Kor. J. Microbiol. Biotechnol.
Vol. 36, No. 1, 82–85(2008)

Antibacterial Activity of HTI Isolated from Oriental Medicine, *Hyungbangjihwang-tang*

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Hyungbangjihwang-Tang (HT), an Oriental herbal formula, has been known to play a role which helps to recover vigor of human in the Orient. In this study, antibacterial substance (HTI) was purified from the ethyl-acetate extracts of HT by using SiO_2 column chromatography and HPLC, and the antibacterial effects of HTI were investigated. By using the CLSI broth micro-dilution assay, the activity of HTI was evaluated against human pathogenic Gram-positive and Gram-negative bacterial strains including the clinical isolates of methicillin-resistant *Staphylococcus aureus*. The results demonstrated that HTI showed broad spectrum antibacterial activities against all bacterial strains tested. In conclusion, HTI is an interesting new molecule for its potential in anti-infective drug discovery and for future studies on activity-structure relationship through analysis of its chemical structure.

Key words: *Hyungbangjihwang-Tang*, antibacterial activity, oriental medicine, methicillin-resistant *Staphylococcus aureus*

The ever-increasing development of pathogenically microbial resistance to traditional antibiotics has reached alarming levels [5]. Specially, the methicillin-resistant *Staphylococcus aureus* (MRSA) strains that appeared in the 1970s are highly resistant not only to β-lactams but also to many other antibiotics [2]. Because of MRSA has spread world-wide, infections caused by it have posed a serious problem for hospitalized patients, especially compromised hosts [8, 11]. Vancomycin (VCM) is the first choice antibiotic for severe MRSA infections; but, its use often evokes unexpected side-effects and the development of microbial resistance to this antibiotic [1, 10]. Therefore, there is an inevitable and urgent medical need for antibiotics with novel antibacterial mechanisms.

Chinese herbal formulas evolved through thousands of years of clinical practice. It appears that many of the ancient combination formulas have sound scientific basis through modern pharmacological evaluation. Significant chemical changes occurred during the decoction process of a prescribed herbal formula. Through decoction process, some toxic ingredients were significantly reduced and new active compounds generated due to the chemical interactions among the ingredients [7].

Hyungbangjihwang-Tang (HT), an oriental herbal formula, has been known to play a role which helps to recover vigor of human in the Orient [6]. It has been used for treatment of various diseases including empyema, rhinitis, and tonsillitis [6]. Though valued biological abilities of HT are known, however, the potential functions of HT as an antibacterial agent still remain unknown.

In this study, we purify antimicrobial substance (HTI) from the ethyl-acetate extracts of HT by using silica column chromatography and finally HPLC with Si60 normal-phase column, and report on the antibacterial effects of HTI toward human pathogenic Gram-positive and Gram-negative bacterial strains including the clinical isolates of methicillin-resistant *Staphylococcus aureus*.

Extraction and Isolation of HTI

An extract of HT was prepared by decocting the dried prescribed nine kinds of oriental medicinal herbs with boiling distilled water (26 g/L). The duration of decoction was about 3 h. After celite filtration water extract was washed with n-hexane and extracted with ethyl acetate, ether and chloroform. The ethyl acetate-soluble fraction

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showed the highest inhibitory activity against Methicillin-resistant *Staphylococcus aureus* (MRSA). The ethyl acetate extract was evaporated to dryness in vacuo to yield 0.4 g of dark brownish crude oil. This crude material was chromatographed on silica gel column using EtOAc:hexane (from 2:1 to 5:1) as elution solvent with stepwise. The fraction with 0.4 Rf value on silica TLC (hexane:ethyl acetate = 1:2) showed the highest inhibitory activity against MRSA. The active fraction was concentrated in vacuo to give partially purified material. The purified active fraction was further purified by preparative HPLC equipped with a Lichrosorb Si60 column (10 μm; Merck, Darmstadt, Germany) with PDA detecter at room temperature. The mobile phase consisted of hexane:ethylacetate (1:1) and the flow rate was 1.0 mL/min. The HPLC instrumentation included an CBM-10A system controller (Shimadzu, Kyoto, Japan), a Shimadzu LC-10AT pump, a Shimadzu DGU-14A degasser, an SIL-10A auto sampler, a Shimadzu SPD-10A detector (set at 280 nm), and an computer running Shimadzu software version CLASS-LC10, and active material (HTI) was obtained 33 mg of light browny oil (final yield: 0.12%), (Fig. 1). HTI was prepared in DMSO and stored at -20°C. For all the experiments, final concentration of 2% DMSO was used as solvent carrier. The ingredients of 26 g HT include 4 g of *Ostericum koreanum,* 4 g of *Aralia contientalis,* 4 g of *Ledebouriella seseloides,* 4 g of *Poria cocos,* 2 g of *Corns officinalis,* 2 g of *Rehmannia glutinosa,* 2 g of *Plantago asiatica,* 2 g of *Alisma canaliculatum, and* 2 g of *Schizonepeta tenuifolia var. japonica.* These plant materials were purchased from the department of pharmacy, Daegu Oriental Hospital of Daegu Haany University, Daegu, Korea, and their voucher specimens have been deposited in the same institution.

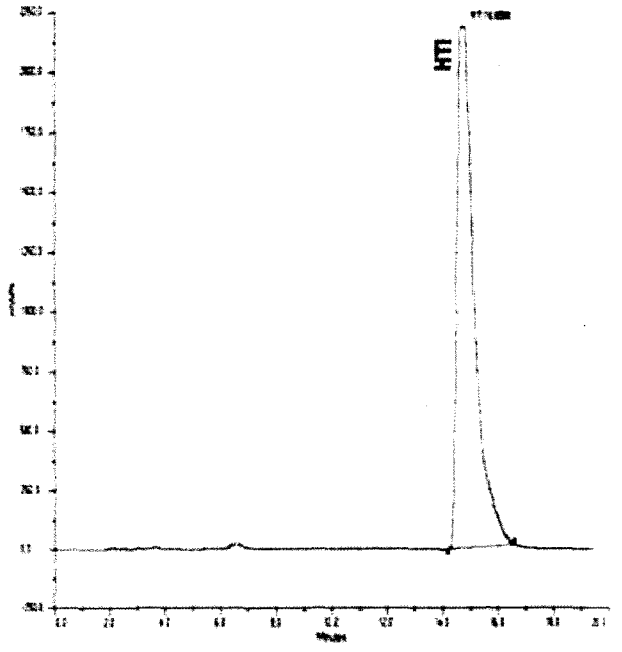

Fig. 1. Silica Si-60 column HPLC chromatogram of the purified HTI isolated from *Hyungbangjihwang-Tang*.

Determining of Antimicrobial Susceptibility

Bacillus subtilis (KCTC 1918), *Staphylococcus aureus* (KCTC 1621), *Staphylococcus epidermidis* (KCTC 1917) and *Escherichia coli* (KCTC 1682) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were clinically isolated from nosocomial patients from the Kyungpook National University Hospital Daegu, Korea and *Escherichia coli* O-157 (ATCC 46895), *Vibrio vulnificus* (ATCC 29307) were obtained from American Type Culture Collection (ATCC) at Manassas, Virginia, USA. Bacterial cells were cultured in a Mueller-Hinton broth containing beef extract powder, acid digest of casein, soluble starch (21 g/L) with aeration at 37°C.

Bacterial cells (2×10^7/mL) were inoculated into a Mueller-Hinton broth and dispensed 0.1 mL/well in 96-well microtiter plates. MICs were determined by a serial two-fold dilution of test compounds, following the recommendations of the Clinical and Laboratory Standards Institute [4]. After 24 h of incubation at 37°C, the minimal compound concentration that prevented the growth of a given test organism was determined and was defined as the MIC. Growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, California, USA) by monitoring absorption at 620 nm. In the current study, propionic acid was used as a positive control; propionic acid is an antibacterial agent that is widely used as a food preservative agent [3]. HTI in MIC values of 20-40 μg/mL showed antibacterial activities against Gram-positive and Gram-negative bacterial strains. HTI exhibited more potent activities than propionic acid, showing the MIC values of 20-80 μg/mL on all bacterial strains. HTI also showed antibacterial activities against clinically isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). HTI exhibited similar activities as those of propionic acid, showing MIC

Table 1. Antibacterial activities of HTI.

Microorganisms		MIC (μg/mL)	
		HTI	propionic acid
Gram-positive bacteria	*B. subtilis*	20	40
	S. epidermidis	20	40
	S. aureus	40	40
	MRSA 1 *	100	100
	MRSA 2	100	100
	MRSA 3	100	100
Gram-negative bacteria	*E. coli*	20	40
	E. coli O-157	20	80
	V. vulnificus	20	40

*MRSA: Methicillin-resistant *Staphylococcus aureus*.

values of 100 μg/mL toward MRSA (Table 1).

Many antimicrobial agents are limited in clinical applications, as they bring about cytolysis of human erythrocytes. To estimate the cytotoxicity of HTI against human erythrocytes, hemolytic activity was evaluated at various concentrations (from 6.25 to 100 μg/mL) of HTI. The hemolytic activity of HTI was evaluated by determining the release of hemoglobin from a 4% suspension of fresh human erythrocytes at 414 nm with an ELISA plate Reader [9]. The hemolysis percentage was calculated using the following equation: Percentage hemolysis = [($Abs_{414\ nm}$ in the HTI solution - $Abs_{414\ nm}$ in a PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 - $Abs_{414\ nm}$ in a PBS)] × 100. The result showed that HTI exhibited no hemolysis activities at all concentration levels (data not shown). In conclusion, these results demonstrate HTI has considerable antimicrobial activity, deserving further investigation on activity-structure relationship through analysis of its chemical structure for clinical applications.

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(Received Jan. 30, 2008/Accepted Feb. 28, 2008)

국문초록

형방지황탕으로부터 분리된 HTI의 항균 활성

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형방지황탕은 동양 고유의 약처방으로 사람의 기력 회복에 도움을 주는 것으로 알려져 있다. 본 연구에서는 SiO_2 컬럼 크로마토그래피와 HPLC를 이용하여 형방지황탕 유래의 항균 물질 (HTI)을 분리하여 그의 항균 활성을 측정하였다. 인체 감염성 그램-양성균, 그램-음성균 및 환자에게서 분리된 메티실린-내성 황색 포도상구균을 대상으로하여 CLSI 방법을 이용하여 HTI의 항균 활성을 측정하였다. 그 결과, HTI은 실험한 모든 세균에 대하여 넓은 범위의 항균 활성을 나타냄을 확인할 수 있었다. 이 결과를 바탕으로하여 HTI이 항균제로서의 발전 가능성을 가진 신물질임을 확인하였으며, HTI의 화학 구조 결정을 통하여 구조와 활성간의 연구를 시행할 것이다.