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Preparation of Susceptibility Test Discs of Huanglian Jiedu Tang and Its Clinical Application on the Antibacterial Effect of *Staphylococcus aureus*

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ABSTRACT

Objective: To prepare in-house susceptibility test discs containing *Huanglian Jiedu Tang* (HLJDT) and evaluate their *in vitro* antibacterial activity against *Staphylococcus aureus* (*S. aureus*). Methods: 1) The content of berberine in HLJDT was quantified using high-performance liquid chromatography (HPLC) to ensure quality control. 2) The antibacterial activity of HLJDT susceptibility discs was assessed against *S. aureus* at varying concentrations, disc thicknesses, and culture times to evaluate their stability, uniformity, and efficacy. 3) The inhibitory effects of the discs were tested on 64 clinical *S. aureus* isolates, and the correlation between inhibition zone diameter and minimum inhibitory concentration (MIC), determined via broth microdilution, was analyzed. Results: 1) The berberine concentration in HLJDT, as determined by HPLC, was 2358.0 μg/mL. 2) In the disc diffusion assay, high (45.0 mg) and medium (22.5 mg) concentration HLJDT discs produced significant inhibition zones against *S. aureus* strains USA300 and ATCC29213, while the low concentration (11.25 mg) discs showed negligible activity. Discs with a thickness of 0.7 mm exhibited significantly better antibacterial effects against MRSA compared to those with a thickness of 1.0 mm ($P < 0.05$). 3) The inhibition zones for methicillin-resistant *S. aureus* (MRSA) were significantly larger than those for methicillin-susceptible *S. aureus* (MSSA) at both high and medium concentrations (15.33 ± 2.37 mm and 11.82 ± 2.48 mm vs. 13.50 ± 1.68 mm and 9.87 ± 1.60 mm, respectively; $P < 0.01$). Furthermore, the inhibition zone diameter for MRSA using high-concentration discs was strongly negatively correlated with MIC values ($r = -0.501$, $P < 0.01$), and moderate negative correlations were observed at medium and low concentrations ($r = -0.327$ and $r = -0.353$, respectively; $P < 0.05$). Conclusion: The HLJDT susceptibility test discs demonstrated reliable quality, good uniformity, and stable antibacterial performance. They showed

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notable antibacterial activity against *S. aureus*, particularly MRSA, with a strong correlation between inhibition zone diameter and MIC. These findings provide a robust methodological foundation for further research and potential clinical applications of HLJDT in the treatment of *S. aureus* infections.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most common Gram-positive pathogens, responsible for a wide spectrum of diseases ranging from mild skin and wound infections to life-threatening sepsis and multiple organ failure [1]. Among these, methicillin-resistant *S. aureus* (MRSA) is a major cause of nosocomial infections. MRSA resists multiple antibiotics through various mechanisms, including cell wall thickening, efflux pump activation, enzymatic degradation, and target site mutations [2, 3]. Except for vancomycin, MRSA displays resistance to most conventional antibiotics, thereby posing a significant challenge to clinical antimicrobial therapy [4-7]. In recent years, with the strong promotion of traditional Chinese medicine (TCM) by government initiatives, increasing attention has been given to the anti-inflammatory and antibacterial properties of Chinese herbal monomers and compound formulations [8, 9]. TCM offers distinct advantages, including low cost, multi-target mechanisms of action, and reduced likelihood of resistance development. These characteristics make TCM a promising avenue for novel drug discovery and for addressing the growing problem of antibiotic resistance. *Huanglian Jiedu Tang* (HLJDT) is a classical TCM compound comprising *Coptis chinensis* (Huanglian), *Scutellaria baicalensis* (Huangqin), *Phellodendron amurense* (Huangbo), and *Gardenia jasminoides* (Zhizi). It is traditionally used to "clear heat and detoxify" and has been widely applied in the treatment of various inflammation-related diseases [10, 11]. Our previous studies have demonstrated that HLJDT and its primary active component, berberine, exhibit notable antibacterial activity against *S. aureus*, particularly MRSA [12-14]. Currently, there is no standardized method for preparing *in vitro* antimicrobial susceptibility test discs for Chinese herbal compounds or decoctions. Existing susceptibility tests for herbal medicines are largely adapted from Western pharmacological standards, such as broth microdilution and disc diffusion assays. Therefore, the present study aims to develop and standardize a method for preparing HLJDT susceptibility test discs. This method will facilitate more reliable *in vitro* evaluations of HLJDT's antibacterial activity against *S. aureus*.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

A total of 64 clinical isolates of *S. aureus* were collected from patient samples at Shanghai Eighth People's Hospital between October 2021 and March 2022. All isolates were identified and classified as either methicillin-sensitive *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) using the VITEK 2 Compact automated microbial identification system (bioMérieux, France) in the hospital's microbiology laboratory. Among the isolates, 18 were MSSA and 46 were MRSA strains. The standard strain USA300 was used as the quality control strain for MRSA, and ATCC 29213 was used as the quality control strain for MSSA. Both strains were obtained from the National Institutes for Food and Drug Control (China).

2.2. Preparation of HLJDT

HLJDT was prepared using the following crude herbal components in a mass ratio of 3:2:2:3: 9 g of Huanglian, 6 g of Huangqin, 6 g of Huangbo, and 9 g of Zhizi. The herbs were combined and soaked in 500 mL of deionized water for 30 minutes, followed by decoction at boiling temperature for 60 minutes. The mixture was then filtered through a nylon mesh. The remaining herbal residue was decocted again in another 500 mL of deionized water for an additional 60 minutes and filtered in the same manner. The two filtrates were combined and concentrated to a final volume of 200 mL by centrifugation, yielding an aqueous extract with a mass concentration of 15% (150 mg/mL). The extract was sterilized by autoclaving at 121˚C

for 15 minutes and stored in sterile glass bottles at 4°C until further use.

2.3. Methods

2.3.1. High-Performance Liquid Chromatography (HPLC) Analysis of Berberine Content in HLJDT

1) Preparation of Standard Berberine Solutions

Approximately 12 mg of berberine reference standard, previously dried at a constant temperature of 100˚C for 5 hours, was accurately weighed and dissolved in methanol in a 10 mL volumetric flask to obtain a stock solution at a concentration of 1.2 mg/mL. The stock solution was serially diluted with methanol to prepare a set of standard solutions with concentrations of 0.01875, 0.0375, 0.0750, 0.1500, 0.3000, and 0.6000 mg/mL.

2) Preparation of Test Sample Solution

One milliliter of the HLJDT aqueous extract was mixed with four volumes of methanol, vortexed thoroughly, and centrifuged. A 1 mL aliquot of the supernatant was then filtered through a 0.45 μm microporous membrane for HPLC analysis.

2.3.2. Chromatographic Conditions

HPLC analysis was performed using a Shimadzu LC-20A system (Shimadzu, Japan) equipped with a reversed-phase C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of 0.03 mg/mL potassium dihydrogen phosphate and acetonitrile in a ratio of 70:30 (v/v). The flow rate was set to 1.0 mL/min, the detection wavelength was 345 nm, the column temperature was maintained at 25˚C, and the injection volume was 20 μL.

2.3.3. Preparation of HLJDT Antimicrobial Susceptibility Test Discs

The aqueous extract of HLJDT was concentrated to prepare three stock solutions with concentrations of 1500 mg/mL, 750 mg/mL, and 375 mg/mL. For each disc, 15 μL of the HLJDT solution was pipetted onto a sterile blank antimicrobial susceptibility test disc (purchased from Beckman Bio, China). After complete absorption, the discs were dried in a 37˚C oven for 18 hours. This process was repeated by applying an additional 15 μL of the same solution onto the same disc, followed by drying under identical conditions. The fully dried discs were then sealed in sterile glass bottles and stored at 4˚C for subsequent use.

2.3.4. Disc Diffusion Method for Measuring Inhibition Zone Diameter

Pure *S. aureus* colonies cultured for 18 - 24 hours were selected for testing. A bacterial suspension was prepared to a turbidity equivalent to 0.5 McFarland standard. Using a sterile cotton swab, the suspension was evenly spread across the entire surface of a Mueller-Hinton (M-H) agar plate. HLJDT antimicrobial susceptibility test discs were then placed on the surface of the inoculated agar. Plates were incubated at 35˚C for 18 hours. The diameter of the inhibition zone was measured using a vernier caliper, and antibacterial activity was assessed based on the size of the inhibition zone.

2.3.5. Broth Microdilution Method

The minimum inhibitory concentration (MIC) of HLJDT against clinically isolated *S. aureus* strains was determined following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018 edition) [CLSI, 2018]. HLJDT was serially diluted in Tryptone Soya Broth (TSB; Oxoid, UK) to achieve final concentrations of 187.5 mg/mL, 93.75 mg/mL, 46.88 mg/mL, and 23.44 mg/mL. A 96-well microtiter plate was used, with each well containing 100 μL of the diluted HLJDT solutions. A bacterial suspension equivalent to 0.5 McFarland was further diluted 1:1000 in TSB, and 100 μL of this dilution was added to each test well, except for the negative control (medium only) and positive control (bacteria without HLJDT). The plate was incubated at 35˚C for 24 hours. The MIC was defined as the lowest concentration of HLJDT that inhibited visible bacterial growth (*i.e.*, no turbidity).

2.4. Statistical Analysis

Quantitative data with a normal distribution were expressed as mean ± standard deviation ($\chi \pm s$).

Paired or unpaired t-tests were applied for comparisons between groups, with a *P*-value < 0.05 considered statistically significant. Correlations between variables were assessed using Spearman's rank correlation analysis. A correlation coefficient (r) < 0.3 was interpreted as weak, 0.3 - 0.5 as moderate, and >0.5 as a strong correlation. Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA), and significance was set at $P < 0.05$.

3. RESULTS

3.1. Distribution of Sample Types of *S. aureus* Isolates

A total of 64 clinical isolates of *S. aureus* were included in this study. The specimens were obtained from various clinical sources, including sputum, throat swabs, bronchoalveolar lavage fluid, pus, wound exudates, intravenous catheters, blood cultures, tissue fluids, and abscesses. Among these, sputum was the most common source, accounting for 24 isolates (37.5%), followed by throat swabs (12 isolates, 18.7%) and bronchoalveolar lavage fluid (5 isolates, 7.8%). These three specimen types together represented 64% of all isolates. Of the 64 isolates, 18 (28.1%) were identified as methicillin-sensitive *S. aureus* (MSSA), and 46 (71.9%) were methicillin-resistant *S. aureus* (MRSA), indicating a predominance of drug-resistant strains among the clinical cases. Among the 18 MSSA isolates, 10 strains (55.5%) were derived from pus and wound secretions, comprising more than half of the MSSA group. In contrast, 36 of the 46 MRSA isolates (78.2%) originated from respiratory tract specimens, including sputum, throat swabs, and bronchoalveolar lavage fluid (Table 1).

Table 1. Types of clinical isolates of *Staphylococcus aureus* (n).

Sample Type	MSSA	MRSA	Total
Sputum	4	20	24
Throat Swab	0	12	12
Bronchoalveolar Lavage Fluid	1	4	5
Wound Secretions	10	3	13
Intravenous Catheter	0	1	1
Blood Culture	1	3	4
Tissue Fluid	2	2	4
Pus	0	1	1
Total	18	46	64

3.2. Preparation of Antimicrobial Susceptibility Test Discs of HLJDT

3.2.1. HPLC Analysis of HLJDT

To ensure the quality and consistency of HLJDT antimicrobial susceptibility test discs, the concentration of berberine—the principal antibacterial constituent of HLJDT—was determined using HPLC for quality control purposes. Under the specified chromatographic conditions, the HLJDT sample was injected, and the corresponding peak area was recorded. A standard calibration curve was constructed by plotting berberine concentration (*x*-axis) against peak area (*y*-axis) (Figure 1(A), Figure 1(B)). The resulting linear regression equation was:

$$y = 638090 + 72955x, r = 0.99992$$

Based on this calibration, the berberine content in the HLJDT extract was determined to be 2358.0 μg/Ml (Figure 1(C)).

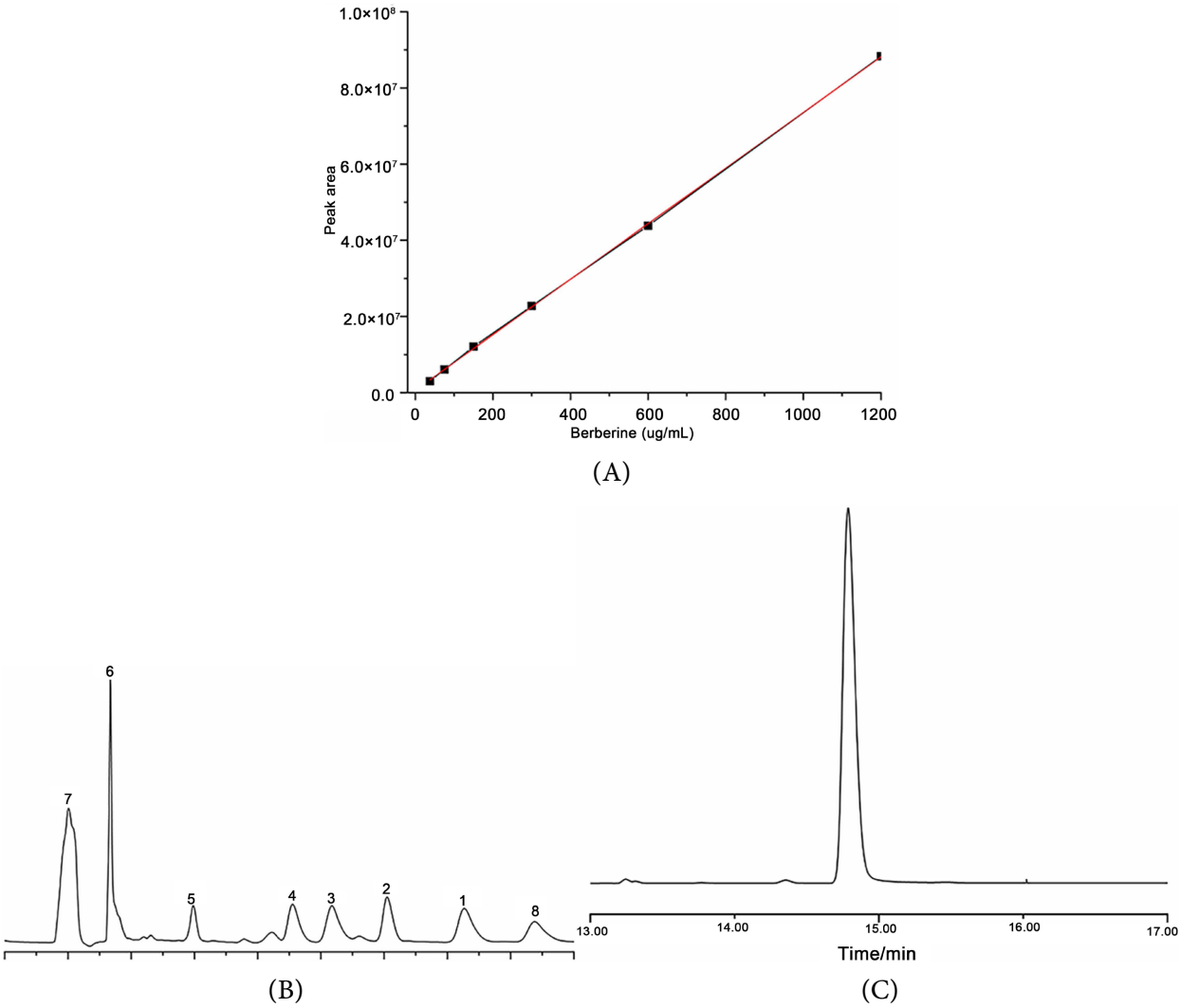

Figure 1. **High-Performance Liquid Chromatography Mass Spectrum Analysis. (A) Standard curve of berberine standard substance, (B) Chromatogram of HLJDT, 1: Berberine, (C) Chromatogram of berberine.**

3.2.2. Antibacterial Activity of HLJDT Susceptibility Test Discs with Varying Drug Contents against Quality Control Strains

To evaluate the antibacterial efficacy of HLJDT at different concentrations, 15 μL of HLJDT extract was initially applied to blank antimicrobial susceptibility test discs, resulting in final drug contents of 22.5 mg, 11.25 mg, and 5.625 mg per disc, respectively. The discs were tested against standard MRSA and MSSA quality control strains—USA300 and ATCC 29213—using conventional 1.0 mm antimicrobial susceptibility test discs. Initial results showed that none of the discs produced significant inhibition zones against either strain (**Figure 2(A)**, **Figure 2(B)**). To increase drug loading, an additional 15 μL of HLJDT was applied to the previously dried discs and then re-dried at 37˚C for 18 hours. This double-application method yielded final effective drug contents of 45 mg, 22.5 mg, and 11.25 mg per disc. Antibacterial tests using the re-prepared discs demonstrated that discs containing 45 mg and 22.5 mg of HLJDT produced clear inhibition zones against *S. aureus*, indicating appreciable antibacterial activity (**Figure 2(C)**, **Figure 2(D)**). Furthermore, the inhibition zone diameter observed for the MRSA strain USA300 was significantly larger than that for the MSSA strain ATCC 29213 (**Table 2**). In contrast, discs with the lowest concentration (11.25 mg) exhibited only small inhibition zones and did not achieve satisfactory antibacterial effects.

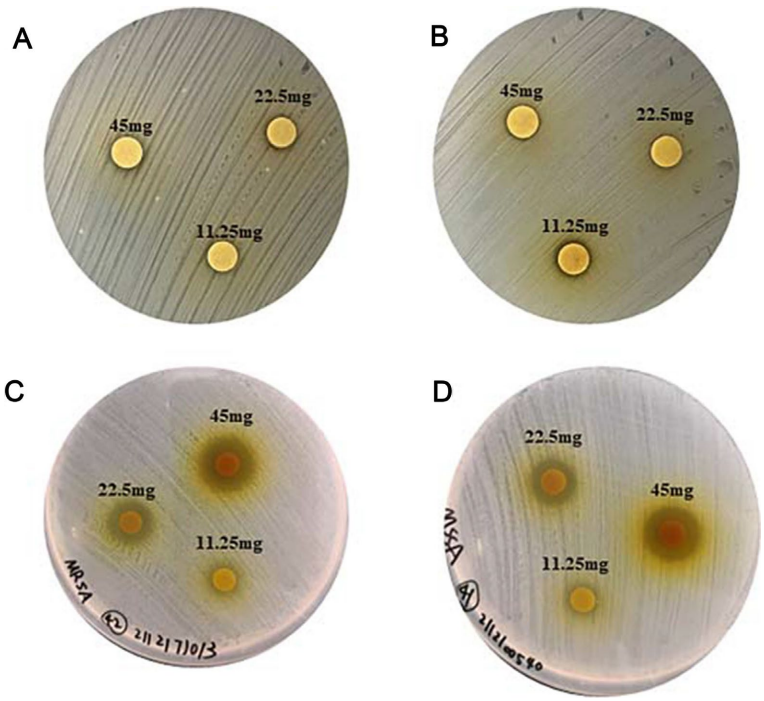

Figure 2. Antibacterial effect of different injection volumes and different contents of susceptibility test disc on quality control strains USA300 and ATCC29213, (A) 15 μL, USA300, (B) 15 μL, ATCC29213, (C) 30 μL, USA300, (D) 30 μL, ATCC29213.

Table 2. Inhibition zone diameter (mm) of susceptibility test disc on quality control strains.

	Inhibition Zone Diameter (mm)		
Strain	45 mg	22.5 mg	11.25 mg
USA300	13.0	11.0	7.0
ATCC29213	12.0	9.5	7.0

3.2.3. Evaluation of Uniformity in HLJDT Susceptibility Test Discs

To assess the uniformity and consistency of the HLJDT susceptibility test discs, six discs, each containing 45.0 mg and 22.5 mg of HLJDT, were randomly selected based on the results from Section 3.2.2. These discs were tested against the MRSA quality control strain USA300. The inhibition zone diameters produced by both concentrations were highly consistent across all samples, demonstrating good batch uniformity and preparation stability. These findings confirm that the HLJDT susceptibility test discs meet quality requirements and are suitable for use in subsequent experiments (Figure 3).

3.2.4. Antibacterial Activity of HLJDT Susceptibility Test Discs with Different Thicknesses

To investigate the effect of disc thickness on antibacterial efficacy, two types of susceptibility test discs—1.0 mm and 0.7 mm—were prepared, each loaded with 30 μL of HLJDT extract. Five MRSA strains (MRSA01-05) and five MSSA strains (MSSA01-05), randomly selected from previously isolated clinical samples, were tested. Each group was exposed to discs of both thicknesses and prepared with three different drug contents: 45 mg, 22.5 mg, and 11.25 mg. The results showed that for MRSA strains, the inhibition zone diameters produced by the 1.0 mm discs were significantly smaller (12.75 ± 2.04 mm, 8.17 ± 1.94 mm, and 6.33 ± 0.52 mm for high, medium, and low concentrations, respectively) compared to those produced by

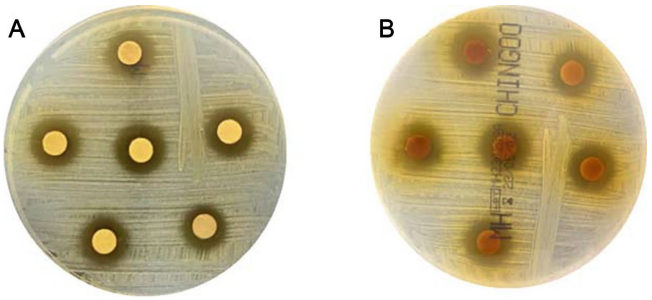

Figure 3. Uniformity verification results of susceptibility test disc. (A) 22.5 mg, (B) 45 mg.

the 0.7 mm discs (16.75 ± 2.04 mm, 13.08 ± 1.69 mm, and 8.00 ± 1.90 mm), with statistically significant differences observed across all concentrations ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively) (Table 3, Figure 4). For MSSA strains, inhibition zone diameters between the two disc thicknesses showed no statistically significant differences ($P > 0.05$) at the high (45 mg) and low (11.25 mg) concentrations (Table 4, Figure 5). However, at the medium concentration (22.5 mg), the 0.7 mm discs (10.09 ± 1.63 mm) produced significantly larger zones than the 1.0 mm discs (8.83 ± 1.51 mm), with a statistically significant difference ($P < 0.05$). These findings suggest that HLJDT susceptibility test discs with a thickness of 0.7 mm exhibit superior antibacterial performance compared to the conventional 1.0 mm discs, particularly against MRSA strains.

Table 3. Size of inhibition zone of HLJDT susceptibility test Disc on MRSA.

	45 mg		22.5 mg		11.25 mg	
	1.0 mm/mm	0.7 mm/mm	1.0 mm/mm	0.7 mm/mm	1.0 mm/mm	0.7 mm/mm
USA300	13.0	11.0	11.0	13.0	13.0	8.0
MRSA01	11.0	7.0	7.0	12	12	6.0
MRSA02	12.0	7.0	7.0	13.5	13.5	9.0
MRSA03	14.0	8.0	8.0	13	13	8.0
MRSA04	10.5	6.0	6.0	11	11	6.0
MRSA05	16.0	10.0	10.0	16	16	11.0
Mean ± SD	12.75 ± 2.04	16.75 ± 2.04	8.17 ± 1.94	13.08 ± 1.69	6.33 ± 0.52	8.00 ± 1.90
p-value	<0.001		<0.001		<0.01	

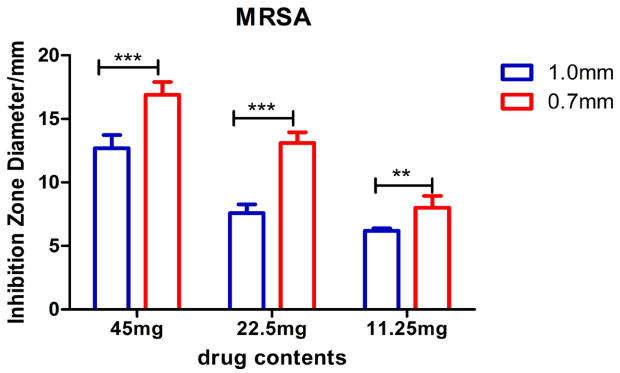

Figure 4. Comparison of inhibition zone diameter of HLJDT susceptibility test Disc on MRSA. ***$p < 0.00\ 1$, **$p < 0.01$, *$p < 0.05$ indicate statistically significant differences.

Table 4. Size of inhibition zone of HLJDT susceptibility test Disc on MSSA.

	45 mg		22.5 mg		11.25 mg	
	1.0 mm/mm	0.7 mm/mm	1.0 mm/mm	0.7 mm/mm	1.0 mm/mm	0.7 mm/mm
ATCC29213	12.0	13.0	9.5	11.5	7.0	7.0
MSSA01	10.0	10.0	6.0	8.0	6.0	6.0
MSSA02	13.0	13.0	10.0	11.0	7.0	7.0
MSSA03	12.0	13.0	10.0	11.0	6.0	8.0
MSSA04	13.0	13.5	8.5	11.0	6.0	7.0
MSSA05	11.0	10.0	9.0	8.0	6.0	6.0
Mean ± SD	11.83 ± 1.17	12.08 ± 1.63	8.83 ± 1.51	10.09 ± 1.63	6.33 ± 0.52	6.83 ± 0.75
P-value	>0.05		<0.01		>0.05	

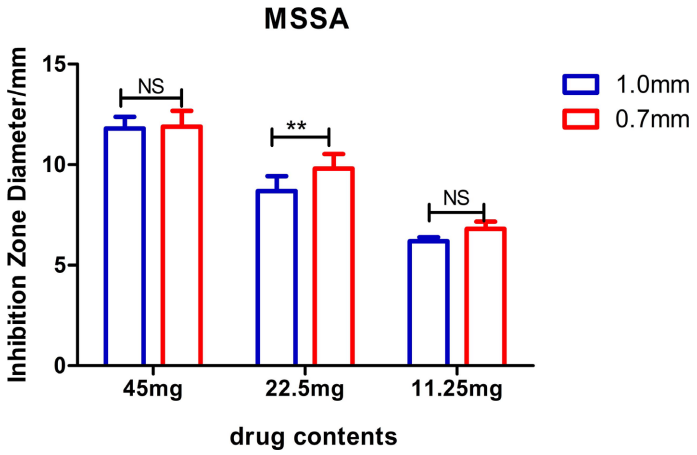

Figure 5. Comparison of inhibition zone diameter of HLJDT susceptibility test Disc on MSSA. $^{**}P < 0.01$ indicates a statistically significant difference.

3.2.5. Effect of Incubation Time on Inhibition Zone Diameter of HLJDT Susceptibility Test Discs

To evaluate the temporal stability of the HLJDT susceptibility test discs, three clinical *S. aureus* strains were randomly selected and tested using discs containing high, medium, and low concentrations of HLJDT. Inhibition zone diameters were measured at four different incubation time points: 18, 20, 22, and 24 hours. The results demonstrated that the variation in inhibition zone diameters across the different time points was minimal, ranging from only 0.5 to 1.0 mm for all three concentration groups (Table 5). These slight fluctuations did not affect the interpretation of the susceptibility results, indicating that the HLJDT discs exhibit good temporal stability under standard incubation conditions.

Table 5. Variation in diameter of inhibition zone of susceptibility test disc at different culture times.

Culture Time	Strain A			Strain B			Strain C		
	45 mg	22.5 mg	11.25 mg	45 mg	22.5 mg	11.25 mg	45 mg	22.5 mg	11.25 mg
18 h	15.0	11.0	7.0	13.5	10.0	6.0	12.0	8.0	6.0
20 h	15.0	11.0	6.5	13.5	10.0	6.0	12.0	8.0	6.0

Continued

22 h	14.5	11.0	6.5	13.0	9.0	6.0	11.5	8.0	6.0
24 h	14.5	11.0	6.5	12.5	9.0	6.0	11.5	8.0	6.0

3.3. Clinical Application of HLJDT Susceptibility Test Discs

3.3.1. Antibacterial Activity of HLJDT Susceptibility Test Discs Against Clinically Isolated *S. aureus* (MRSA and MSSA)

Based on the findings in Section 3.2.4, susceptibility test discs with a thickness of 0.7 mm demonstrated superior antibacterial efficacy compared to those with a thickness of 1.0 mm. Therefore, all subsequent experiments were conducted using the 0.7 mm discs. Susceptibility testing was performed on 46 MRSA strains, 18 MSSA strains isolated from clinical specimens, and two quality control strains. HLJDT susceptibility test discs at three different concentrations (45 mg, 22.5 mg, and 11.25 mg) were used to evaluate *in vitro* antibacterial activity. The results showed that HLJDT exhibited measurable inhibitory effects on both MRSA and MSSA strains, with inhibition zone diameters increasing in a concentration-dependent manner. This indicates a dose-response relationship between HLJDT concentration and antibacterial efficacy. At high and medium concentrations (45 mg and 22.5 mg), HLJDT susceptibility test discs produced significantly larger inhibition zones against MRSA strains (15.33 ± 2.37 mm and 11.82 ± 2.48 mm, respectively) compared to MSSA strains (13.50 ± 1.68 mm and 9.87 ± 1.60 mm), with statistically significant differences ($P < 0.01$). However, no statistically significant difference was observed between MRSA and MSSA for the low concentration group (11.25 mg, $P > 0.05$) (Figure 6).

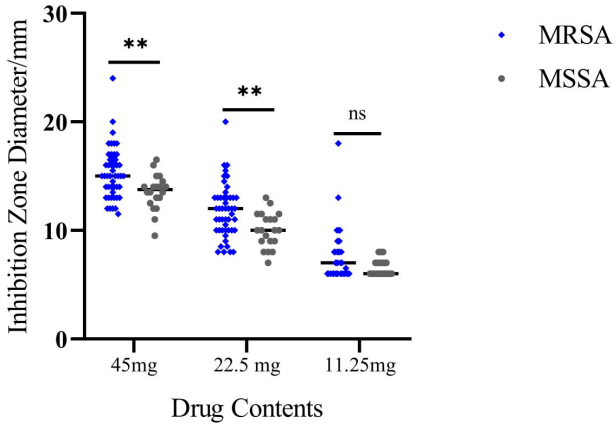

Figure 6. Comparison of the inhibition zone diameters of *Staphylococcus aureus* using different concentrations of HLJDT on susceptibility test disc. **$P < 0.01$ indicates a statistically significant difference.

3.3.2. Correlation Analysis between Disc Diffusion Method and Microbroth Dilution Method

The MIC of HLJDT was determined for all 64 clinical *S. aureus* isolates using the microbroth dilution method. Correlation analysis was then conducted to compare MIC values with the inhibition zone diameters obtained from the HLJDT susceptibility test discs (disc diffusion method). For MRSA strains, the inhibition zone diameter of the high-concentration (45.0 mg) HLJDT discs showed a strong negative correlation with MIC values ($r = -0.501$, $P < 0.01$), indicating that larger inhibition zones were associated with lower MICs. At medium (22.5 mg) and low (11.25 mg) concentrations, the correlations were moderate ($r = -0.327$, $P < 0.05$; $r = -0.353$, $P < 0.05$, respectively). In contrast, no statistically significant correlation was observed between inhibition zone diameters and MIC values for MSSA strains across all three HLJDT concentrations ($r = 0.015$, $r = 0.386$, $r = -0.034$; $P > 0.05$) (Table 6). These results suggest that for MRSA, the HLJDT

susceptibility test disc—particularly the 0.7 mm disc containing 45.0 mg of the drug—provides a reliable and predictive measure of antibacterial activity, supporting its potential use in clinical and pharmacological research.

Table 6. Correlation analysis between susceptibility test disc method and microbroth dilution method.

	MRSA (46 cases)		MSSA (18 cases)	
Susceptibility Test Disc	r-value	*P*-value	r-value	*P*-value
45.0 mg	−0.501	0.000	0.015	0.952
22.5 mg	−0.327	0.025	0.386	0.103
11.25 mg	−0.353	0.015	−0.034	0.889

Note: $^{**}P < 0.01$ indicates a statistically significant difference; $P > 0.05$ indicates no statistically significant difference.

4. DISCUSSION

In recent years, the identification of antibacterial components within TCM has become a major area of research interest. Previous studies have demonstrated that various TCM ingredients—such as Huanglian, Huangqin, Lianqiao, Dahuang, and Huangbo—exhibit differing degrees of antibacterial activity against *S. aureus* [12]. Furthermore, combinations of muscone, quercetin, cefuroxime, and imipenem have been reported to produce synergistic antibacterial effects against methicillin-resistant *S. aureus* (MRSA) [13]. In our previous studies, HLJDT demonstrated potent antibacterial activity, particularly against Gram-positive bacteria, including *S. aureus*, as determined by the microbroth dilution method [14-16]. The microbroth dilution method remains a gold standard for determining MICs, it involves discontinuous dilution steps, requires technical expertise, and is susceptible to variability due to factors such as inoculum density, drug batch consistency, and subjective interpretation of results. Consequently, it is not yet feasible to fully replace the paper disc diffusion method in routine clinical practice [17]. The disc diffusion method, on the other hand, is user-friendly, cost-effective, and provides visually intuitive results. Additionally, susceptibility test discs are easy to manufacture and store, and they show good stability over time. However, in clinical settings, only commercially available antibiotic discs are widely used, with few options available for TCM formulations or monomers. A growing body of evidence supports the antibacterial efficacy of individual TCM components and compound prescriptions [18, 19]. In this context, the development and validation of self-prepared HLJDT susceptibility test discs, as described in our study, not only provide a practical method for evaluating antibacterial activity but also lay an important methodological foundation for future research and potential clinical applications of HLJDT and similar TCM-based therapies.

HLJDT is a complex TCM formulation comprising multiple herbal components with varying concentrations, making it challenging to standardize the effective antibacterial constituents and their concentrations when preparing susceptibility test discs. Previous studies have identified berberine—a key alkaloid monomer in HLJDT—as one of its primary antibacterial agents [14-16]. Using the microbroth dilution method, the MIC of berberine against MRSA has been reported to range from 32 to 256 μg/mL, corresponding to an effective drug quantity of 3.2 to 25.6 μg. In the present study, HPLC analysis determined the berberine concentration in the aqueous extract of HLJDT to be 2358.0 μg/mL, equivalent to 15.72 mg/g. This finding is consistent with results reported by Kwok *et al.* [20], who analyzed ten batches of HLJDT from various sources and found that only one batch met the established quality standards, with a berberine content of 16.49 mg/g. These results support the conclusion that the HLJDT aqueous extract prepared in this study was of high quality and suitable for susceptibility test disc preparation. Utilizing berberine content as a quantitative standard for HLJDT extract ensures consistency in the preparation of susceptibility test discs and minimizes inter-batch variability. This standardization is essential for improving reproducibility and clinical reliability.

Currently, limited research has been conducted on the preparation of susceptibility test discs for

compound TCM decoctions. One possible reason is the difficulty in achieving sufficient drug loading onto the paper discs during preparation. In this study, we optimized the disc preparation method by introducing a two-step loading process—applying the HLJDT solution in two stages, each followed by drying at 37°C. This approach increased the total drug content per disc to 45 mg. Subsequent HPLC analysis showed that the effective berberine content per disc reached 707.4 μg. Experimental validation demonstrated that the self-prepared HLJDT discs exhibited strong antibacterial activity against the quality control strains USA300 (MRSA) and ATCC 29213 (MSSA), meeting the basic performance requirements for potential clinical application.

The research results demonstrated that sensitivity paper discs containing 45.0 mg and 22.5 mg of the drug produced significant inhibition zones against the quality control strains USA300 and ATCC29213, indicating strong antibacterial activity against *S. aureus*. These findings are consistent with our previous studies [14-16]. To further optimize the quality of HLJDT, two types of paper discs with different thicknesses (0.7 mm and 1.0 mm) were evaluated. The 0.7 mm discs exhibited superior antibacterial effects compared to the 1.0 mm discs. As the performance of the disc diffusion method can be influenced by the material, diameter, and thickness of the paper discs—which affect the drug diffusion rate and the accuracy of the susceptibility test results [17]—thinner paper discs are more conducive to drug penetration and diffusion in the agar medium, resulting in better antibacterial effects. Additionally, we assessed the performance of the self-prepared paper discs in terms of uniformity, stability, and incubation time. The results confirmed that the 0.7 mm self-made discs showed stable performance and are suitable for batch production.

In this study, a total of 64 clinical *S. aureus* isolates were collected, comprising mainly drug-resistant strains, including 46 MRSA and 18 MSSA isolates. The susceptibility results showed that HLJDT discs at different concentrations exhibited antibacterial activity against both MSSA and MRSA. Notably, the inhibition zone diameters for MRSA were significantly larger than those for MSSA when using high- and medium-concentration discs. Moreover, the inhibition zone size for MRSA strains was significantly negatively correlated with MIC, whereas no such correlation was observed for MSSA strains, suggesting that HLJDT has stronger antibacterial activity against MRSA. Consistent with our previous research, HLJDT has demonstrated potent antibacterial effects against MRSA. Electron microscopy and RNA-seq analyses further revealed that the active component berberine significantly upregulates the expression of cell wall hydrolysis genes ssaA and lytM, enhancing cell wall hydrolytic activity and resulting in cell wall lysis [14-16].

In addition, since MRSA expresses the mecA gene, which encodes PBP2a (penicillin-binding protein 2a)—a membrane protein located on the bacterial surface—it can regulate the synthesis of peptidoglycan, the major component of the bacterial cell wall, thereby conferring resistance to all β-lactam antibiotics. It is speculated that berberine may alter the cell wall structure of MRSA, further affecting the expression and activity of PBP2a, thereby influencing peptidoglycan synthesis, effectively inhibiting MRSA drug resistance and exerting antibacterial effects [2]. Furthermore, in this study, there was no observed correlation between the disc diffusion results and MIC values for MSSA, which may be due to the relatively small number of MSSA isolates. We will continue to investigate the antibacterial mechanisms of HLJDT against MRSA in future studies.

5. CONCLUSION

In summary, this study established a method for preparing susceptibility test discs for the traditional Chinese medicine compound HLJDT and successfully produced discs with a thickness of 0.7 mm and a drug content of 45.0 mg. These discs demonstrated good stability, uniformity, and antibacterial activity, meeting the requirements for clinical application. This provides a convenient and reliable methodological basis for further research and clinical use of HLJDT's antibacterial effects.

6. LIMITATIONS OF THE STUDY

This research is classified as methodological experimental research. It is acknowledged that minor errors may occur during the preparation of each drug sensitivity disc due to the involvement of multiple

preparers. In addition, the number of bacterial isolates included was limited, and the small sample size may have masked true correlations due to random error. In future studies, we will expand the sample size and further analyze the correlation between inhibition zone size and MIC in MSSA isolates.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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