



Article A Screening Model of Antibacterial Agents Based on Escherichia coli Cell-Division Protein

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Abstract: Pathogenic Escherichia coli cannot be killed by most antibiotics (including colistin, a last-resort drug) due to the rapid development of antibiotic resistance. A highly conserved prokaryotic mitotic protein, filamenting temperature-sensitive protein Z (FtsZ) with GTPase activity, plays a key role in cell division and has become a promising target for screening novel antibacterial agents. In this study, the amplified *ftsZ* gene was inserted into cloning/expression vectors and recombinantly produced in E. coli; the recombinant FtsZ protein was purified by the Ni²⁺-NTA affinity column and then was used to screen for natural antibacterial agents. The results showed that the ftsZ gene with a size of 1170 bp was successfully amplified from E. coli and inserted into the pET-28a expression vector. After induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), FtsZ was expressed in E. coli BL21 as inclusion bodies. After purification, the recombinant FtsZ protein showed GTPase activity. The highest GTPase activity (0.998 nmol/mL/min) of FtsZ was observed at a GTP concentration of 1.25 mM. Several alkaloids were screened by a constructed model of FtsZ inhibitors. Sanguinarine chloride exhibited higher antibacterial activity against E. coli and Salmonella enteritidis (with minimum inhibitory concentrations (MICs) of 0.04-0.16 mg/mL and minimum bactericidal concentrations (MBCs) of 0.16–0.32 mg/mL) than tetrandrine (0.16–0.32 mg/mL) and berberine hydrochloride (0.32–0.64 mg/mL). Berberine hydrochloride prevented FtsZ polymerization in a concentrationdependent manner and bound to FtsZ protein by hydrogen bonding interaction. This study suggested that the FtsZ-based E. coli screening model could be exploited for the development of novel antibacterial agents for clinical applications.

Keywords: FtsZ; expression; purification; GTPase; alkaloid; antibacterial activity

1. Introduction

Pathogenic *Escherichia coli* can cause severe outbreaks of diarrheal and extraintestinal diseases in both humans and animals [1-3]. The global incidence of diarrheal infection due to *E. coli* is up to 1.7 billion cases, with a mortality of 760,000 children per year; the in-hospital mortality of *E. coli* bloodstream infections ranged from 9.6% to 11% [4]. It has been noted that some *E. coli* strains are not killed by colistin, a last-resort antibiotic, due to the rapid development of antibiotic resistance [5]. Therefore, it is very urgent to develop novel antibacterial drugs to fight against pathogenic *E. coli*.

Filamenting temperature-sensitive protein Z (FtsZ) is a highly conserved prokaryotic protein that is a homolog of eukaryotic tubulin with approximately 7% sequence identity and plays an important role in prokaryotic cell division [6,7]. FtsZ protein is present in almost all bacteria and shares 40–50% sequence similarity among most bacteria but is notably absent in eukaryotes; FtsZ is also a GTP enzyme that polymerizes into single-stranded filaments in a GTP-dependent manner and further assembles into the Z-ring



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). on the inside of the bacterial cytoplasmic membrane [8]. FtsZ is the first molecule to assemble at the division site and recruits other proteins (including FtsA, ZapA (YgfE), etc.) involved in bacterial cell division [9–13]. It has been demonstrated that abnormalities in FtsZ assembly and GTPase activity may inhibit Z-ring and septum formation, leading to abnormal cell division and even cell death [14]. Therefore, targeting FtsZ or inhibiting GTPase activity for antibacterial therapy is a promising avenue; FtsZ has recently become a hopeful target for the development of novel antibacterial drugs [8].

Natural products are seen as a rich source of potential drugs to treat bacterial infections. It is estimated that approximately 60% of drugs are derived from natural products. Currently, there are a multitude of natural compounds being evaluated as bacterial FtsZ inhibitors, including polyphenols, phenylpropanoids, and terpenoids [15]. Some polyphenols such as resveratrol, curcumin, and viriditoxin could inhibit FtsZ expression and Z-ring formation in *Bacillus subtilis, E. coli, Staphylococcus aureus,* and *Enterococci* [8,16–20]. Other compounds (including cinnamaldehyde, emodin, alkaloids, etc.) prevented the assembly of FtsZ profilaments, GTPase activity, and Z-ring formation in *E. coli* [8,19,20]. Moreover, these natural product molecules exhibited prominent antibacterial activity, and they had low toxicity to eukaryotes and even good pharmacokinetic properties [8]. It has been proven that virtual screening, a colorimetric (Alamar blue) microdilution broth assay, and computational, biochemical, and *in vivo* cell-based assays could be used to screen active inhibitors of FtsZ from *Staphylococcus aureus, Salmonella Typhi, Mycobacterium tuberculosis*, etc. [21–24]. However, rapid and effective screening methods based on FtsZ GTPase from *E. coli* have rarely been reported.

In this study, to construct an efficient screening assay for novel antibacterial agents against *E. coli*, the *ftsZ* gene was amplified from *E. coli*, cloned into the pET-28a vector, and then expressed in *E. coli* BL21 in the presence of isopropyl β -D-1-thiogalactopyranoside (IPTG). After purification with the Ni²⁺-NTA affinity column, GTPase activity of the recombinant FtsZ protein was determined, and a rapid screening model of antibacterial agents was mainly constructed *in vitro* based on the FtsZ GTPase activity. In addition, several active alkaloid molecules were obtained by this screening model and they had potent antibacterial activity against pathogenic *E. coli* and other bacteria.

2. Materials and Methods

2.1. Strains, Plasmids, and Reagents

E. coli strains DH5α and BL21 were purchased from TransGen Biotech (Beijing, China). The pMD19-T and pET-28a vectors were supplied by TAKARA Bio (Dalian, China) and Miaoling plasmid platform (Wuhan, China), respectively. *Senteritidis enteritidis* ATCC14028 and *Staphylococcus aureus* ATCC43300 were purchased from the American Type Culture Collection (ATCC); *E. coli* CVCC1515, *E. coli* CVCC195, and *S. enteritidis* CVCC3377 were provided by the China Veterinary Culture Collection Center (CVCC). Both *Xho* I and *Nco* I restriction endonucleases were provided by Baolaibo Technology (Beijing, China). The primers were supplied by Sangon Biotech (Shanghai, China).

TIANprep Mini Plasmid Kit, TIANamp Bacteria DNA Kit, TIANgel Midi Purification Kit, and DNA Marker were purchased from Tiangen Biotech (Beijing, China). Twelvepercent TGX Stain-freeTM FastCastTM acrylamide solutions were provided by Bio-Rad Laboratories (Beijing, China). His-Tagged Inclusion Body Protein Purification Kit was supplied by CWBIO (Beijing, China). Malachite Green Phosphate Assay Kit was provided by Cayman Chemical Company (Beijing, China). BCA Protein Assay Kit was purchased from Beyotime Biotechnology (Beijing, China). PageRulerTM Prestained Protein Ladder and GTP were provided by Thermo Fisher Technology (Beijing, China). All natural products were purchased from Solarbio Biotechnology (Beijing, China). All other biochemical reagents were of analytical grade.

2.2. Cloning of the ftsZ Gene and Construction of the Recombinant Plasmids

The *ftsZ* of *E. coli* CVCC1515 was amplified by polymerase chain reaction (PCR) with a pair of specific primers (F: 5'-CATGCCATGGGCATGTTTGAACCAATGGAAC-3' and R: 5'-CCGCTCGAGATCAGCTTGCTTACGCAGGA-3') with restriction sites of *Nco* I and *Xho* I. The *ftsZ* PCR product was purified by TIANgel Midi Purification Kit and cloned into the pMD-19T vector to produce the pMD-ftsZ plasmid according to the manufacturer's instructions [25].

The positive pMD-ftsZ plasmids were digested by *Xho* I and *Nco* I; the obtained *ftsZ* DNA fragment was ligated into the pET-28a expression vector digested with the same endonucleases, followed by transformation into *E. coli* DH5 α . The recombinant pET-ftsZ plasmids were identified by DNA sequencing and enzyme digestion methods [26].

2.3. Recombinant Expression of FtsZ Protein

The pET-ftsZ plasmids were transformed into *E. coli* BL21 (DE3) and incubated overnight (37 °C) on LB plate (containing 50 μ g/mL kanamycin). A single grown colony was inoculated in LB liquid medium and incubated overnight at 37 °C (250 rpm). The culture was then transplanted to LB medium with 50 μ g/mL kanamycin and cultivated at 37 °C (200 rpm) until the optical density (OD) at 600 nm was 0.6. Following induction by 0.4 mM IPTG at 37 °C for 5 h, cultures were concentrated at 5000× g for 10 min at 4 °C. The pellets were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [27].

To optimize expression conditions, the cultures were incubated at 37 °C (200 rpm) to $OD_{600} = 0.6$ and induced by different concentrations of IPTG (0.2, 0.4, and 0.6 mM) at different temperatures (16, 25, and 37 °C) for different time periods (1, 3, 5, and 7 h). The bacterial samples (2 mL) collected at each time point were centrifuged at $5000 \times g$ for 10 min at 4 °C, lysed, and analyzed by 12% SDS-PAGE.

2.4. Isolation and Purification of the Recombinant FtsZ Protein

To isolate the expressed protein, 2 mL lysis buffer (containing 1 μ L 1000 U/mL DNase I and 2 μ L 50 mg/mL lysozyme) was added to 100 mg cell pellets and homogenized at 4 °C for 10 min on an ultrasonicator (JY92-IIN) (including 3 cycles, 5's per cycle, and 10's interval) with power levels of 227.5 W, 162.5 W, and 97.5 W, respectively. The bacterial solution was kept in an ice bath during the ultrasonic process. The supernatant and precipitate were collected by centrifugation at 4 °C (10,000 × *g*, 15 min). The recombinant proteins were analyzed by 12% SDS-PAGE and Image Lab software.

The recombinant FtsZ protein was separated and purified with a His-tagged Inclusion Body Protein Purification Kit (CWBIO). The precipitate was resuspended in a binding buffer and centrifuged at 4 °C (10,000 × g, 20 min). The supernatant was separated, filtered by a filter membrane (0.22 μ m pore size), and then loaded onto the Ni²⁺-NTA affinity column. FtsZ protein was eluted with different concentrations of imidazole (ranging from 250 to 750 mM), dialyzed with ultra-pure water at a magnetic stirrer (MS-01H) for 24–48 h, and dried in a vacuum freeze-drying machine (LGJ-18) for 72 h. The recombinant FtsZ protein concentration was qualified by the BCA Protein Assay Kit in accordance with the manufacturer's instructions.

2.5. Determination of the GTPase Activity of the Recombinant FtsZ Protein

GTPase activity of the recombinant FtsZ protein was determined by the Malachite Green Phosphate Assay by measuring the enzyme's ability to release free phosphoric acid (Pi) from GTP [28]. Briefly, 3 μ M recombinant FtsZ protein was added to each well of a 96 well half-volume transparent plate (20 μ L/well). GTP stock solution (5 mM) was diluted two-fold with a buffer (containing 50 mM Tris, pH 7.5, 250 mM KCl, and 5 mM MgCl₂) and also added to the plate. After incubation for 30 min at 37 °C, MG acid reagent (5 μ L/well) was added to the plate and incubated for 10 min at room temperature (RT). MG blue reagent was added to the reaction and incubated for 20 min at RT. The group without

GTP solution was used as the negative control. The free Pi release was measured at OD_{620} and GTPase activity of the recombinant FtsZ protein (nmol/mL/min) in the reaction was calculated according to the standard phosphoric acid curve. Three independent replicates were performed.

2.6. Screening of Alkaloids Based on FtsZ GTPase Activity

The recombinant FtsZ protein was incubated for 30 min at 37 °C with various natural products, including sophoridine, matrine, theophylline, betaine, betonicine, tetrandrine, sanguinarine chloride, sinomenine hydrochloride, nonivamide, and berberine hydrochloride. In brief, different concentrations (3.13–50 µgmL) of alkaloids were added to a 96 well half-volume transparent plate containing 3 µM FtsZ (10 µL/well) and 0.625 mM GTP (15 µL/well). After incubation for 30 min at 37 °C, MG acid reagent was added to the plate (5 µL/well), and the reaction was conducted for 10 min at RT. After incubation with MG blue reagent for 20 min at RT, effects of alkaloids on FtsZ GTPase activity were assessed by OD₆₂₀. The group without GTP solution was used as the negative control. Those that showed >20% inhibition of GTPase activity were initially considered active [23]. This experiment was repeated three times.

2.7. Antibacterial Activity of Alkaloids

The antibacterial activity of alkaloids against pathogenic *E. coli* (CVCC1515 and CVCC195), *S. enteritidis* (ATCC14028 and CVCC3377), and *S. aureus* ATCC43300 was detected by microtiter broth dilution method [29]. Briefly, the bacteria tested grew to the exponential growth stage. After dilution, bacterial cells (10^5 CFU/mL, 90μ L/well) were inoculated into 96 well plates. A series of two-fold dilutions of alkaloids were added to the plates (10μ L/well). The plates were incubated for 18–24 h at 37 °C until visible turbidity was observed in the negative control. The minimum inhibitory concentration (MIC) of alkaloids against the tested strains is the lowest concentration that can completely inhibit bacterial growth.

The minimum bactericidal concentration (MBC) of alkaloids was determined by the lowest concentration (>99.9% killing rate) on fresh agar plates. This experiment was repeated three times.

2.8. Cytotoxicity of Alkaloids

The cytotoxicity of three alkaloids (including sanguinarine chloride, tetrandrine, and berberine hydrochloride) was evaluated using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) method. Briefly, RAW 264.7 cells (2.5×10^5 cells/mL) were added into a 96 well plate and cultured in DMEM medium (containing 10% fetal bovine serum and 0.1% penicillin-streptomycin) at 37 °C (5% CO₂, 95% saturated humidity) for 24 h. The medium was then removed and the cells were washed twice with PBS. The alkaloid solutions (from 1 to 2560 µg/mL) were then added to the plates (100 µL/well), incubated for 24 h, and washed twice with PBS. Next, 5 mg/mL MTT (100 µL/well) was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) (150 µL/well) was added to dissolve the crystals in the bottom of wells, and the absorbance was measured at 570 nm. The cell viability was calculated according to the following formula: cell viability (%) = (Abs _{alkaloid}/Abs _{PBS}) × 100%.

2.9. Interaction of Berberine Hydrochloride and the Recombinant FtsZ Protein

The recombinant FtsZ protein ($128 \ \mu g/mL$) was assembled in an MES buffer (containing 50 mM Tris, pH 7.5, 5 mM MgCl₂, and 250 mM KCl), in the absence or presence of berberine hydrochloride according to the previous method [30]. GTP (0.25–8 mM) was added to the recombinant FtsZ solution and incubated for 30 min. Two methods were used to detect the effects of berberine hydrochloride (1.56, 6.25, and 25 $\mu g/mL$) on FtsZ aggregation: (i) two procedures: berberine hydrochloride and FtsZ protein were preincubated for 30 min and the mixtures were then incubated with GTP for another 30 min. The reaction

solution consisted of 10 μ L FtsZ (128 μ g/mL), 1 μ L GTP (20 mM), and 5 μ L berberine; (ii) one procedure: berberine hydrochloride and FtsZ were incubated directly with GTP for 1 h. The effect of berberine on FtsZ polymerization was detected by 12% SDS-PAGE.

Molecular docking (MD) was performed by AutoDock software to analyze the binding of berberine hydrochloride (a ligand) to *E. coli* FtsZ (receptor) [30]. Briefly, the native ligand of FtsZ protein was removed, and its polar hydrogen atom and Kollman unified-atom partial charges were added. Active torsion groups and Gasteiger-Marsilli atomic charge of berberine hydrochloride were defined. Grid maps were calculated with a $70 \times 80 \times 80$ point spacing of 0.375 Å, centered at the point using Autogrid4. The docking was performed with a flexible ligand using Lamarckian Genetic Algorithm (LGA) with a translation step of 0.2 Å, quaternion one of 5°, and torsional one of 5°. Other parameters were the default values.

3. Results

3.1. Construction of Recombinant Plasmids

Figure 1A shows the construction procedure for recombinant plasmids. The *ftsZ* DNA fragment (1170 bp) was successfully amplified from *E. coli*, ligated into the pMD-19T vector, and finally produced the recombinant pMD-ftsZ plasmid. After digestion with *Xho* I and *Nco* I, the *ftsZ* gene was obtained from the pMD-ftsZ plasmid (Figure 1B) and then inserted into pET-28a expression vector. The resulting recombinant pET-28a-ftsZ plasmid was digested with the same enzymes, and the size of the *ftsZ* DNA fragment was approximately 1170 bp (Figure 1B). Meanwhile, DNA sequencing results showed that the recombinant pET-28a-ftsZ plasmid contained the *ftsZ* gene. The plasmid pET-28a-ftsZ was then transformed into *E. coli* BL21, and positive transformants were obtained by colony PCR.

3.2. Optimization of FtsZ Expression

To obtain a high expression level of FtsZ protein, positive transformants were selected to cultivate in LB medium to an OD₆₀₀ of 0.6–0.8. Cultures were then induced by different concentrations of IPTG (0.2, 0.4, and 0.6 mM) at different temperatures (16, 25, and 37 °C) for different time periods (1, 3, 5, and 7 h). The results showed that the expression level of FtsZ at 37 °C was significantly higher than those at 16 °C and 25 °C (Figure 1C). Moreover, after induction at 37 °C for 5 h, the FtsZ level was higher than at other time periods (including 1 h, 3 h, and 7 h) (Figure 1D); after induction with 0.2 mM IPTG for 5 h, a higher level of FtsZ protein was observed than with other IPTG concentrations (Figure 1D). Overall, the highest expression level of FtsZ protein was induced by 0.2 mM IPTG at 37 °C for 5 h (accounting for approximately 50% of total cell proteins).

The molecular weight (MW) of the recombinant FtsZ protein was approximately 41 kDa (Figure 1D), which is consistent with the theoretical value of 41.13 kDa, indicating that the FtsZ protein was successfully produced in *E. coli*. Additionally, FtsZ protein was expressed as inclusion bodies in *E. coli* BL21 (Figure 2).



Figure 1. Construction of recombinant plasmids and FtsZ protein expressed in *E. coli* under different conditions. (**A**) Construction of recombinant plasmids. (**B**) Digestion of recombinant plasmids with endonucleases. Lane 1: digestion of pMD-ftsZ plasmids with *Nco* I and *Xho* I; lane 2: digestion of pET-28a-ftsZ plasmids with *Nco* I and *Xho* I. M: marker. (**C**) Expression of FtsZ at different temperatures. M: marker. (**D**) Different induction times (1, 3, 5, and 7 h) and IPTG concentrations ranging from 0.2 to 0.6 mM. PC: bovine serum albumin; M: marker.



Figure 2. Isolation and purification of the recombinant FtsZ protein at different ultrasonic powers and imidazole concentrations. (**A**) Isolation of FtsZ protein at ultrasonic power 227.5 W. Lane 1: sediment after ultrasound; lane 2: supernatant after ultrasound; M: marker. (**B**) Isolation of FtsZ protein at 162.5 W. Lane 1: sediment after ultrasound; Jane 2: supernatant after ultrasound; lane 2: supernatant after ultrasound; M: marker. (**C**) Isolation of FtsZ protein at 97.5 W. Lane 1: sediment after ultrasound; lane 2: supernatant after ultrasound; M: marker. (**D**) Effect of imidazole concentrations on elution of FtsZ. Lane 1: flow-through liquid; lane 2: 250 mM imidazole; lane 3: 300 mM imidazole; lane 4: 350 mM imidazole; lane 5: 400 mM imidazole; lane 6: 450 mM imidazole; lane 7: 500 mM imidazole; lane 8: 550 mM imidazole; lane 9: 600 mM imidazole; lane 10: 650 mM imidazole; lane 11: 700 mM imidazole; lane 12: 750 mM imidazole; M: marker. (**E**) Elution times of FtsZ. Lane 1: sediment after ultrasound; lane 2: supernatant after ultrasound; lane 3: flow-through liquid; lane 4–9: 10 mL 500 mM imidazole from first elution to sixth; M: marker.

3.3. Isolation and Purification of the Recombinant FtsZ Protein

After ultrasonication at 97.5 W power, the amount of FtsZ protein was higher than that in the samples prepared at 227.5 W and 162.5 W (Figure 2A–C), indicating that ultrasonication treatment at a proper power can improve the target protein level.

The recombinant FtsZ protein was observed in both sediment and supernatant. After ultrasound, the level of FtsZ protein in sediment was significantly higher than that in supernatant (Figure 2A–C). After purification by an Ni²⁺-NTA column, dialysis, and lyophilization, FtsZ protein exhibited more distinct target bands (Figure 2), indicating that Ni²⁺-NTA affinity chromatography was effective for purification of the target protein. As shown in Figure 2D, no FtsZ protein was observed in the flow-through fluid when the elution buffer contained 250–300 mM imidazole (lanes 2–3), suggesting that the recombinant FtsZ protein was fully combined with the Ni²⁺-NTA column. Meanwhile, the elution buffer containing 500 mM imidazole produced a higher yield of FtsZ protein than that obtained in the elution buffer containing other concentrations of imidazole.

Additionally, compared to more or less elution times, higher levels of FtsZ protein with no impurity bands were observed after three elution times (Figure 2E). The purified FtsZ protein was estimated to be approximately 41 kDa, with a purity of >90%. It indicated that the recombinant FtsZ protein was effectively isolated and purified by Ni²⁺-NTA affinity column.

3.4. The GTPase Activity of the Recombinant FtsZ Protein

Bacterial FtsZ has GTPase activity, which can hydrolyze GTP to GDP and free Pi [31]. Malachite green reagent can combine with Pi to form a green complex, and the complex amount is positively correlated with the OD_{620} value [32]. GTPase activity of the recombinant FtsZ protein was detected by the Pi release amount in the reaction system. The results showed that the recombinant FtsZ protein exhibited GTPase activity at different concentrations of GTP ranging from 0.31 to 5.00 mM (Figure 3A). FtsZ protein had the highest GTPase activity (0.998 nmol/mL/min) at 1.25 mM GTP. It indicated that the recombinant FtsZ protein produced in *E. coli* BL21 showed GTPase activity.



Figure 3. GTPase activity of the recombinant FtsZ protein and its inhibitors. (**A**) Determination of GTPase activity of the recombinant FtsZ protein. (**B**) Effects of 10 alkaloids on GTPase activity.

3.5. A Screening Model of Antibacterial Agents Based on FtsZ GTPase Activity

The hydrolysis rate of GTP depends on the dynamics of FtsZ polymerization [22,33]. The effects of alkaloids on the activity of FtsZ GTPase were measured by the malachite green phosphate method. The results showed that most alkaloids significantly inhibited FtsZ GTPase activity, and the inhibition rate was over 20% (Figure 3B), suggesting that these alkaloids may be recognized as antibacterial agents or FtsZ inhibitors. Among them, sophoridine showed the most potent inhibitory effect on the activity of FtsZ GTPase, with an inhibitory rate of more than 60% at a concentration of 3.13 μ g/mL. However, 50 μ g/mL of tetrandrine and 3.13 μ g/mL of sanguinarine chloride inhibited FtsZ GTPase activity by less than 20% (Figure 3B). It indicated that an effective screening model for antibacterial drugs was initially established based on the GTPase activity of the recombinant FtsZ protein. The inhibitory effects of alkaloids on GTPase activity may result in disruption of *E. coli* FtsZ polymerization [22].

3.6. Antibacterial Activity of Alkaloids

To further detect the antibacterial activity of the above screened alkaloids against pathogenic bacteria, MIC and MBC values were determined for each alkaloid. Among these alkaloids, sanguinarine chloride and tetrandrine displayed the highest antibacterial activity against *E. coli, S. enteritidis*, and *S. aureus*; their MIC and MBC values were 0.04–0.16, 0.16–0.32, and 0.16–0.32 mg/mL, respectively (Table 1). Berberine hydrochloride had potent antibacterial activity (with MICs and MBCs of 0.32–0.64 mg/mL, except *S. enteritidis* ATCC14028); theophylline, sophoridine, and matrine had moderate antibacterial activity (with MICs of 2.56–10.24 mg/mL), but other alkaloids had no antibacterial activity against the strains tested.

Table 1. MIC and MBC values of alkaloids against different bacterial pathogens.

| | Sangui- narine Chloride | | Tetrandrine | | Berberine Hydro- chloride | | Theophylline Sophoridine | | | Matrine | | Sinomenine Hydrochloride | | Betaine | | Betonicine | | Nonivamide | | |
|-----------------------------|-------------------------------|------|-------------|------|---------------------------------|-------|--------------------------|-------|-------|---------|-------|-----------------------------|-------|---------|--------|------------|--------|------------|-------|-----|
| (mg/mL) | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| E. coli CVCC1515 | 0.04 | 0.16 | 0.16 | 0.16 | 0.64 | 0.64 | 2.56 | >5.12 | 5.12 | 10.24 | 5.12 | >5.12 | 8.88 | 5.12 | >10.24 | - | >10.24 | Į - | >5.12 | - |
| E. coli CVCC195 | 0.16 | 0.32 | 0.16 | 0.16 | 0.64 | >0.64 | 2.56 | >5.12 | 5.12 | 10.24 | 5.12 | >5.12 | >5.12 | >5.12 | - | - | - | - | - | - |
| S. enteritidis CVCC3377 | 0.04 | 0.16 | 0.32 | 0.32 | 0.32 | 0.64 | 2.56 | >5.12 | 10.24 | >10.24 | >5.12 | >5.12 | >5.12 | >5.12 | - | - | - | - | - | - |
| S. enteritidis ATCC14028 | 0.16 | 0.32 | 0.32 | 0.32 | >0.64 | - | 2.56 | >5.12 | 5.12 | 10.24 | 5.12 | >5.12 | >8.88 | 8.88 | >10.24 | - | >10.24 | ŧ - | >5.12 | - |
| S. aureus ATCC43300 | 0.16 | 0.32 | 0.32 | 0.32 | 0.32 | 0.64 | 2.56 | >5.12 | 10.24 | >10.24 | >5.12 | >5.12 | >8.88 | >8.88 | >10.24 | - | >10.24 | Į - | - | - |

Note: -, no detection.

3.7. Cytotoxicity of Alkaloids

The MTT result showed that the cell viability of RAW 264.7 cells when exposed to berberine hydrochloride was higher than those with sanguinarine chloride and tetrandrine (Figure S1), indicating that berberine hydrochloride had markedly less cytotoxicity than other alkaloids. Thus, berberine hydrochloride was used in the subsequent experiment.

3.8. Interaction of Berberine Hydrochloride and the Recombinant FtsZ Protein

The effects of berberine hydrochloride on FtsZ polymerization were analyzed by SDS-PAGE. As shown in Figure 4A, the amount of FtsZ pelleted increased slightly with GTP concentrations ranging from 0.25 to 1 mM, but decreased with increasing GTP concentrations (from 2 to 8 mM). The amount of FtsZ pelleted was the highest in the presence of 1 mM GTP. As shown in Figure 4B, compared to the control, FtsZ polymerization was inhibited by berberine hydrochloride in a concentration-dependent manner. Meanwhile, one procedure in the reaction was superior to two (Figure 4B).



Figure 4. Interaction of berberine hydrochloride and the recombinant FtsZ protein. (**A**) Effects of different concentrations of GTP on FtsZ aggregation. Lane 1: 0 mM GTP; lane 2: 0.25 mM GTP; lane 3: 0.5 mM GTP; lane 4: 1 mM GTP; lane 5: 2 mM GTP; lane 6: 4 mM GTP; lane 7: 8 mM GTP; M: marker. Lane numbers indicate the relative intensity of the bands. (**B**) Effect of berberine hydrochloride on FtsZ aggregation. Lane 1–3: berberine hydrochloride was first incubated for 30 min with FtsZ protein and then incubated for another 30 min with GTP; lane 4–6: berberine hydrochloride was incubated directly with FtsZ protein and GTP for 1 h; berberine hydrochloride concentrations were 1.56 (lane 1 and 4), 6.25 (lane 2 and 5), and 25 (lane 3 and 6) μ g/mL; lane 7: FtsZ and GTP solution; lane 8: FtsZ solution; M: marker. Lane numbers indicate the relative intensity of the bands. (**C**,**D**) Binding of berberine hydrochloride to FtsZ. Molecular docking was performed by using AutoDock software to analyze the binding of berberine hydrochloride (a ligand) to FtsZ (receptor).

Molecular docking showed that one hydrogen bond was formed between berberine hydrochloride and FtsZ protein and the binding energy was -26 kJ/mol; berberine hydrochloride was predicted to bind to the Asp253 residue of FtsZ (Figure 4C,D).

4. Discussion

Many pathogens, including *E. coli*, cannot be killed by conventional antibiotics (including colistin, a last-resort drug) due to the emergence of antibiotic resistance, which urges the development of novel antibacterial drugs [1–3]. Bacterial FtsZ protein plays an important role in cell division and may be used as one of the targets for antibacterial agents [34]. Although recent studies have revealed some natural products that may inhibit FtsZ GTPase activity and kill bacteria, there are limited known natural products used to fight *E. coli* [35]. In this work, the recombinant FtsZ protein was expressed in *E. coli* and

purified by the Ni²⁺-NTA column; a rapid and efficient screening method of antibacterial agents was mainly constructed based on the GTPase activity of FtsZ protein.

The *E. coli* expression system has been a preferred microbial cell factory for the production of many recombinant proteins (such as FtsZ, rhGH, TusA, etc.) due to its advantages, such as simple procedure, low cost, scale, or high quantities [36-38]. Heterologous proteins produced in *E. coli* usually result in soluble and insoluble proteins, also known as inclusion bodies. Expression form of heterologous proteins in E. coli is related to culture and induction temperature [39]. A temperature of 37 °C or higher during cultivation, which causes a larger growth rate of bacteria and cell density, is more prone to form inclusion bodies, which can accumulate near the cytoplasm (or inner membrane) and block protein translocation across channels in E. coli [40]. In contrast, proteins expressed in E. coli at lower temperatures in the range of 18 °C to 25 °C can form aggregates in the inner membrane or periplasmic space and are released to the supernatant in soluble formation; several advantages of low temperature include reduced proteolytic activity, simpler purification protocols due to fewer host proteins, correct formation of disulfide bonds, etc. [41]. Additionally, lower temperatures can stabilize refolded enzymes and lead to lower protein expression rates, so that newly transcribed recombinant proteins have enough time to fold correctly or properly [42]. As a consequence, low temperature can enhance the refolded yield of recombinant proteins produced in *E. coli* and is therefore usually selected to induce the production of heterogenous proteins [43–46]. However, some previous studies have demonstrated that lower temperatures may limit bacterial cell growth by disrupting an early step in protein synthesis, which leads to low recombinant protein yields [47,48]. In our study, FtsZ protein was produced as an inclusion form after induction with IPTG at 16–37 °C (Figure 1C); the highest expression level (approximately 1 mg/mL) of FtsZ protein was obtained after induction with 0.2 mM IPTG for 5 h at 37 °C (Figure 1D). Furthermore, ultrasonic lysis of bacterial cells at 97.5 W was more effective than at 162.5 W or 227.5 W (Figure 2A–C), which may be linked to an increase in protein denaturation [49].

Proper concentrations of imidazole may result in a higher purity of the eluted sample. In our work, the recombinant FtsZ protein was eluted by a solution supplemented with 500 mM imidazole, with a higher yield of 25 μ g/mL and purity of >90% (Figure 2D); this result was different from those in previous studies, in which higher protein yield and purity were achieved with 300–350 mM imidazole [50,51]. The purified FtsZ protein from *E. coli* exhibited GTPase activity at a concentration of 3 μ M, converting approximately 13 nmol of GTP to GDP per mg of FtsZ per min (Figure 3A), lower than the FtsZ GTPase activity from *B. subtilis*, which may be related to FtsZ from different bacteria [52]. Bhattacharya et al. also found that plumbagin inhibited GTPase activity and FtsZ assembly from *B. subtilis in vitro*; however, plumbagin did not affect GTPase activity and the assembly of FtsZ from *E. coli*. The data indicated that FtsZ protein from different bacteria may have different binding properties of ligand compounds [52].

It has been demonstrated that berberine can bind to FtsZ, inhibit GTPase activity, and disrupt FtsZ assembly or polymerization *in vitro* [20]. In our study, some alkaloids such as sophoridine and berberine hydrochloride were initially screened by an established screening model based on the recombinant FtsZ protein (Figure 3B). These alkaloid products exhibited high antibacterial activity against pathogenic *E. coli*, *S. enteritidis*, and *S. aureus* strains, with MICs and MBCs of 5.12–10.24 mg/mL (Table 1). Meanwhile, several alkaloids also inhibited GTPase activity and FtsZ assembly and bound to *E. coli* FtsZ protein (Figure 4B). The data suggested that an efficient screening system for antibacterial agents was successfully constructed based on GTPase activity of the recombinant FtsZ protein produced in *E. coli*. Furthermore, berberine hydrochloride was predicted to bind to the Asp253 residue of FtsZ protein from *E. coli* by hydrogen bonding interaction (Figure 4C,D). Comparably, in the previous study, berberine could bind to more active sites (including Thr132, Pro134, Phe135, Ile163, Pro164, Phe182, and Leu189) of FtsZ from *Methanococcus jannaschii* by hydrophobic interactions; this discrepancy can be attributed to: (i) different chemical structures of the ligands berberine and berberine hydrochloride and (ii) low sequence

homology (<50%) and different structures of FtsZ from *E. coli* and *M. jannaschii* [8,20]. FtsZ is highly divergent across bacterial species; FtsZ from *E. coli* shares approximately 46% and 53% amino acid identity with those from *M. tuberculosis* and *B. subtilis*, respectively [53,54]. Therefore, the same compound may interact with FtsZ proteins from widely divergent bacterial species by different modes of action.

5. Conclusions

The *ftsZ* gene was obtained from *E. coli* and the recombinant FtsZ protein in *E. coli* was induced by IPTG. The purified FtsZ protein displayed GTPase activity. An effective screening model for antibacterial agents was primarily constructed based on the recombinant FtsZ protein and was used to screen alkaloids. Several alkaloids inhibited GTPase activity and FtsZ aggregation and displayed potent antibacterial activity against *E. coli* and other pathogens. This study suggested that the FtsZ-based *E. coli* screening model could be exploited in future studies for the development of more potent novel antibacterial agents for clinical applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13074493/s1, Figure S1: Cytotoxicity of three alkaloids to mouse macrophages (RAW264.7).

Author Contributions: X.W., H.L. and Q.F. conceived and designed the study. Q.F. conducted the experiments, analyzed the data, and wrote the manuscript. X.W. supervised the project. H.L. and X.W. revised the manuscript. Q.F., B.X., C.L. and J.W. contributed to result collection and processing of partial results. All authors have read and agreed to the published version of the manuscript.

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