

Microdilution inhibition test of Chinese herbs to assess their effect against clinical strains of *Ureaplasma urealyticum in vitro*

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Abstract

Objective: To explore the antibacterial effect of Chinese crude drugs against clinical strains of *Ureaplasma urealyticum*(UU), including eight pure herbs and three compound herbs, and determine their minimal inhibitory concentrations(MICs). **Methods:** Isolates were collected from clinical patients with UU infection, and cultured in UU broth. In order to test the different effects on clinical strains of UU, the assays were performed by microdilution inhibition tests, and MICs of the herbs against the clinical strains of UU were calculated. **Results:** The MICs of eight pure herbs against clinical strains of UU were as follows: Galla Chinensis: 0.313~1.25 g/L; Fructus Forsythiae: 1.25~5.00 g/L; Cortex Phellodendri: 1.25~5.00 g/L; Radix Paeoniae Rubra: 1.25~2.50 g/L; Semen Plantaginis: 2.50~10.00 g/L; Herba Lysimachiae: 5.00~20.00 g/L; Rhizoma Coptidis: 5.00~20.00g/L, Herba Houttuyniae: 10.00~20.00 g/L. The MICs of compound herbs were: Liuheji: 0.625~2.50 g/L; Bazhengsan: 5.00~20.00 g/L; Wulinsan: 2.50~20.0 g/L. **Conclusion:** Galla Chinensis, Fructus Forsythiae, Cortex Phellodendrim, Radix Paeoniae Rubra, and Semen Plantaginis, exerted the stronger antibacterial effect against clinical strains of UU, whereas Herba Lysimachiae, Rhizoma Coptidis and Herba Houttuyniae, had relatively weaker activity against UU. Compound herbs, Bazhengsan and Wulinsan, and particularly Liuheji, also had antibacterial effects against UU. Further studies of the effects and mechanisms of action of Chinese crude drugs against UU infections are worthwhile.

Keywords: *Ureaplasma urealyticum*; microdilution inhibition test; Chinese herbs; minimal inhibitory concentration; antibacterial

INTRODUCTION

Presently, *Ureaplasma urealyticum*(UU) is a common sexually transmitted disease(STD) pathogen that may cause non-gonococcal urethritis, prostatitis, epididymitis^[1], sterility^[2,3], bacterial vaginosis^[4], chorioamnionitis^[5], premature delivery^[6], chronic lung disease in neonates^[7] and other medical problems. Thus UU is harmful to health and an infection must be treated effectively. With the widespread use of antibiotics in clinical practice, drug-resistance of UU has become more serious^[8], and the treatment of UU is facing a challenge. Hence, it is both urgent and necessary to develop new drugs to cure UU infection. In this paper, we report the results of

research on the antibacterial effect of Chinese crude herbal drugs against UU.

MATERIALS AND METHODS

Collection of clinical Strains

The bacterial species were obtained from patients in the STD department with patients' permission. All cases had symptoms of urinary frequency and urgency, and swabs were used to obtain urethral secretions in males, or cervical epithelial cell scrapings in female.

UU standard strains and UU broth

UU standard strains(ATCC27815, ATCC27816) were kindly provided by the Capital Institution of Pediatrics. UU broth was bought from Biomereux Company(France).

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UU culture and purification

Isolates were inoculated in UU broth at 37°C for 48h. The positive UU broth was filtered and then sub-cultivated up to the third generation. The concentration of UU in the broth was adjusted to 10⁶ color change unit (CCU), and stock samples were stored at -20°C.

Preparation of Chinese crude herbs

All Chinese medicinal herbs were from the Chinese Medicine Pharmacies of the First Affiliated Hospital of Anhui Medical University and were certified by herb experts. Compound herbs Wulinsan was composed of the following ingredients: Glabrous Greenbrier Rhizome 9g, Radix Angelica Sinensis 7g, Radix Glycyrrhiza 7g, Paeoniae Rubra 15g, Fructus Gardenia 15g. Compound Bazhengsan included: Semen Plantaginis, Dianthus Superbus L., Herba Polygoni Avicularis, French Chalinoplasty, Fructus Gardenia, Radix Glycyrrhiza, Caulis Akebiae and Rheum Officinale Baill each 9g. Compound Liuheji included: Galla Chinensis, Fructus Gardenia, Terminalia chebula Retz. and Garden Burnet each 9g; Radix Paeoniae Rubra 12g; Radix Glycyrrhiza 6g. Crude herbs were made by the distilled water boiling method, and the final concentration was 0.4 g/L.

Process of extracting Chinese herbs

① Herbs(100 g) were added to 200 ml distilled water, soaked for 30 min, and boiled for 30 min.

② The drug was filtered through gauze, 300 ml water added, and boiled for 30 min. An additional 250 ml water was added and the solution mixed.

③ After centrifugation at 4 000 r for 5min, the supernate was removed, placed into a 100ml saline bottle, and adjusted pH to 6.0 with 2 mol/L NaHCO₃ and 1 mol/L HCl.

④ The preparation was autoclaved at 121°C for 20 min, and the herb extract was stored at -4°C. The same manufacturing procedure was used for the compound herbs, Wulinsan, Bazhengsan and Liuheji.

Inhibition test of Chinese herbs against UU clinical isolates

Inhibition tests were performed using 96 well plates by the following dilution method. In the first well 40 μl of the herbal mixture was added to 160 μl culture broth, and 1:1(100 μl contents:100 μl culture broth) serial dilutions were used sequentially in the second to ninth well. To the final volume of 100 μl in each well was added 100 μl of inoculated UU broth(10⁶ CCU). The 10th to 12th wells were used as blank, negative and positive control, respectively. Plates were incubated at 37°C for 48h. Twenty replicate microdilution inhibition tests were performed with each herb, with 20 UU isolates from different clinical cases, and the two

standard UU strains.

Determination of experimental end points

When all of controls were valid, the results were recorded. UU growth was judged as positive when the color of the broth changed from yellow to red. MICs were calculated by conventional microbial criteria.

Statistical analysis

MIC50 and MIC90 were calculated using SPSS 13.0 statistical software.

RESULTS

MICs of eleven herbs against clinic UU isolates

The MIC values of the herbal preparations used in assays against UU clinical isolates are summarized in *Table 1*.

Table 1 Minimal inhibitory concentrations of Chinese crude drugs against UU clinical isolates *in vitro*

Drugs	MIC(g/L)	MIC(g/L)	
		50%	90%
Galla Chinensis	0.313~1.25	0.625	1.25
Radix Paeoniae Rubra	1.25~2.50	1.25	2.5
Fructus Forsythiae	1.25~5.00	2.5	5.0
Cortex Phellodendri	1.25~5.0	1.25	5.0
Semen Plantaginis	2.5~10.0	2.5	5.0
Herba Lysimachiae	5.00~20.0	10	20
Rhizoma Coptidis	5.00~20.0	10	20
Herba Houttuyniae	10.0~20.0	10	20
Bazhengsan	5.00~20.00	10	20
Wulinsan	2.50~20.0	10	10
Liuheji	0.625~2.50	1.25	2.5

Inhibitory effects of herbal preparations against UU standard strains

The two standard UU strains were sensitive to a MIC50 concentration of Galla Chinensis, Radix Paeoniae Rubra, Fructus Forsythiae, Cortex Phellodendri, Semen Plantaginis and Liuheji. When a MIC90 concentration was used, the standard strains were sensitive to all of the herbal preparations tested.

DISCUSSION

Nowadays, UU plays an important role in genital duct infectious disease. For the past few years, the percentage of STDs attributable to UU infection has increased, as the total number of UU infections has increased^[8]. At present, the most commonly used drugs for the clinical treatment of UU infections are macrocyclic lactones, tetracyclines, and quinolones. Unfortunately, due to the selective pressure and inductive functions of antibacterial agent, some UU isolates have become resistant to the above agents^[9]. In addition, the side effects of antibacterial agents are also a disadvantage to their clinical application. Thus there is a significant and urgent need to develop new drugs to control UU

infections. Compared with western drugs, Chinese herbs have a greater safety advantage. In fact, in ancient times, some herbs were used to treat genital duct infectious diseases and STDs in China. For example, the Chinese compound drugs Wulinsan and Bazhengsan from the Chinese medicine books, Hejijufang^[10], were used to treat STD with reported good results. Considering that some herbs have antibacterial and antiviral properties^[11], we carefully referred to a great many of Chinese tradition medicine books before selecting the above herbs and used microdilution inhibition tests to assess their effectiveness against clinical strains of UU *in vitro*. Based on Chinese traditional theory and previous experimental results, we also made the herbal compound Liuheji.

Our research results showed that *Galla Chinensis*, *Fructus Forsythiae*, *Cortex Phellodendri*, *Radix Paeoniae Rubra*1, and *Semen Plantaginis* exerted the stronger antibacterial effect on clinical strains of UU, whereas *Herba Lysimachiae*, *Rhizoma Coptidis*, and *Herba Houttuyniae* had weaker activities in inhibiting UU clinical isolates. Of the compound Chinese herbal medicines tested, Liuheji was the most effective, while Bazhengsan and Wulinsan had weaker antibacterial effects on the UU isolates.

Galla Chinensis nicknamed meretrix, is *cecidum* injured by parasitic aphids in the leaf or leafstalk toxicodendron of *Rhus chinensis* Mill, contains tannin, galic acid, acid resina and other compounds. Modern medicine has proved that water extractions of gallnut can improve the expression of interleukin-6 by monocyte^[12]. Gallnut apozem can inhibit or kill staphylococci, pneumococci, streptococcus, typhoid bacillus, *Bacillus dysenteriae*, *Bacillus diphtheriae*, anthrax bacillus, *Pseudomonas aeruginosa* and others^[11-13]. Additionally, Gallnut apozem can also strongly inhibit influenza virus infection in vaccinated chick embryos. For *Fructus Forsythiae*, *Cortex Phellodendri*, *Radix Paeoniae Rubra*1, *Semen Plantaginis*, *Herba Lysimachiae*, *Rhizoma Coptidis*, and *Herba Houttuyniae* and others, prior experiments confirmed their antiviral and antimicrobial properties^[11].

Compared to pure herbs, compound herbs, Wulinsan and Bazhengsan had higher MIC values, but they are reputed to strengthen immunity and improve the elimination of toxicants. Liuheji inhibited the growth of UU clinical isolates in our *in vitro* microdilution assay, and its inhibition was stronger than Wulinsan or Bazhengsan.

In addition, our experiments demonstrated that the above herbs had various degrees of inhibitory effect against UU standard strains.

In brief, some Chinese herbs can inhibit growth of UU clinical isolates, offering *in vitro* evidence and encouragement to physicians to develop new drugs from Chinese herbs to treat UU infectious disease.

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