanism of MDR of *H. pylori* has become a hot research topic. Bacterial efflux pumps play an important role in the development of MDR. Liu *et al*[12] found that high expression of the *hefA* gene, which encodes a member of the active efflux system, resulted in the development of MDR in *H. pylori*. Zhang *et al*[13] artificially induced MDR and, for the first time, found that efflux pump inhibitors could partially reverse MDR. Studies have shown that traditional Chinese medicines emodin and baicalin have an obvious inhibitory effect on *H. pylori*[14-17]. The present study investigated the possible inhibitory effect of emodin, baicalin, schizandrin and berberine on MDR of *H. pylori* strains and the relationship between efflux pump *hefA* mRNA expression and reduced minimum inhibitory concentrations (MICs) of antibiotics, with an aim to explore the effect of Chinese herbs on *H. pylori* efflux pumps and to provide a theoretical basis for reversing MDR.

MATERIALS AND METHODS

Materials

H. pylori strains were obtained from the Department of Gastroenterology, Affiliated Hospital of Youjiang Medical University for Nationalities. For isolating *H. pylori* strains, gastric mucosal specimens were collected, ground, inoculated on Columbia blood agar plates containing 5% fresh rabbit blood, and cultured at 37 ℃ under micro-aerobic conditions (850 mL/L N_2, 100 mL/L CO_2, 50 mL/L O_2) in > 98% relative humidity for 3 to 5 d. The isolated strains were confirmed as *H. pylori* by biochemical tests (urease, catalase, oxidase), hematoxylin and eosin staining, and morphological analysis. Antibiotics used in this study included: amoxicillin (Lot No. 10807; Sichuan Pharmaceutical, Inc., China), clarithromycin (Lot No. 111202; Harbin Pharmaceutical Group Sixth Pharm Factory, China), levofloxacin (Lot No. 120120, Xinchang Pharmaceutical Factory of Zhejiang Pharmaceutical Co., LTD., China), tetracycline (Lot No. 20110902, Guangdong Taicheng Pharmaceutical Co., LTD., China), and metronidazole (Lot No.10091544, Zhejiang Jimin Pharmaceutical Co., LTD., China). Emodin (Lot No. 120908; purity, 98%), berberine (Lot No. 120810; purity, 97%), schizandrin (Lot No. 120908; purity, 98%), and baicalin (Lot No. 120908; purity, 90%) were purchased from Shaanxi Angsheng Biological Technology Co., LTD (China). Other reagents or kits used included Trizol reagent kit (Shanghai Invitrogen, China); RevertAid First Strand cDNA Synthesis Kit and DNase Ⅰ (Fermentas); 2 × SYBRGreen quantitative PCR (qPCR) Mix (Beijing Zhuangment Co., LTD, China). PCR primers were designed based on the *H. pylori hefABC* gene

Table 1 Resistance of multidrug resistance strains to the five antibiotics

No.	Levofloxacin	Amoxicillin	Clarithromycin	Tetracycline	Metronidazole
23	R	R	R	R	R
40	R	R	R	R	R
41	S	R	R	R	R
42	R	R	I	R	R

R: Resistant; S: Sensitive; I: Intermediate.

Table 2 Minimum inhibitory concentrations of the four Chinese medicines against multidrug resistance strains (mg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin
23	12.5	50	200	50
40	12.5	100	100	100
41	12.5	100	100	100
42	12.5	25	100	50

sequences deposited in GenBank. Through sequence homology analysis, the primers were selected in the conserved region. 16S rRNA was used as an internal control. The primers were synthesized by Shanghai Sangon Biotech (China).

Screening of MDR strains

The double dilution method was used to determine the MICs of emodin, baicalin, schizandrin, berberine, clarithromycin, metronidazole, tetracycline, amoxicillin and levofloxacin against *H. pylori* strains. According to the Clinical and Laboratory Standards Institute (NCCLS) criteria, *H. pylori* strains that could grow in medium containing three or more of amoxicillin (≥ 4 μg/mL), levofloxacin (≥ 8 μg/mL), clarithromycin (≥ 1 μg/mL), metronidazole (≥ 8 μg/mL), and tetracycline (≥ 4 μg/mL) were identified as MDR strains.

Determination of antibiotic susceptibility of MDR strains before and after treatment with traditional Chinese medicines

Based on the method described previously[18], the MICs of the traditional Chinese medicines against MDR strains were calculated, and the next concentration below MIC was 1/2 MIC. The screened MDR stains were treated with emodin, baicalin, schizandrin or berberine at a 1/2 MIC concentration for 48 h to determine their effect on the MICs of amoxicillin, tetracycline, levofloxacin, metronidazole and clarithromycin. A positive control (10 mg/mL pantoprazole) and a negative control (culture medium) were also run at the same time.

Reverse transcription-PCR for detection of hefA mRNA expression in MDR strains with reduced MICs of amoxicillin

MDR strains with reduced MICs of amoxicillin after treatment with emodin, baicalin, schizandrin and berberine at a 1/2 MIC concentration were screened, and total RNA was prepared with Trizol reagent according to the manufacturer's instructions. Reverse transcription was then performed in a 20-μL system containing 5 μL total RNA, 1 μL random primer p(dN)6 (0.2 μg/μL), 5 μL RNase-free ddH$_2$O, 4.0 μL, 5 × reaction buffer, 2.0 μL dNTP mix (10 mmol/L), 1.0 μL Rnase inhibitor (20 U/μL) and 2.0 μL AMV reverse transcriptase (10 U/μL). The reaction parameters were 37 ℃ for 5 min, 42 ℃ for 60 min, and 70 ℃ for 10 min. PCR was then performed in a 20-μL system containing 10 μL SybrGreen qPCR Master Mix, 1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L), 7 μL ddH$_2$0, and 1 μL template (cDNA; 1:6 dilution). Cycling parameters were pre-denaturation at 95 ℃ for 2 min, and 40 cycles of denaturation at 95 ℃ for 10 s and annealing at 60 ℃ for 40 s.

Statistical analysis

Statistical analyses were performed using SPSS12.0. The differences in *hefA* mRNA expression were compared using analysis of variance (ANOVA) and Tukey tests.

RESULTS

Screening of MDR strains

A total of four MDR strains were screened, of which two were resistant to amoxicillin, clarithromycin, levofloxacin, tetracycline, and metronidazole, one was resistant to amoxicillin, clarithromycin, tetracycline, and metronidazole, and one was resistant to amoxicillin, levofloxacin, tetracycline, and metronidazole. These MDR strains are shown in Table 1.

MICs of the four traditional Chinese medicines against MDR strains

The MICs of emodin, baicalin, schizandrin and berberine against MDR strains are shown in Table 2.

Changes in MICs of the determined antibiotics against MDR strains after treatment with the four traditional Chinese medicines

After the four MDR strains were treated with emodin, baicalin, schizandrin and berberine at a 1/2 MIC concentration or pantoprazole, the MICs of amoxicillin and tetracycline against some strains were decreased compared with before treatment or the negative control group. Compared with 10 mg/mL pantoprazole,

Table 3 Minimum inhibitory concentrations of amoxicillin against the four multidrug resistance strains after treatment with the four traditional Chinese medicines (μg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin	Pantoprazole	NC
23	32	16	64	32	32	64
40	16	8	16	16	16	32
41	64	64	21	64	32	64
42	32	16	32	32	32	32

Pantoprazole was used as a positive control, and culture medium was used as a negative control. NC: Negative control.

Table 4 Minimum inhibitory concentrations of tetracycline against the four multidrug resistance strains after treatment with the four traditional Chinese medicines (μg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin	Pantoprazole	NC
23	64	32	64	64	32	64
40	16	16	32	32	32	32
41	32	32	32	64	16	32
42	32	16	32	32	32	32

NC: Negative control.

Table 5 Minimum inhibitory concentrations of metronidazole against the four multidrug resistance strains after treatment with the four traditional Chinese medicines (μg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin	Pantoprazole	NC
23	128	128	128	128	64	128
40	64	64	64	64	32	64
41	64	64	64	64	32	64
42	64	64	64	64	64	64

NC: Negative control.

Table 6 Minimum inhibitory concentrations of clarithromycin against the four multidrug resistance strains after treatment with the four traditional Chinese medicines (μg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin	Pantoprazole	NC
23	64	64	64	64	32	64
40	32	32	32	32	16	32
41	32	32	32	32	16	32
42	32	32	32	32	32	32

NC: Negative control.

the effects of the traditional Chinese medicines were comparable or superior (Tables 3 and 4). However, the MICs of clarithromycin, levofloxacin, and metronidazole against MDR strains showed no significant changes (Tables 5-7).

Expression of hefA mRNA in MDR strains with significantly changed MICs of amoxicillin

Sixteen sub-cultured strains with significantly reduced MICs of amoxicillin after treatment with emodin, baicalin, schizandrin, berberine or pantoprazole (Table 3) were selected and used to detect the *hefA* mRNA expression by reverse transcription-PCR. After No. 23 MDR strain was treated with emodin, schizandrin or berberine, both MICs of amoxicillin and *hefA* mRNA expression were decreased. After No. 40 MDR strain was treated with emodin, baicalin, schizandrin and berberine, both MICs of amoxicillin and hefA mRNA expression were decreased. For No. 41 MDR strain, treatment with baicalin decreased MIC of amoxicillin but increased *hefA* mRNA expression. For No. 42 MDR strain, treatment with berberine decreased MIC of amoxicillin but increased *hefA* mRNA expression, while treatment with pantoprazole did not significantly change MIC of amoxicillin but increased *hefA* mRNA expression. The amplification curves, melting curves, PCR products and quantitative results for the 16 sub-cultured strains are shown in Figures 1-3 and Table 8, respectively.

Table 7 Minimum inhibitory concentrations of levofloxacin against the four multidrug resistance strains after treatment with the four traditional Chinese medicines (μg/mL)

No.	Emodin	Berberine	Berberine	Schizandrin	Pantoprazole	NC
23	64	64	64	64	32	64
40	32	32	32	32	16	32
41	32	32	32	32	16	32
42	64	64	64	64	64	64

NC: Negative control.

Table 8 Real-time PCR quantitative results for *hefA* gene expression in 16 sub-cultured strains with significantly changed minimum inhibitory concentration of amoxicillin

No.	hefA	16S rRNA	ΔCt	ΔΔCt	$2^{-(\Delta\Delta Ct)}$
1	31.0933170	14.457372	16.63595	0	1
2	30.2871494	15.427569	14.85958	-1.77637	3.425620
3	29.8203506	16.133926	13.68642	-2.94952	7.724926
4	30.2641807	16.449774	13.81441	-2.82154	7.069158
5	33.8933296	15.226003	18.66733	2.031382	0.244621
6	32.0969762	15.256840	16.84014	0.204191	0.868025
7	29.9665985	15.307595	14.65900	-1.97694	3.936578
8	31.2955170	14.817378	16.47814	-0.15781	1.115590
9	31.6979313	14.758531	16.93940	0.303455	0.810309
10	30.7486935	14.488201	16.26049	-0.37545	1.297247
11	34.3626747	18.408577	15.95410	-0.68185	1.604193
12	36.2199669	18.414414	17.80555	1.169608	0.444542
13	33.9337616	17.102884	16.83088	0.194932	0.873614
14	31.4263000	14.820235	16.60606	-0.02988	1.020928
15	32.9468956	14.884178	18.06272	1.426772	0.371962
16	31.6464024	15.465043	16.18136	-0.45459	1.370390

DISCUSSION

In recent years, there have been more and more studies on traditional Chinese medicines. As a result, the mechanisms of action of many traditional Chinese medicines have been gradually elucidated. Heat-

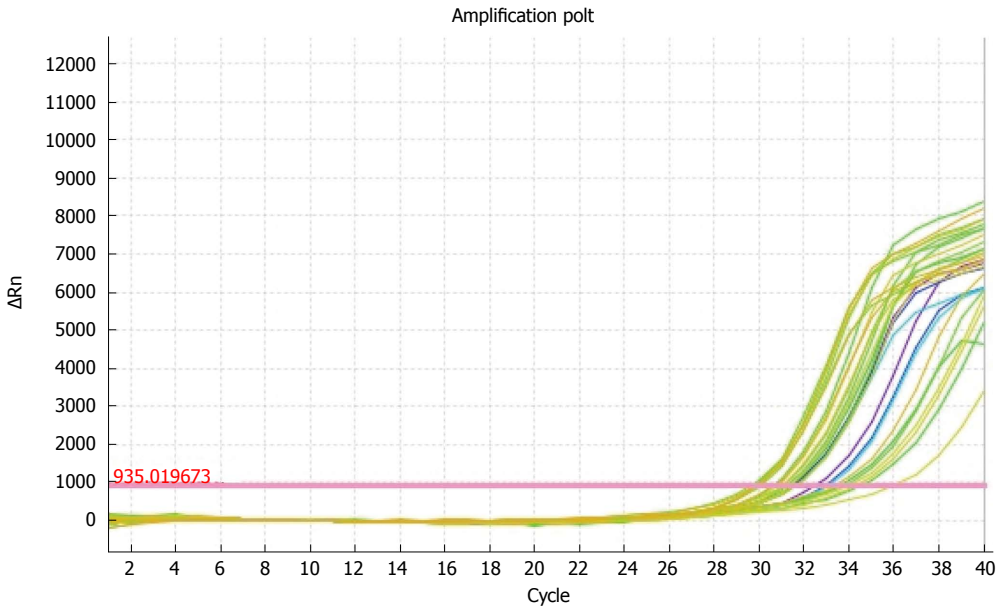

Figure 1 Amplification curves for the *hefA* gene in 16 sub-cultured strains with significantly changed minimum inhibitory concentration of amoxicillin.

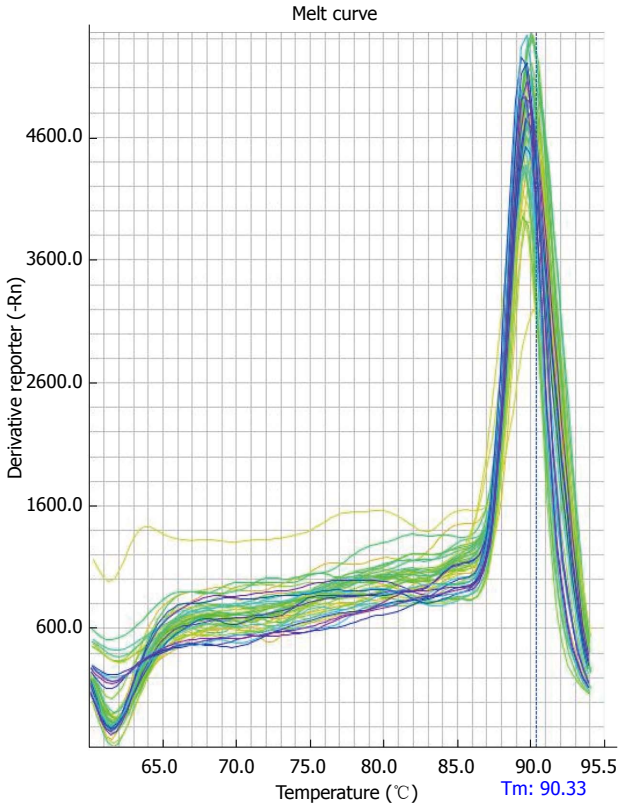

Figure 2 Melting curves for the *hefA* gene in 16 sub-cultured strains with significantly changed minimum inhibitory concentration of amoxicillin.

clearing and detoxicating Chinese medicines have strong antibacterial effects and are therefore called plant antibiotics; these include *Rhizoma coptidis*, *Radix scutellariae*, *Andrographis paniculata*, rhubarb, and *Radix isatidis*[17,19-24]. Baicalin is the main effective ingredient of *Radix scutellariae*, and its bacteriostatic mechanisms include destroying bacterial cell membrane, inhibition of bacterial DNA, RNA and protein biosynthesis, and degradation of endotoxins[25]. Berberine, also called berberine hydrochloride, is the main ingredient of *Rhizoma coptidis*. It exerts bacteriostatic effects probably by inhibiting bacterial growth and respiration, suppressing the oxidation of glucose and sugar metabolic intermediates, especially deoxidization reactions[26]. Rhubarb consists mainly of anthraquinone compounds including emodin, rhein, and chrysophanol. Emodin has purgative, antibacterial, antitumor and hemostatic effects, and its antibacterial effects are associated with inhibiting bacterial nucleic acid biosynthesis and breathing processes, because it can cause bacterial DNA damage and result in the production of small pieces of DNA[27,28]. However, some studies found that the inhibitory effect of emodin on *H. pylori* is related to the inhibition of aromatic amine-N-acetyl transferase activity[29]. Berberine, emodin, schizandrin, and baicalin have inhibitory and killing effects against *H. pylori*, even in drug-resistant strains. Currently, there have been no other reports of the effects of traditional Chinese medicines on MDR and the underlying mechanisms.

The results of the present study, together with our previous findings, showed that berberine, emodin, schizandrin, and baicalin have certain inhibitory and killing effects against MDR *H. pylori* strains. Additionally, emodin, baicalin, schizandrin or berberine at a 1/2 MIC concentration could reduce the MICs of amoxicillin and tetracycline against some MDR strains. Compared with 10 mg/mL pantoprazole, the effects of the traditional Chinese medicines were comparable or superior; however, they could not reduce the MICs of clarithromycin, levofloxacin, and metronidazole against MDR strains. The possible reasons are: (1) different MDR strains may have different drug resistance

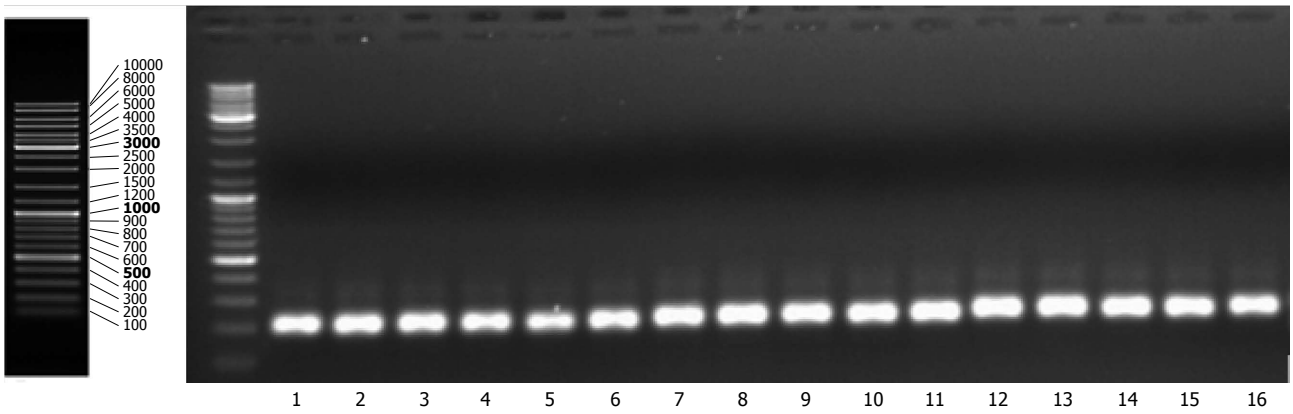

Figure 3 Agarose gel electrophoresis analysis of PCR products for *hefA* in 16 sub-cultured strains with significantly changed minimum inhibitory concentration of amoxicillin.

patterns, thus resulting in different MICs; (2) different traditional Chinese medicine ingredients have different bacteriostatic effects. Berberine has obvious, relatively stable bacteriostatic effects, while the other three medicines have unobvious, unstable antibacterial effects; and (3) bacterial strains came from different sources and had different growth environments, which may also affect MICs.

While exploring the mechanism by which emodin, baicalin, schizandrin, berberine and pantoprazole reduced the MICs of amoxicillin against MDR strains, we found that both MICs of amoxicillin and *hefA* mRNA expression were reduced in No. 23 and No. 40 MDR strains, suggesting that *hefA* mRNA expression may be positively correlated with the effects of emodin, baicalin, schizandrin, berberine and pantoprazole in reducing the MICs of amoxicillin against MDR strains. However, in No. 41 MDR strain, treatment with baicalin decreased the MIC but increased *hefA* mRNA expression; for No. 42 MDR strain, treatment with berberine decreased the MIC but increased *hefA* mRNA expression, and treatment with pantoprazole did not significantly change the MIC but increased *hefA* mRNA expression. On one hand, this may be associated with drug resistance patterns of MDR strains. Both No. 23 and No 40 MDR strains had the same pattern of resistance to levofloxacin, clarithromycin, amoxicillin, tetracycline, and metronidazole, while No. 41 was sensitive to levofloxacin, and No. 42 was moderately sensitive to clarithromycin. On the other hand, the existence of multiple efflux pump gene families may result in the above discrepancy. Since the *hefA* gene is only one member of one of the five efflux pump families, other members of the five efflux pump families may mediate the MIC reduction.

The present study showed that berberine, baicalin, emodin, and schizandrin all have antibacterial activities against MDR, and can reduce the MICs of amoxicillin and tetracycline, possibly *via* mechanisms associated with altering *hefA* gene expression. These findings provide a new idea and new method for solving the problem of increasingly serious MDR. However, it remains to be investigated why these Chinese medicines only reduced the MICs of amoxicillin and tetracycline, and did not alter those of levofloxacin, clarithromycin and metronidazole, and why *hefA* gene expression was not correlated with MIC reduction in some strains.

COMMENTS

Background

There are different mechanisms of *Helicobacter pylori* (*H. pylori*)-induced resistance to various antibiotics, but its multidrug resistance may be closely linked to the activity of efflux pumps. However, there is not yet an accredited method to prevent or reverse multidrug-resistance induced by *H. pylori*. Our research group has found that herbal extracts (especially emodin) have an effective inhibitory effect on *H. pylori*-induced multi-drug resistance, and that they reduced the minimum inhibitory concentration (MIC) of some antibiotics. Thus, the authors further explored the molecular mechanism of emodin lowering of antibiotic MIC by assessing the expression of the efflux pump gene *hefA*.

Research frontiers

The issue of *H. pylori*-induced drug resistance has become one of the current research directions, and seeking to optimally measure its resistance is an urgent scientific project. Chinese herbal medicine has pronounced characteristics, such as anti-inflammatory, immunomodulatory, anti-tumor roles and it lowers side effects. Therefore, developing some substitutes extracted from Chinese herbals would provide new ideas or methods to solve the drug resistance.

Innovations and breakthroughs

A class of fever-reducing and detoxification herbals generally has antibacterial effects, and some herbals, such as berberine, play an inhibitory role against *H. pylori*-induced drug resistance; however, its mechanism remains unclear. This study found that berberine had an effective inhibitory effect on *H. pylori*-induced drug resistance and decreased the MIC of amoxicillin antibiotic, *etc*. The authors also report that the mechanism may be related to reduction of efflux pump *hefA* gene expression.

Applications

This study found that berberine has an effective inhibitory effect on *H. pylori*-induced multi-drug resistance, and lowered the MIC of amoxicillin antibiotic *etc*. The findings suggest new ideas or methods to solve the drug-resistance induced by *H. pylori*, thereby aiming to cure this disease.

Terminology

Minimum inhibitory concentration is the lowest antimicrobial concentration that can inhibit the growth of bacteria; Multidrug resistance is a condition enabling disease-causing microorganisms (bacteria, viruses, fungi or parasites) to resist

distinct antimicrobials.

Peer-review

The present study revealed that berberine *etc.* has an effective inhibitory effect on *H. pylori*-induced multi-drug resistance and lowered the MIC of amoxicillin antibiotic *etc.*; the mechanism may be related to down-regulation of efflux pump *hefA* gene expression. The findings demonstrate that use of berberine has a relatively large practical value, and it would provide the scientific theory for developing effective medication against resistance induced by *H. pylori*.

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