

RESEARCH ARTICLE



Modified Zhibai Dihuang pill alleviated urinary tract infection induced by extended-spectrum β -lactamase *Escherichia coli* in rats by regulating biofilm formation

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ABSTRACT

Context: Zhibai Dihuang pill (ZD), a traditional Chinese medicine nourishes Yin and reduces internal heat, is believed to have therapeutic effects on urinary tract infections (UTIs).

Objective: To explore the effects and mechanism of modified ZD (MZD) on UTI induced by extended-spectrum β -lactamase (ESBLs) *Escherichia coli*.

Materials and methods: Thirty Sprague-Dawley rats were randomly divided into control, model (0.5 mL 1.5×10^8 CFU/mL ESBLs *E. coli*), MZD (20 g/kg MZD), LVFX (0.025 g/kg LVFX), and MZD + LVFX groups (20 g/kg MZD + 0.025 g/kg LVFX), $n = 6$. After 14 days of treatment, serum biochemical indicators, renal function indicators, bladder and renal histopathology, and urine bacterial counts in rats were determined. Additionally, the effects of MZD on ESBLs *E. coli* biofilm formation and related gene expression were analyzed.

Results: MZD significantly decreased the count of white blood cells (from 13.12 to 9.13), the proportion of neutrophils (from 43.53 to 23.18), C-reactive protein (from 13.21 to 9.71), serum creatinine (from 35.78 to 30.15), and urea nitrogen (from 12.56 to 10.15), relieved the inflammation and fibrosis of bladder and kidney tissues, and reduced the number of bacteria in urine (from 2174 to 559). In addition, MZD inhibited the formation of ESBLs *E. coli* biofilms (2.04-fold) and decreased the gene expressions of *luxS*, *pfS* and *ompA* (1.41–1.62-fold).

Discussion and conclusion: MZD treated ESBLs *E. coli*-induced UTI inhibited biofilm formation, providing a theoretical basis for the clinical application of MZD. Further study on the clinical effect of MZD may provide a novel therapy option for UTI.

ARTICLE HISTORY

Received 29 September 2022

Revised 4 March 2023

Accepted 2 April 2023

KEYWORDS

Traditional Chinese medicine; antibacterial; bacteria biofilm; quorum sensing

Introduction

Urinary tract infections (UTIs) are infectious diseases caused by the abnormal reproduction of various pathogenic microorganisms in the urinary tract (Gupta et al. 2017). Complicated UTIs often lead to prolonged infection and eventually cause renal function damage and serious health hazards (Tandogdu and Wagenlehner 2016; Millner and Becknell 2019). Uropathogenic *Escherichia coli* (UPEC) is a typical bacterial UTI pathogen (Herrmann et al. 2002). Various virulence factors in the pathological group of UPEC provide more opportunities for bacterial survival (Behzadi et al. 2020). Currently, the main intervention measures for the clinical treatment of UTI are antibacterial drugs (Flores-Mireles et al. 2019). Approximately 60% of antibiotics are β -lactams, which have various chemical structures and are often used to treat different types of bacterial infectious diseases (Behzadi et al. 2020). The application of antimicrobials has led to β -lactamase production, resulting in drug-resistant bacterial strains (Issakhanian and Behzadi 2019). In a clinical study, 86% of ESBL-producing strains were isolated from UPEC, and there was neither a pattern of resistance nor ESBL production in UTI (Khonsari et al. 2021). The presence of extended-spectrum

β -lactamase (ESBLs) *E. coli* makes the most common antimicrobial agent less effective in treating UTIs (Zowawi et al. 2015). Therefore, there is an urgent clinical need for alternative treatment options that target UTI pathogenesis.

Bacterial biofilms are a special survival mode for bacteria to adapt to the environment (Vuotto et al. 2017), which can resist host immunity and reduce antibiotic sensitivity (Pavithra and Doble 2008). When human body resistance is decreased, the remaining bacteria in the biofilm proliferate and release again, resulting in repeated attacks and prolonged infections (Tremblay et al. 2014). Studies have shown that biofilms are positively correlated with ESBL production and play a key role in UTIs (Shrestha et al. 2019). Destruction of this natural barrier of bacterial biofilm may be an effective way to overcome bacterial resistance and treat chronic clinical infections (Kostakioti et al. 2013).

An increasing number of studies have confirmed that traditional Chinese medicine (TCM) can effectively improve clinical symptoms and reduce the UTI recurrence rate (Yu et al. 2017; Li et al. 2019; Liu et al. 2019). TCM compounds have multi-directionality, multi-layer and multi-target characteristics, which make it difficult for bacteria to simultaneously produce multiple

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mutations against multiple antibacterial components (Zhang et al. 2013). Zhibai Dihuang pill (ZD) is a traditional compound TCM that nourishes Yin and reduces internal heat and is used to treat Yin-deficiency-heat (YDH) syndrome (Yi et al. 2020). Recently, ZD has been used to treat the accompanying symptoms of hyperthyroidism, nephrotic syndrome, UTI, and prostatitis (Liu et al. 2018). According to the literature, we adjusted ZD to obtain a compound TCM with a better curative effect against UTI. By observing the effects of the components of modified ZD (MZD) on the blood biochemical indices, histopathology, and urine bacterial content of the UTI rat model induced by ESBLs *E. coli*, and the effects of the medicated serum on the formation of ESBLs *E. coli* biofilms, the role and possible mechanism of MZD against ESBLs *E. coli* to treat UTI were explored.

Materials and methods

Preparation of MZD

The MZD used in this study was prepared by experts of the Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University, conformed with the compatibility principle, and was prepared uniformly by the pharmacy department of the hospital. Briefly, MZD is the fusion of Chinese classical prescriptions Zhibai Dihuang Pill and Bazheng Powder, including 15 g *Phellodendron amurense* Rupr. (Rutaceae), 10 g *Anemarrhena asphodeloides* Bunge (Liliaceae), 10 g *Alpinia oxyphylla* Miq. (Zingiberaceae), 12 g *Cuscuta chinensis* Lam. (Convolvulaceae), 15 g *Plantago asiatica* L. (Plantaginaceae), 12 g *Polygonum aviculare* L. (Polygonaceae), 12 g *Dianthus superbus* L. (Caryophyllaceae), 12 g *Gardenia jasminoides* J.Ellis (Rubiaceae), 20 g *Astragalus mongholicus* Bunge (Fabaceae), 15 g *Poria cocos* (Schw.) Wolf. (Polyporaceae), 12 g *Atractylodes macrocephala* Koidz. (Asteraceae), 10 g *Scutellaria baicalensis* Georgi (Lamiaceae) and 12 g *Achyranthes bidentata* Blume (Amaranthaceae). Water was added to the MZD mixture at a 10:1 ratio (water: plant material, v/w). After 30 min of soaking in water, the plant material was boiled twice for 1 h each and filtered. The filtrate was then concentrated to 3.33 g/mL using a rotary evaporator (RF02C, Shanghai Kangsheng Industrial Co., Ltd., China) and stored at 4 °C until use.

ESBLs *E. coli* culture

The ESBLs *E. coli* was provided by the Clinical Laboratory of the Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University. The cryopreserved ESBLs *E. coli* strain was regenerated by inoculation onto an agar plate and incubated in an incubator at 37 °C for 18 h. The regenerated bacterial strain was picked and inoculated into a lysogeny broth (LB) liquid medium and cultured at 37 °C at 150 rpm for 16–24 h (Hou et al. 2019), and the bacterial solution concentration was adjusted to 1.5×10^8 CFU/mL as seed liquid.

Animal experiments

Thirty 8-week-old specific-pathogen-free female Sprague-Dawley (SD) rats, weighing 200–220 g, were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (license number: SCXK (Sichuan) 2020-030). Rats were kept at a room temperature of 22 °C, with a humidity of 50–60%, with good ventilation, under a 12 h light/dark cycle, and had free access to food and water. This study was performed in strict accordance with the Guide

for the Care and Use of Laboratory Animals of the National Institutes of Health (Bayne 1996). All experimental protocols of this study were approved by the Animal Ethics Committee of Southwest Medical University (NO.:20221118-018; Luzhou, China), and all efforts were made to minimize animal suffering and reduce the number of animals used. The rats were randomly divided into five groups ($n=6$): control group, model group, MZD group, LVFX group and MZD + LVFX group. After 24 h of water inhibition, rats were anesthetized by intraperitoneal injection of 0.8 mg/100 g 1% pentobarbital. The rats were supine, the urethral intubation was inserted into the urethra, and the lower abdomen of the rats was pressed to empty the urine. Then 0.5 mL 1.5×10^8 CFU/mL ESBLs *E. coli* was slowly injected into the bladder with a 1 mL sterile syringe to establish a rat model of UTI (Johansson et al. 1987). The control group was injected with an equal volume of sterile normal saline solution. After the establishment of the model, each group was treated with corresponding drugs for 14 days (Xue et al. 2017). The MZD group was intragastrically administered 20 g/kg MZD, the LVFX group was intragastrically administered 0.025 g/kg LVFX, and the MZD + LVFX group was intragastrically administered 20 g/kg MZD and 0.025 g/kg/d LVFX, while the control and model groups were intragastrically administered the same dose of normal saline every day. After treatment, the rats were sacrificed by cervical dislocation, urine was collected for bacterial colony culture, and serum was collected for subsequent microbial experiments. Additionally, blood biochemical indexes were measured, and a histopathological examination of the kidney and bladder was performed.

Serum biochemical indicators assays

Blood was collected from the rats, and biochemical indices, such as white blood cells (WBC), the proportion of neutrophils (NEUT%), and renal function indices serum creatinine (Scr) and urea nitrogen (BUN) were determined a HITACHI 7600-020 automatic biochemistry analyzer (Shandong, China). Additionally, blood samples were centrifuged at 3000 rpm for 15 min to obtain serum. Serum C-reactive protein (CRP) expression was determined using enzyme-linked immunosorbent assay (ELISA). All operations were performed in strict accordance with the manufacturer's instructions and were repeated at least thrice for each sample.

Histopathological staining

Bladder and kidney tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, paraffin-embedded, and sectioned at 3 μm. Histopathological changes, inflammation, and fibrosis were observed by HE and Masson staining. Sections of bladder and kidney tissues were dewaxed with xylene and ethanol gradient and then stained with hematoxylin and eosin (HE) or Masson staining (Ozawa and Sakaue 2020). Histological analyses were performed using a light microscope (Nikon). Each tissue section was evaluated histologically, and images were taken at $\times 400$ magnification.

Urine bacterial culture

The urine of different treatment rats was extracted from the bladder with sterile needles, and continuously diluted to 10^{-4} , coated with 100 μL diluent on the plate, and cultured upside

down in an incubator at 37 °C for 24 h, then the number of bacterial colonies was counted using light microscopy (Nikon).

Antibiotic resistance and MS-MZD sensitivity

Experimental antibiotics, including ampicillin (AMP, CAS: 7177-48-2), cefazolin (CFZ, CAS: 25953-19-9), cefotaxime (CTX, CAS: 64485-93-4), gentamicin (GEN, CAS: 1403-66-3), tetracycline (TE, CAS: 60-54-8), chloramphenicol (CAP, CAS: 56-75-7), and levofloxacin (LVFX, CAS: 100986-85-4), were all obtained from McLean Biochemical Technology Co., Ltd. (Shanghai, China). Stock solutions of experimental antibiotics were prepared at a concentration of 10.24 mg/mL, filtered, and sterilized at 0.22 µm. Medicated serum of MZD (MS-MZD) was obtained from previous animal experiments. In brief, abdominal aorta blood was collected 1 h after the last drug administration in rats and centrifuged at 3000 rpm for 15 min to separate the serum, which was filtered and sterilized a 0.22 µm before use. The minimal inhibitory concentration (MIC) of MS-MZD against ESBLs *E. coli* was determined using the broth microdilution method (Jorgensen and Ferraro 2009), which was recommended by the American Society for Clinical and Laboratory Standards (CLSI) for drug sensitivity testing. The MS-MZD dilution times were 1:1 to 1:2048, and the concentrations of antibiotics were 256–0.125 µg/mL, both of which were double-gradient dilutions.

Crystal violet dyeing

To determine the effect of MS-MZD on *E. coli* biofilm formation, five groups were randomly set up: control, negative serum control (SHRN), medicated serum of the MZD (MS-MZD, MIC₅₀), levofloxacin (LVFX, MIC₅₀), and MS-MZD + LVFX (MIC₅₀). ESBLs *E. coli* was cultured overnight in LB liquid medium at 37 °C, and the concentration was adjusted to 1 × 10⁹ CFU/mL. Then, 10 µL of ESBLs *E. coli* solution and 200 µL of media with different drugs were added to 96-well plates (Coffey and Anderson 2014). The culture solution was added to the control group, and each strain was made in three duplicate wells, and the culture was static at 37 °C. After 72 h of culture, 50 µL of 0.25 g/L crystal violet dye was added and stained at room temperature for 15 min. After rinsing with normal saline and air drying, 150 µL of 95% ethanol was added to each well and decolorized at room temperature for 10 min. The absorbance of each well was measured at 570 nm wavelength using a microplate reader (Bio-Rad Laboratories, Inc.).

Scanning electron microscopy (SEM)

Ultrastructural changes in the biofilm were observed using SEM (Leoney et al. 2020). Approximately 10⁷ CFU/mL ESBLs *E. coli* solution was inoculated into the culture medium containing the different drugs. After incubation for 10 h, the supernatant was

discarded after centrifugation at 5000 rpm for 5 min, 2.5% dialdehyde was added for fixation at room temperature for 48 h, and 1% osmic acid was added for 4 h, washed 10 times with PBS, dehydrated with an ethanol gradient, and replaced with acetone 10 times. The centrifugally concentrated liquid was dried using a CO₂ critical point dryer (Emitech K850, Quorum, UK), and the dried bacterial powder was observed by SEM (FEI Quanta 200 F, Shanghai, China).

Real-time quantitative polymerase chain reaction (RT-PCR)

The levels of *luxS*, *pfS* and *ompA* in ESBLs *E. coli* were measured by RT-PCR (Sun et al. 2019). Briefly, the total RNA of ESBLs *E. coli* was obtained using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was prepared using HiScript III^{RT} SuperMix for qPCR (with gDNA wiper) (Vazyme Biotech Co., Ltd.). qPCR was performed using ChamQTM SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) with a CFX96TM RT-PCR detection system (Bio-Rad Laboratories, Inc.) at a final volume of 20 µL using the standard protocol as follows: 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 60 °C for 30 s, 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Relative *luxS*, *pfS*, and *ompA* mRNA expression levels were determined using the comparative 2^{-ΔΔC_t} method with *gapA* as an internal reference gene. Primer sequences used in this study are listed in Table 1.

Statistical analysis

All data were analyzed using SPSS software (version 22.0; IBM Corp.) and are shown as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison between the groups, Dunnett's test was used for paired comparison (*post hoc*), and *p* < 0.05 was considered to indicate a statistically significant difference.

Results

Effects of MZD on UTI induced by ESBLs *E. coli* in rats

To investigate the effects of MZD on UTI in rats induced by ESBLs *E. coli*, the blood biochemical indices and renal function indices of rats were analyzed. Compared with the control group, WBC, NEUT%, CRP, Scr, and BUN of rats in the model group were significantly increased, showing an inflammatory response and renal function damage (*p* < 0.001, Table 2). Rats in the MZD, LVFX, and MZD + LVFX groups exhibited significantly lower WBC, NEUT%, CRP, Scr, and BUN than those in the model group, suggesting that both MZD and LVFX can improve the inflammatory response and renal function damage in rats with UTI (*p* < 0.001, Table 2).

The histopathological analysis of the bladder and kidney was performed, and as shown in Figure 1(A), in the model group,

Table 1. The primer sequences used for real-time PCR assay.

Genes	Function	Length (bp)	Primers	Sequence	Reference
<i>luxS</i>	quorum sensing regulatory gene	116	<i>luxS-F</i>	5'-TGCCACACTGGTAGACGTTTC-3'	(Chen et al. 2018)
			<i>luxS-R</i>	5'-TGATTGGTACGCCAGATGAG-3'	
<i>pfS</i>	quorum sensing regulatory gene	169	<i>pfS-F</i>	5'-CGGCAACAGCCAGGAATCA-3'	(Sun et al. 2019)
			<i>pfS-R</i>	5'-GCGAAAATCCGCCAACACTT-3'	
<i>ompA</i>	outer membrane protein regulates gene	152	<i>OmpA-F</i>	5'-TCCAGAGCAGCCTGACCTTC-3'	(Sun et al. 2019)
			<i>OmpA-R</i>	5'-GCTGAGCCTGGGTGTTCTC-3'	
<i>gapA</i>	housekeeping gene	104	<i>gapA-F</i>	5'-GAAATGGGACGAAGTTGGTG-3'	(Chen et al. 2018)
			<i>gapA-R</i>	5'-AACCACTTCTTCGCCACCA-3'	

Table 2. Blood biochemical and renal function indexes of rats.

	WBC ($\times 10^9/L$)	NEUT% (%)	CRP ($\mu g/mL$)	Scr ($\mu mol/L$)	BUN (mmol/L)
Control	5.13 \pm 1.01	20.15 \pm 1.33	9.01 \pm 0.23	26.56 \pm 2.98	9.25 \pm 1.25
Model	13.12 \pm 1.23***	43.53 \pm 2.15***	13.21 \pm 1.03***	35.78 \pm 2.35***	12.56 \pm 1.21***
MZD	9.13 \pm 0.98###&	23.18 \pm 0.96###	9.71 \pm 1.12##	30.15 \pm 1.96###	10.15 \pm 1.08###
LVFX	8.45 \pm 0.56###	22.98 \pm 1.56###	10.15 \pm 2.10##	27.56 \pm 1.66###	9.05 \pm 0.95###
MZD + LVFX	7.33 \pm 0.79###	21.01 \pm 1.32###	9.32 \pm 1.56###	26.98 \pm 1.26###	9.43 \pm 1.19###

Note. Data are shown as mean \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with the control group; # p < 0.05, ## p < 0.01, ### p < 0.001, compared with the model group; & p < 0.05, && p < 0.01, &&& p < 0.001, compared with the MZD + LVFX group. MZD: modified Zhibai Dihuang pill; LVFX: levofloxacin; WBC: white blood cells; NEUT%: proportion of neutrophils; CRP: C-reactive protein; Scr: serum creatinine; BUN: urea nitrogen.

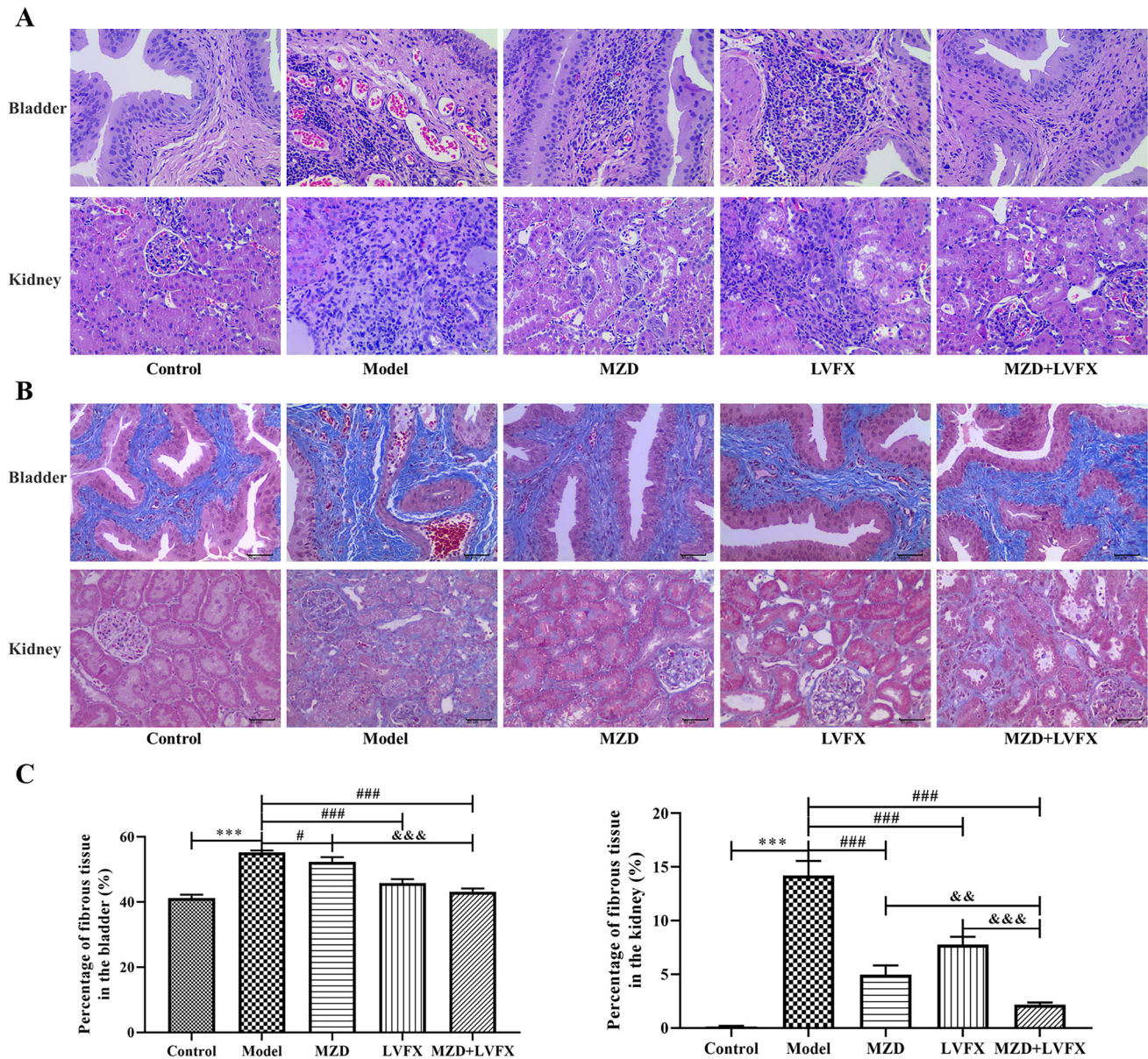


Figure 1. Effects of MZD on tissue morphology of UTI induced by ESBLs *E. coli* in rats. (A) Representative images showing HE staining of bladder and kidney in rats. Magnification, $\times 400$. (B) Representative images showing Masson staining of bladder and kidney in rats. Magnification, $\times 400$. (C) Percentage of fibrotic tissue in the bladder and kidney of rats stained by Masson. Data were shown as mean \pm SD. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with the control group; # p < 0.05, ## p < 0.01, ### p < 0.001, compared with the model group; & p < 0.05, && p < 0.01, &&& p < 0.001, compared with the MZD + LVFX group. MZD: modified Zhibai Dihuang pill; LVFX: levofloxacin.

there were vascular hyperplasia, dilation, and inflammatory cell infiltration in the bladder tissue lamina propria, the thickening and edema of the muscle and outer membrane were obvious,

and blood vessels, lymphocytes, neutrophils, and macrophages were visible in the proliferative fibrous connective tissue. For kidney tissue, the local glomerular morphology and structure

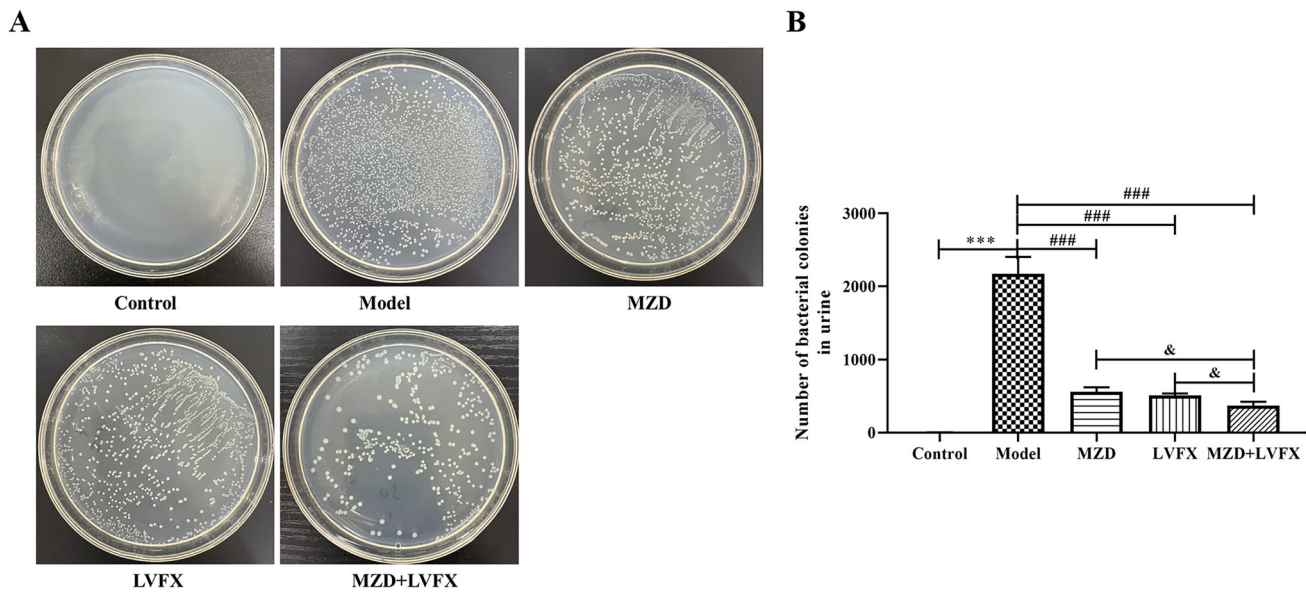


Figure 2. Urine bacterial culture of rats with UTI caused by ESBLs *E. coli*. (A) Representative plate showing the bacterial culture of urine. (B) The number of bacterial culture colonies in the urine of rats. Data are shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, compared with the model group; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$, compared with the MZD + LVFX group. MZD: modified Zhibai Dihuang pill; LVFX: levofloxacin.

were fuzzy, the renal tubules were denatured and necrotic, the epithelial cell nuclei were flattened, lysed, or irregular, some lumens were slightly dilated, the surrounding interstitial fibrosis was more obvious, a large number of fibroblasts were hyperplastic, and lymphocyte infiltration was observed in the necrotic area. Inflammatory infiltration and necrosis of the bladder and kidney tissue were alleviated in the MZD, LVFX, and MZD + LVFX groups. The therapeutic effect of MZD combined with LVFX was significantly better than that of a single alone. There was no obvious degeneration, necrosis, or inflammatory cell infiltration in the bladder and kidney tissues of the MZD + LVFX group (Figure 1(A)). Additionally, as shown in Figure 1(B), Masson staining was used to analyze bladder and kidney fibrosis. The percentage of fibrous tissue in the bladder and kidney in rats of the model group was significantly higher than that in the control group, fibrosis was decreased after MZD or LVFX treatment, and the curative effect of the MZD + LVFX group was better than that of the MZD or LVFX groups ($p < 0.05$, Figure 1(C)).

Urine bacterial culture of rats with UTI caused by ESBLs *E. coli*

Bladder urine from rats in each group was collected, cultured on agar plates, and counted. The results showed that there were no bacteria in the control group, and urine bacteria in the model group were significantly increased. MZD, LVFX, and MZD + LVFX eliminated urine bacteria in the model group ($p < 0.05$, Figure 2), and the effect of MZD + LVFX was significantly better than that of MZD or LVFX ($p < 0.05$, Figure 2).

Antibiotic resistance and MS-MZD sensitivity

As shown in Table 3, ESBLs *E. coli* showed different degrees of resistance to seven antibiotics. CFZ, CTX, GEN, CAP, and LVFX showed relatively low MICs of 1, 0.25, 16, 8, and 2 $\mu\text{g/mL}$, respectively, indicating good antibacterial effects. AMP and TE

Table 3. MIC ($\mu\text{g/mL}$ or dilution ratio) of ESBLs *E. coli*.

	Medicine							
	AMP	CFZ	CTX	GEN	TE	CAP	LVFX	MS-MZD
MIC ($\mu\text{g/mL}$)	256	1	0.25	16	128	8	2	1:2

Note. AMP: ampicillin (CAS: 7177-48-2); CFZ: Cefazolin (CAS: 25953-19-9); CTX: cefotaxime (CAS: 64485-93-4); GEN: gentamicin (CAS: 1403-66-3); TE: tetracycline (CAS: 60-54-8); CAP: chloramphenicol (CAS: 56-75-7); LVFX: levofloxacin (CAS: 100986-85-4); and MS-MZD: medicated serum of modified Zhibai Dihuang pill.

showed MIC levels of 256 and 128 $\mu\text{g/mL}$, respectively. The MIC of MS-MZD against ESBLs *E. coli* strains was a dilution ratio of 1:2 (Table 3).

Effects of MS-MZD on the formation of ESBLs *E. coli* biofilm

Biofilm formation further promotes the development and spread of bacteria, which are important causes of persistent bacterial infections. To further clarify the effects of MZD on ESBLs *E. coli*, we analyzed the biofilm-forming ability of ESBLs *E. coli*. As shown in Figure 3(A), crystal violet staining showed that the biofilm formation ability in the SHRN group was the same as that in the control group ($p > 0.05$, Figure 3(A)), indicating that serum did not affect biofilm formation. The biofilm formation ability of ESBLs *E. coli* in the MS-MZD, LVFX, and MS-MZD + LVFX groups was significantly lower than that in the model group, and the MS-MZD + LVFX group had the lowest value, which was lower than that in the MS-MZD or LVFX groups ($p < 0.01$, Figure 3(A)). Additionally, the ultrastructure of ESBLs *E. coli* was observed by SEM, as shown in Figure 3(B), ESBLs *E. coli* in the control and SHRN group showed a complete and smooth elongated structure. After different treatments, the cell lengths of the MS-MZD, LVFX, and MS-MZD + LVFX groups significantly increased, and all groups showed a certain bacteriostatic effect.

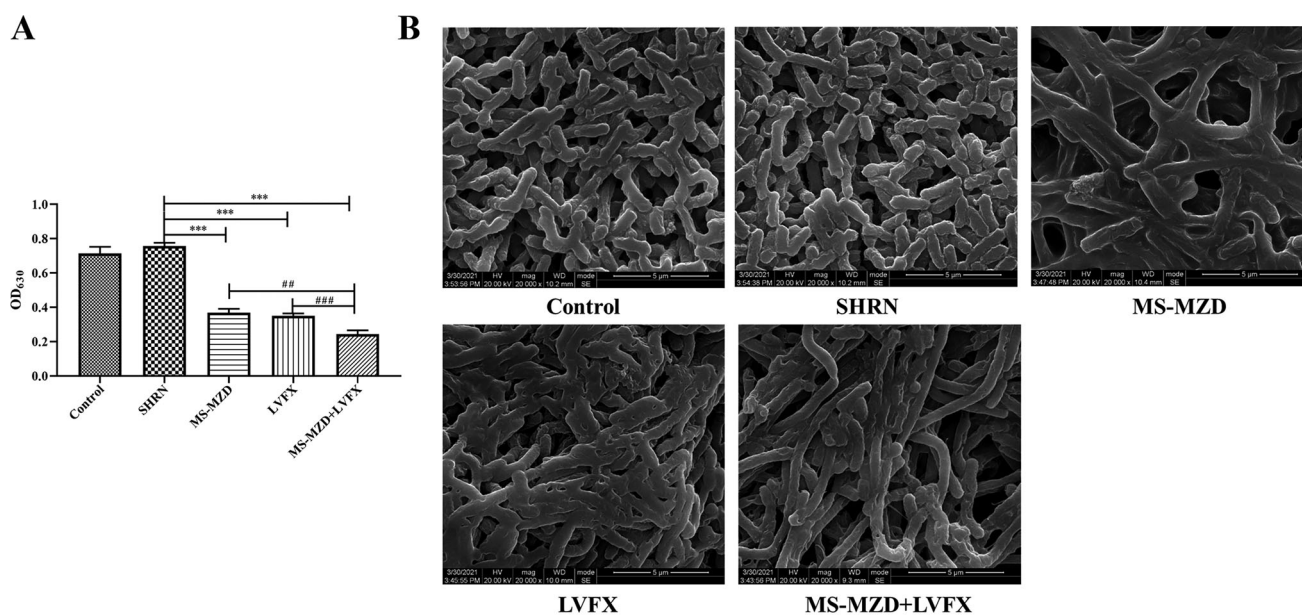


Figure 3. Comparison of the biofilm formation ability of ESBLs *E. coli*. (A) The biofilm formation was quantitatively determined by crystal violet staining. (B) Representative images showing biofilm formation observed by laser scanning electrolysis. Magnification, $\times 20,000$. Data are shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the SHRN group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, compared with the MS-MZD + LVFX group. SHRN: negative serum control group; MS-MZD: medicated serum of modified Zhibai Dihuang pill group; LVFX: levofloxacin group.

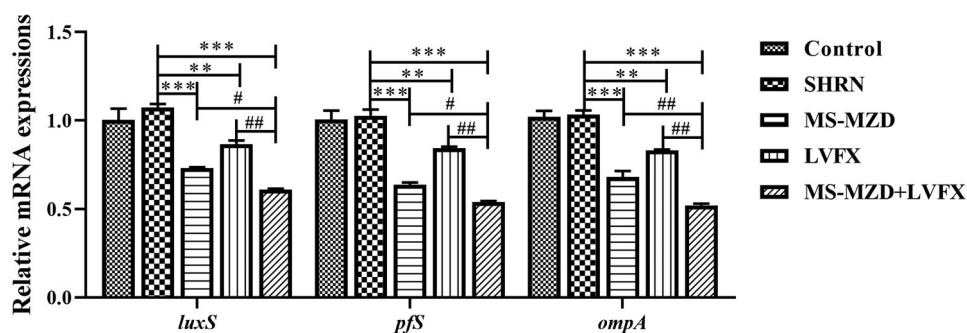


Figure 4. Effects of MS-MZD on the relative gene expression of *luxS*, *pfS*, and *ompA* in ESBLs *E. coli*. Data are shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the SHRN group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, compared with the MS-MZD + LVFX group. SHRN: negative serum control group; MS-MZD: medicated serum of modified Zhibai Dihuang pill; LVFX: levofloxacin.

Effects of MS-MZD on the gene expression of ESBLs *E. coli*

The *luxS*, *pfS*, and *ompA* expression in bacteria are closely associated with bacterial biofilm formation. In this study, the gene expression of *luxS*, *pfS*, and *ompA* in the SHRN group was not significantly different from that in the control group ($p > 0.05$, Figure 4), suggesting that the serum did not affect gene expression. MS-MZD and LVFX significantly reduced the gene expression of *luxS*, *pfS*, and *ompA*, and the difference was statistically significant compared to that of the SHRN group ($p < 0.05$, Figure 4). The MS-MZD + LVFX group further reduced the gene expression of *luxS*, *pfS*, and *ompA* compared with the MS-MZD or LVFX alone groups, and the effects were better than those of the single group ($p < 0.05$, Figure 4).

Discussion

UTIs are among the most common chronic and recurrent infectious diseases caused by various microbial pathogens (Choe et al. 2018). Studies have shown that 13 different microbial agents are involved in UTIs, including *Escherichia coli*, *Streptococcus*,

Klebsiella, *Staphylococcus*, etc. (Behzadi and Behzadi 2008). Among these, the UPEC strain is the main strain causing different types of UTI and contains various virulence genes (Behzadi and Behzadi 2017; Hozzari et al. 2020). The presence of these virulence genes led to the rapid growth of ESBLs and metal- β -lactamase (MBL) of UPEC origin, but studies have shown that there is no significant correlation between the characteristics of ESBL production and bacterial antibiotic resistance (Khonsari et al. 2021). With the emergence of bacterial resistance, the use of antibiotics to treat complex UTIs caused by drug-resistant bacteria has become challenging (Waller et al. 2018). Due to the poor efficacy of antibiotic therapy strategies in treating UTIs associated with urinary pathogens, several new alternatives have been developed, including anti-adhesion therapy (Sarshar et al. 2020), anti-biofilm formation, etc. In modern medicine, TCM has a certain effect on the treatment of infectious diseases, and there are few reports of adverse reactions (Ma et al. 2019). In this study, ESBLs *E. coli* was used to construct a UTI rat model. After intragastric administration of MZD or LVFX in UTI rats, the renal function indices were significantly improved, pathological damage of the bladder and kidney was alleviated, and the

number of bacteria in the urine was significantly inhibited. Additionally, MZD medicated serum significantly inhibited the formation of ESBLs *E. coli* biofilms and significantly decreased the *luxS*, *pfs*, and *ompA* gene expression in ESBLs *E. coli*, indicating that MZD may be an effective drug for UTI treatment.

UTI belongs to the category of drench syndrome in TCM, and TCM has great advantages in treating this disease due to its multi-target characteristics, integrity, and small toxicity and side effects (Liu et al. 2017). MZD, including *Phellodendri chinensis cortex*, *Anemarrhenae rhizoma*, *Alpiniae oxyphyllae fructus*, *Cuscutae semen*, *Plantaginis semen*, *Polygoni avicularis herba*, *Dianthi herba*, *Gardeniae fructus*, *Astragali radix*, *Poria*, *Atractylodis macrocephalae rhizoma*, *Scutellariae radix* and *Achyranthis bidentatae radix*, were used in this study. Modern pharmaceutical research shows that *Phellodendri chinensis cortex* has an inhibitory effect on pathogenic bacteria such as *dysentery Bacillus* and *Staphylococcus aureus* (Sun et al. 2019), *Anemarrhenae rhizoma* has an inhibitory effect on *E. coli*, *Proteus genus* and other bacteria (Park et al. 2003), *Dianthi herba* has strong inhibitory activities against urogenital *Chlamydia trachomatis* (Li et al. 2000), *Gardeniae fructus* can inhibit *Helicobacter pylori* infection (Chang et al. 2017), *Scutellariae radix* protects mice from methicillin-resistant *Staphylococcus aureus* infection (Shi et al. 2020). Additionally, *Cuscutae semen*, *Plantaginis semen*, *Polygoni avicularis herba*, *Astragali Radix*, *Poria*, *Atractylodis macrocephalae rhizoma*, and *Achyranthis bidentatae radix* are commonly used to tonify the kidney and can significantly improve renal function (Zhao et al. 2013; Zhang et al. 2014; Saremi et al. 2018; Wang et al. 2021; Zhao et al. 2021). The combination of these TCM accords with the idea of TCM syndrome differentiation treatment, which can not only treat the pathogen but also improve the symptoms and regulate the immunity of the body (Barrett et al. 2015). This study found that MZD had a good therapeutic effect on UTI caused by ESBLs *E. coli*, mainly manifested as improved renal function, alleviated inflammation and fibrosis of the bladder and kidney tissue, and significantly reduced the number of bacteria in the urine of rats, showing a strong antibacterial effect.

Gram-negative bacteria communicate with each other through the production and perception of diffused signaling molecules, a phenomenon known as quorum sensing (QS), in which bacterial biofilms play an important role in regulating many bacterial activities (Kai 2018). The structural characteristics of bacterial biofilms make them insensitive to antibiotics and the immune system, and biofilm formation can aggravate antibiotic resistance and make it difficult to treat bacterial infections (Chen and Wen 2011). Bacterial biofilm formation inhibition is considered a potential target for antibacterial drugs (Rabin et al. 2015). Studies have shown that Yunnan Baiyao can affect biofilm formation by *Pseudomonas aeruginosa*, inhibiting the virulence of bacteria (Zhao et al. 2013). Citrus flavonoids can inhibit biofilm formation and bacterial cell-cell signal transduction in *E. coli* (Vikram et al. 2010). Numerous studies have shown that TCM has antibacterial effects and can inhibit biofilm formation (Zhang et al. 2020). This study also found that MZD significantly inhibits the formation of ESBLs *E. coli* biofilms. Additionally, the formation of bacterial biofilms is regulated by various signaling pathways, and the QS system is one of the regulatory mechanisms closely associated with the pathogenicity of pathogens (Muhammad et al. 2020). In *E. coli*, QS inter-acoustic signaling molecules secreted by the bacteria are transported into the cells by *luxS* and then phosphorylated. Signaling molecules bind to the receptor protein *luxS* to regulate the formation and structure

of biofilms (Williams 2002). Studies have shown that the inactivation of *luxS* and *pfs* significantly reduces the ability of *E. coli* to generate QS and induce biofilm formation (Wang et al. 2016). Moreover, *ompA* is a major regulatory gene for the formation of outer membrane proteins in *E. coli*, which regulates biofilm formation in *E. coli* and is closely related to the virulence of *E. coli* (Ma and Wood 2009). In this study, it was found that medicated serum of MZD significantly reduced the *luxS*, *pfs*, and *ompA* gene expressions, and showed an inhibitory effect on QS. The combined effect of MS-MZD and LVFX was better, suggesting that MZD might play a therapeutic role by inhibiting bacterial biofilm formation.

Conclusions

This study demonstrated that MZD could improve renal function, alleviate inflammation and fibrosis of bladder and kidney tissue, reduce the number of bacteria in urine, and show a good therapeutic effect on UTI induced by ESBLs *E. coli*. In addition, MZD medicated serum inhibited the formation of ESBLs *E. coli* biofilms and reduced the expression of *luxS*, *pfs*, and *ompA*, suggesting that MZD plays a therapeutic role in rats with UTI caused by ESBLs *E. coli* by inhibiting bacterial biofilm formation.

Author contributions

Kaifa Chen and Xin Liu conceived and designed the experiments, Kaifa Chen, Yongsheng Zhu and Hongwei Su performed the experiments, Kaifa Chen and Xin Liu analyzed the data, Hao Jiang contributed reagents/materials/analysis tools. Kaifa Chen and Xin Liu wrote the manuscript. All authors read and approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Scientific research Fund of Luzhou Science and Technology Bureau under Grant [No. 2019-JY]-67].

Data availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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