ANTIBACTERIAL EFFECT OF THE WATER EXTRACT OF *HOUTTUYNIA CORDATA* WATER EXTRACT AGAINST MULTI-DRUG RESISTANT *ESCHERICHIA COLI*

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Abstract. This study aimed to investigate the antibacterial activity of the water extract of *Houttuynia cordata* (HCWE) against multi-drug resistant (MDR) *Escherichia coli* isolates harboring the *AcrA* gene in order to determine its susceptibility for potential therapy. We examined 18 *E. coli* strains that exhibited resistance to at least three different classes of antibiotics. The antibacterial effect, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and transcriptional level of the *AcrA* gene were assessed by using the agar well diffusion technique, tube dilution method and real-time PCR analyses, respectively. The water extract of *H.cordata* had antibacterial activity against MDR *E.coli* isolates tested with the highest and lowest zone diameters of inhibition (ZDI) of 29 and 13 mm at concentrations of 500 and 50 mg/ml, respectively. The MIC and MBC of HCWE against MDR *E.coli* isolates were 400 and 500 mg/ml, respectively. The expression of the *AcrA* gene was inhibited (0.39-, 0.29- and 0.16- fold) in a dose dependent manner by the HCWE when cultured with 25 mg/ml, 50 mg/ml and 100 mg/ml HCWE. Our results show HCWE has activity *in vitro* against MDR *E.coli*. Further studies are needed to determine if HCWE can be developed as a therapeutic agents against MDR *E. coli*.

Keywords: *Houttuynia cordata,* minimum bactericidal concentration, multi-drug resistant, *Escherichia coli*

INTRODUCTION

Escherichia coli is an essential, commensal bacterium present as microflora in human and animal intestinal tracts (Kaper *et al*, 2004). *E. coli* can cause infections in both humans and animals (Naganandhini *et al*, 2015). *E. coli* infections can be treated by a variety of antibiotics, but some strains have developed resistance to antimicrobials and treatment costs have increased. Antimicrobial-resistant strains of *E. coli* has become a serious problem in hospital environments worldwide (Erb *et al*, 2007). Effort has gone into reducing inappropri-

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ate use of antimicrobials to prevent the emergence of drug resistant bacteria. At the same time, it is also important to develop new antimicrobial agents to treat drug resistant strains.

Multidrug resistant (MDR) strains of *E. coli* are becoming more common among clinical isolates (Erb *et al*, 2007). This resistance is mainly associated with the major multi-drug efflux pump (AcrAB-TolC); secondary transporter AcrB and outer-membrance channel Tolc) in resistance-nodulation-division (RND) family and is found in the chromosomes of *E.coli* (Nikaido and Zgurskaya, 2001). This natural efflux system consists of drug transporters which secrete antibodies and toxic chemicals and enable *E.coli* to survive under harsh environments by hydrolyzing ATP and using the proton gradient as a source of energy (Nikaido and Zgurskaya, 2001; Kumar and Schweizer, 2005; Marquez, 2005). In *E.coli*, the AcrAB pump works with TolC to remove from the cell a broad range of antimicrobial compounds, including antibiotics, dyes, and detergents (Ma *et al*, 1993). AcrB is a drug proton anti-porter that captures its substrates in the inner membrane and transports them via the OM channel TolC to the external media (Zgurskaya and Nikaido, 1999). However, the AcrB-TolC cooperation is interceded by the periplasmic protein AcrA. These three genes (AcrAB-TolC) are needed for efficient transport and a disturbance in any of these genes results in hyper-susceptibility of *E.coli* to different substrates including antibiotics (Okusu *et al*, 1996). In our experiment, the water extract of *Houttuynia cordata* (HCWE) was used to attempt to limit expression of the *AcrA* gene.

Traditional herbal medicine has been used to treat a variety of diseases. *Houttuynia cordata* Thunb is an important medicinal plant widely distributed in East and Southeast Asia, containing groups of chemical components such as flavones, essential oils and alkaloids (Bauer *et al*, 1996). These components exhibit a strong antibacterial effect against gram-positive bacteria, including *Streptococcus aureus* and *S. ureae* (Kwon *et al*, 1996). *H.cordata* is frequently used for its antiviral, antimicrobial and anti-inflammatory properties in traditional medicine (Park *et al*, 2005; Lu *et al*, 2006). However, scientific studies evaluating the antimicrobial effects of this herb against drug resistant pathogens are lacking.

In the present study, we investigated the antibacterial effects of HCWE against multi-drug resistant (MDR) *E.coli* isolates *in vitro*, to assess its potential for development as an antimicrobial agent. To the best of our knowledge, this is the first report of the antibacterial effect and transcriptional regulation of resistant genes by HCWE against MDR pathogens.

MATERIALS AND METHODS

Materials and reagents

The agar and broth used for this study were purchased from GE Hangwei Medical Systems (Beijing, China). The *H. cordata* was obtained from Yuan Cheng Medicine Company (Wuhan, China) and positively identified by the Department of Botany, Huazhong Agriculture University, China. The plant specimens were stored in the Department of Clinical Medicine, Huazhong Agriculture University Wuhan, China.

Water extraction of *Houttuynia cordata*

The water extraction of *Houttuynia cordata* was conducted following Kim *et al* (2008) with minor modifications. The

H. cordata was washed with deionized water; lyophilized and pulverized into powder form. Four grams of this HC powder was then extracted with 100 ml distilled water by stirring at room temperature for 8 hours. The supernatant was centrifuged at 5,000 rpm for 10 minutes and then filtered through Whatman No. 1 filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK). The resultant filtrate was concentrated with a rotary evaporator (Beijing, China) at 54°C at 10 rpm and the extract was dissolved in distilled water. Concentrations of 50, 100, 250, 400 and 500 mg/ml were prepared and sterilized using a Corning syringe filter of 0.2 μm size (Pall Life Sciences, Port Washington, NY) and stored at -20°C until use.

Bacterial strains

Eighteen MDR *E.coli* strains (1×10^8 CFU/ml) were obtained from stock culture isolated from free ranging Tibetan yaks during 2015-2016 and kept at the Department of Clinical Veterinary Medicine, HZAU Wuhan, China. MDR was determined based on the presence of the *AcrA* gene and phenotypic resistance to at least 3 different classes of antibiotics, including beta-lactams, trimethoprim, chloramphenicol, gentamicin, ofloxacin and tetracycline, following National Committee for Clinical Laboratory Standards guidelines (CLSI, 2014). Identification of the strains was performed using an API 20E (Api-bioMérieux Systems). *E.coli* ATCC 25922 was used as the quality control strain for MIC determination. *E.coli* BW5104 (expression at basal level) and *E.coli* ATCC-700603, were used as reference strains. All studied strains were maintained in a nutrient broth and stored in a refrigerator until used.

Antibacterial assay of *H. cordata* water extract

The antibacterial activity of the HCWE was determined using the agar well diffusion technique with slight modification (Peni *et al*, 2010). Briefly, the studied strains were inoculated onto Muller-Hinton Agar (MHA) and incubated at 37°C for 24 hours. After incubation, the nutrient broth, which had a McFarland turbidity of 0.5, was used to suspend the studied strain while MHA plates were inoculated with the prepared bacterial suspensions. For the agar well diffusion technique, a sterile cork borer was used to bore the surface of MHA plate with wells of 6 mm diameter. Zero point two milliliters of HCWE (500, 400, 250, 100 and 50 mg/ml) were placed in the wells. The test solution was allowed to diffuse into the agar for 1 hour at room temperature then incubated at 37°C for 18 hours. The wells without the HCWE or *E.coli* were used as controls. The zone diameter of inhibition (ZDI) around the test wells was observed and a total zone diameter of 12 mm was considered sensitive (CLSI, 2014).

Determining the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC of the HCWE was determined by using the tube dilution method (Bukar *et al*, 2010) with modifications. Briefly, 1 ml of the various concentrations of HCWE (500, 400, 250, 100 and 50 mg/ml) was added to 9 ml Muller-Hinton or nutrient broth. One milliliter of a standardized inoculum of the MDR *E.coli* was also added. This was then incubated at 37ºC for 2 hours using the lowest concentration of HCWE inhibiting visible growth; this was considered the MIC. Tubes without the HCWE or bacteria were incubated as controls. Each test was repeated in triplicate.

The antibacterial activity of HCWE

was determined on a fresh drug free solid medium by sub-culturing the tested dilution (100 μl) lacking visible growth for 24 hours. The lowest concentration of HCWE resulting in > 99.9% decrease in the initial inoculum was considered to be the minimal bactericidal concentration (MBC).

Reverse transcription quantitative-real time polymerase chain reaction (RT-qPCR)

The *Escherichia coli* strain BW5104 was grown in Mueller-Hinton broth at 37°C with graded sub-inhibitory concentrations of HCWE to the post-exponential growth phase. The RNA was harvested using Trizol reagent (Tian Gen, China) following the manufacturer's instructions. A final volume of 1 μg of total RNA was transcribed into the cDNA using the first-strand reverse transcription (RT) cDNA kit (Tian Gen, China) and 10% of the RT product was included in all the PCR reactions using already described primers sequences (Viveiros *et al*, 2007). The RT-qPCR (20 μl final volume) was performed in quadruplet with the Step One-Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) for the *AcrA* gene. Amplification was performed using the following thermal cycling parameters: 95°C for 10 minutes; 35 amplification cycles at 95°C for 8 seconds, 59°C for 30 secconds; and 72°C for 35 seconds. The PCR reaction system contained 10 μl of SYBR quantitative real-time polymerase chain reaction (qPCR) Mix (Transgen biotech, China), specific forward and reverse primers (1 μl each), 2 μl of cDNA, and nuclease-free water to give a total volume of 6 μl. The relative quantification of the genes was calculated using the delta Ct ($\Delta\Delta$Ct) method and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control to normalize the levels of expression among samples.

Statistical analyses

Data were expressed as means ± standard deviations (SD). A *p*-value < 0.05 was considered statistically significant. The independent Student's *t*-test was used to compare significant differences. All analyses were conducted using Stata 11 software (StataCorp LP, College Station, TX).

RESULTS

Antibacterial effects of the water extract of *Houttuynia cordata*

The MDR *E.coli* strains (1×10^8 CFU/ml) were incubated with different concentrations of *H. cordata* water extract (500, 400, 250, 100 and 50 mg/ml) in MH or nutrient broth for 24 hours at 37°C. Of the 18 MDR *E. coli* isolates tested, the highest ZDI of 29 mm (29.3±1.3), and 28 mm (28.1±1.6) were seen at concentrations of 500 mg/ml and 400 mg/ml, respectively, followed by 19 (19.4±1.3), 17 (16.7±0.7) and 13 (12.9±0.7) mm at 250, 100 and 50 mg/ml, respectively.

The MIC of the MDR *E.coli* isolates and *E.coli* standard strains by HCWE was 400 mg/ml, except in 2 isolates (Table 1). The HCWE had antibacterial activity against MDR *E.coli* strains in a dose-dependent for up to 24 hours. The MBL of HCWE against the tested *E.coli* clinical isolates and standard strains was 500 mg/ml. The control without HCWE continued to grow uncheck for 24 hours.

Transcriptional level of the *AcrA* gene measured by RT-qPCR

RT-qPCR analysis was performed to assess the transcriptional level of *AcrA* gene expression involved in the multidrug efflux pump system of *E.coli.* The *E.coli* BW5104 and ATCC 700603 was positive and negative for the *AcrA* gene,

Table 1
Antibacterial activity of the water extract of *Houttuynia cordata* (HCWE) against multi-drug resistant *Escherichia coli* isolates.

Strain	HCWE (mg/ml)				
	500	400	250	100	50
BW5104	-	-	+/ -	+	+
ATCC-700603	-	-	+/ -	+	+
E.coli TY 01	-	-	-	+	+
E.coli TY 21	-	-	+/ -	+	+
E.coli TY 49	-	-	+/ -	+/ -	+
E.coli TY 89	-	-	+/ -	+	+
E.coli TY 101	-	-	+/ -	+	+
E.coli TY 111	-	-	-	+	+
E.coli TY 165	-	-	+/ -	+	+
E.coli TY 166	-	-	+/ -	+	+
E.coli TY 167	-	-	+/ -	+	+
E.coli TY 199	-	-	+/ -	+/ -	+
E.coli TY 221	-	-	+/ -	+	+
E.coli TY 223	-	-	+/ -	+	+
E.coli TY 239	-	-	+/ -	+	+
E.coli TY 321	-	-	+/ -	+	+
E.coli TY 399	-	-	+/ -	+/ -	+
E.coli TY 401	-	-	+/ -	+	+
E.coli TY 402	-	-	+/ -	+	+
E.coli TY 432	-	-	+/ -	+	+

+, good growth; - , no growth; +/ - , slight growth.

respectively. All MDR *E.coli* isolates expressed the *AcrA* gene. After treatment with a sub-inhibitory level of HCWE, the expression of *AcrA* gene was inhibited in a dose dependent approach by the HCWE (Fig 1). The HCWE significantly inhibited the transcription of *AcrA* in the standard strain of *E.coli*. The transcriptional levels of *AcrA* in the *E.coli* BW5104 strain were decreased by 0.39-, 0.29- and 0.16- fold, when cultured with 25 mg/ml, 50 mg/ml, and 100 mg/ml HCWE, respectively. These results suggest the antibacterial activity of HCWE against MDR *E.coli* strains is related to the major multi-drug efflux pump, AcrAB-TolC.

DISCUSSION

Antibiotic resistance is a natural phenomenon. The presence of MDR organisms is one of the biggest challenges for researchers and practitioners for treating and preventing bacterial infections in the hospital environment. New antimicrobial agents are urgently needed to manage this problem. We investigated the antibacterial activity of HCWE against MDR *Escherichia coli.* The HCWE had antibacterial activity against the 18 tested MDR *E.coli* strains and the control *E.coli* BW5104 (expressing *AcrA* at basal level). Our findings are in line with the previous studies showing

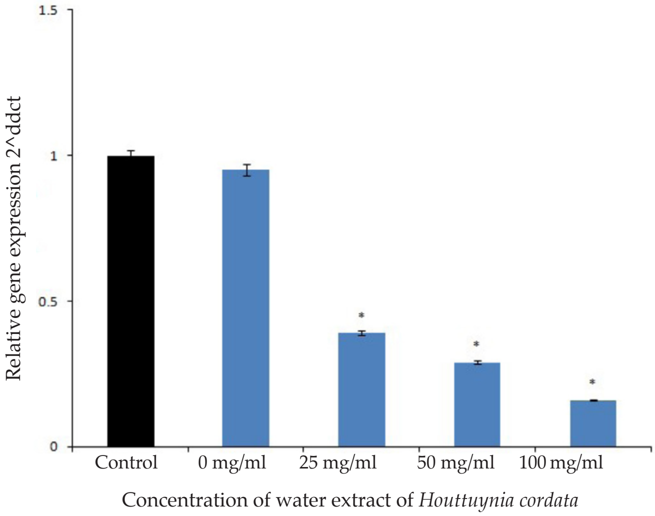

Fig 1–Relative expression of the *AcrA* gene in *E.coli* BW5104 treated with various concentrations of the water extract of *Houttuynia cordata*. Values represent the mean and standard error of three independent experiments. *: $p < 0.05$.

H. cordata has antibacterial and antiviral activity against variety of organisms (Kwon *et al*, 1996; Zhang *et al*, 2007; Meng *et al*, 2008). *H. cordata* plant extracts are widely used in homeopathic medicine as anti-cancer, antioxidant, anti-SARS, and anti-inflammatory drugs (Park *et al*, 2005; Lu *et al*, 2006). These data suggest *H. cordata* may be useful to be developed as a potential antimicrobial to treat *E.coli* and MDR *E.coli* infections.

To better understand the molecular mechanisms HCWE has against MDR *E.coli* strains, we used RT-qPCR analysis of one of the major multi-drug efflux pump, *AcrA* gene expression. The standard strain of *E.coli* (BW5104) expressing *AcrA* was used as a resistant gene for regulation of HCWE. The expression of *AcrA* was affected in a dose dependent manner by HCWE at the transcriptional level. Our results showed the HCWE is potentially useful to treat MDR *E.coli* infections since it can increase the susceptibility of drugs by effecting the expression of the *AcrA* gene. The periplasmic protein AcrA mediates the cooperation between AcrB and TolC and all three of these components are compulsory for effective transport, because hyper-susceptibility of *E.coli* to different substrates (basic dyes, detergents and antibiotics) is linked with disruption of any of these genes (Ma *et al*, 1993; Okusu *et al*, 1996). The antibacterial effect of HCWE may be linked to disruption of the inter-membrane AcrAB-TolC complex, increasing the hyper-susceptibility of *E.coli* to different antibiotics. HCWE is easily obtainable and an attractive therapeutic agent since this plant exist in most parts of the Asia and is known to modulate *E.coli* resistance.

In conclusion, HCWE may be a potential antimicrobial agent against MDR *E.coli*, even though an extensive clinical trial is needed to evaluate its therapeutic efficacy *in vivo*. Further studies are needed to assess the potential use of HCWE against other MDR pathogens.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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