**Biomedical Engineering**

Proteomic Analysis and Antibacterial Effects of Lithospermi Radix against Common Bacteria from Human Infected Wounds

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Proteomic Analysis and Antibacterial Effects of Lithospermi Radix against Common Bacteria from Human Infected Wounds

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Abstract

The prevention and treatment of infections of human wounds are an important issue. In this investigation five herbal plants were selected, and their antibacterial effects were elucidated. Of these five herbal plants, the morphological changes of the one with the strongest antibacterial effects were observed using a scanning electron microscope (SEM) and the differential expression of bacterial proteins treated with them was determined by two-dimensional gel electrophoresis (2D-GE). Two proteins of weakly expressed spots from the highest matching percentage of 2D-GE were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF). The results indicated the Lithospermi radix have the best minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The SEM examination reveals different bactericidal morphological changes and colony distributions obtained using Lithospermi radix. 2D-GE reveals that Staphylococcus aureus treated with Lithospermi radix has the highest matching percentage (90.93%). Then identification of two proteins that are weakly expressed in Staphylococcus aureus by MALDI-TOF/TOF reveals that they are Holliday junction ATP-dependent DNA helicase RuvB and D-alanine:D-alanine ligase (DDI). These results are noteworthy to suggest that Lithospermi radix may be used as an antibacterial medicine to treatment of human wound infection.
Keywords: Wound infection; Antibacterial effects; Herbal plant; Mass spectrometry;

Scanning electron microscope
1. Introduction

All wounds contain bacteria and a limited amount of bacteria will be present even if a wound is healing normally. However, if the number of bacteria rises, the wound may become infected. Bacterial overload in a wound can cause a serious infection that requires antibiotic or surgical treatment. A survey sponsored by the World Health Organization demonstrated a prevalence of nosocomial infections from 3 to 21%, 5-34% of which are caused by wound infections.\(^1,2\) Accordingly, the prevention of wound infection is important. Generally, the use of antibiotics is the most simple and common method of treating or preventing wound infection. However, the emergence of antibiotic resistant bacterial strains is a growing problem,\(^3,4\) which has led to an urgent global call for new antimicrobial drugs, particularly from natural sources. The use of herbal medicines in the US has increased by 20% annually since the 1990s and the sale of non-prescribed herbal medicines is estimated to be US$ 4 billion per year.\(^5\)

The bacteria that are most commonly isolated from infected wound include *Staphylococcus aureus*, Coagulase-negative *staphylococci*, *enterococci*, *Escherichia coli* and *Pseudomonas aeruginosa*.\(^6,7\) Since *S. aureus* and *E. coli* are the most common gram-positive and gram-negative bacteria that cause wound infection and *P. aeruginosa* is an important nosocomial and highly prevalent opportunistic pathogen,\(^8,9\) these three bacteria are selected as the targets in the present study. Based on previous
studies, five herbal plants (Aloe barbadensis, Mentha piperta, Lithospermi radix, Callicarpa japonica and Forsythia suspensa) were selected to evaluate their antibacterial activities toward to S. aureus, E. coli and P. aeruginosa. The morphological changes and colony distributions of the tested bacteria were examined using a scanning electron microscope (SEM). The differential expressions of the proteins are compared using two-dimensional gel electrophoresis (2D-GE); the proteins with the best matching results are identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF) and their functions are evaluated.
2. Materials and Methods

2.1 Preparation of plant extracts

The five selected plant samples that were adopted herein were purchased in January 2009 from a local market. Parts of the plants (whole plant, bark, root, leaves, tubers and a mixture of these) were chopped into small pieces, dried in air at room temperature (25 °C) in the shade and pulverized using a laboratory mill, yielding 300–400 g. The fine powders were separately soaked in 75% methanol (3 × 500 mL) for four hours at room temperature to produce extracts. The extracts were filtered and concentrated using a rotary evaporator at 50 °C and 100 mbar. Further steps in the preparation were taken from a method described in a previous study. The extracts were transferred into freeze-drying equipment (Freeze dryer FDU-1200).

2.2 Bacterial strains and cultures

Reference isolates S. aureus (ATCC 25923), E. coli (ATCC 25922), and P. aeruginosa (ATCC 27853) were obtained from the Food Industry Research and Development Institute of Taiwan. Luria-Bertani medium (LB) were used for propagation and maintenance of bacterial cultures. Broth cultures were incubated under aerobic conditions at 37°C, whereas solid cultures were incubated at 37°C and 5% CO₂.
2.3 Evaluation of antibacterial activity

The antibacterial activity of the extracts was evaluated using a micro-dilution bioassay in 96-well micro-plates. For each, minimum inhibitory concentration (MIC) was determined. The broth dilution method was used to determine the MIC of reference bacterial strains. The bacterial strains were cultured overnight with incubation at 37 °C (FIRSTEK MODEL: S300). The test strains were suspended in sterile physiological saline to yield a final density of $1 \times 10^6$ cfu/mL. A serial of two-fold diluted plant extracts was obtained using Muller Hinton broth (BBL™ Muller Hinton Broth). A microtiter plate was set up and an extract of the herbal plants at a starting concentration of 50 mg/ml was transferred into the first well. Serial dilutions were conducted to yield concentrations of 25, 12.5, 6.4, 3.2, 1.6, 0.8, 0.4 and 0.2 mg/ml; the last well contained Muller Hinton broth without extract as a negative control. An inoculum of a prepared bacteria culture with approximately $1 \times 10^6$ cells was added to each of the wells and incubated at 37°C for 24 h. The MIC was taken as the lowest concentration that inhibited any visible bacterial growth on the culture plates. 10 μL from each tube of MIC broth with visible growth was even deposited onto nutrient agar plates. Following overnight incubation, the plates were examined for colony growth. Lack of growth indicated that the tested drug was bactericidal, while growth indicated that the drug was bacteriostatic at the relevant dilution. To
establish minimum bactericidal concentration (MBC), 10 μL of each culture medium without visible growth was used. After 16-24 h of aerobic incubation at 35 °C, the surviving organisms were counted. The MBC was the lowest concentration at which 99.9% of the bacteria are killed.

2.4 Scanning electron microscopy (SEM)

The bacteria that were susceptible to the plant extracts were prepared for examination using a SEM. Samples were prepared as described elsewhere. The samples were fixed in 2.5% glutaraldehyde in 0.1 M Tris buffer (pH 7.3) at 4°C for 1 hr. After they had been washed twice in a Tris buffer with 5% sucrose, they were post-fixed in 1.0% osmium-tetroxide solution in the same buffer for 1 hr. The resulting osmium-treated samples were then washed with 0.1 M Tris buffer and dehydrated by successive extractions with 50, 70, 80, 90 and 95% ethanol, each for a period of 10 min, and finally with 100% ethanol for 15 min twice. They were then dried using the CO₂ critical-point drying technique, coated with gold and examined using a SEM (Hitachi S-3000N, Japan).

2.5 Two-dimensional gel electrophoresis (2D-GE) and MALDI-TOF/TOF MS

Bacteria were grown on Luria-Bertani medium (LB) and maintained at 37°C. They
were collected for sonication on ice using a microtip with the power level set between 4 and 5 at 20% duty, with a 10s short burst followed by a 10s interval, repeated for 20 minutes. The total proteins were precipitated using 10% trichloroacetic acid (TCA) and were separated by 2D-GE, as follows. The protein sample (600μg) was loaded onto 18cm-long Readystrip IPG strips (Amersham Biosciences, UK) at a pH range of 3–10NL (nonlinear) and layered with 0.8 ml of covering oil to prevent drying of the gel or crystallization of the urea. The gel was then run on an Ettan IPGPhor II (Amersham Biosciences, UK) at 30 V to rehydrate the gel strip for 16 h; then programs with 500 V for 1 h (500 Vh), 1000 V for 1 h (1000 Vh), and 8000 V for 8 h (64000 Vh) were run. The voltage ramped automatically owing to the increase in resistance of the strip as excess ions exited it. After the first-dimension IEF, the strip was washed to remove the cover oil and then equilibrated for 12~15 min in 5 ml of equilibration buffer, which contained 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 1% DTT. The strip was then subjected to a second equilibration, using 5 ml equilibration buffer, which was like the first, but with the DTT replaced by 1.5% iodoacetamide, for an additional 12~15 min. Next, SDS-PAGE was run using a PROTEAN II xi cell tank (Bio-Rad, USA) at 200 V for 4 h. The results were further analyzed by calculating software (Image Master 2D Platinum, GE Healthcare). Following electrophoresis, the gel was stained with 0.25% (w/v) coomassie R-250
(Amersham Biosciences, UK), and spots that revealed variation of expression among
strains were selected manually and digested by trypsin for subsequent
MALDI-TOF/TOF analysis. Mass spectrometry (MS) and protein identification were
conducted by Mission Biotech Co., Ltd., Taiwan. Tandem MS was carried out on a
QSTARXL (Applied Biosystems-Sciex, Ontario, Canada) hybrid
quadropole-time-of-flight mass spectrometer. Mascot software (Matrix Sciences Inc.,
Beachwood, OH, USA) was used to identify proteins against the NCBInr protein
database of the National Center for Biotechnology Information at the National
Institutes of Health.
3. Results

3.1 The antimicrobial activities of plant extracts.

The MICs and MBCs of the five herbal plants (*Aloe barbadensis, Mentha pipert*, *Lithospermum radix, Callicarpa japonica and Forsythia suspensa*) against *S. aureus, E. coli* and *P. aeruginosa*, were determined (Table 1). Of these five herbal plants, *Lithospermum radix* exhibit potent antibacterial activities. Accordingly, *Lithospermum radix* was used in further tests.

3.2 Preparation of samples and two-dimensional gel electrophoresis (2D-GE)

Changes in the extracellular proteins of the three aforementioned bacteria upon exposed to *Lithospermum radix* were determined. Table 2 shows the final concentrations used in 2D-GE. The capacity of *Lithospermum radix* to induce or suppress the expression of various extracellular proteins was determined by examining the resulting culture supernatants using 2D-GE and IEF from pH 3 to pH 10. Figures 1-3 present typical 2D gel images of each bacterium with/without exposure to *Lithospermum radix*. The molecular weights of most expressed proteins are between 29 kDa to 97 kDa. Although the antibacterial effect of *Lithospermum radix* is evident, most proteins are preserved and the matching percentage range is 79.98 % to 90.93 % (Table 3). The increased spots are more than decreased spots in *S. aureus* and *P. aeruginosa.*
The increased spots are a little bit more than decreased spots while *E. coli* treating with *Lithospermi radix*. The proteins of *S. aureus* and *P. aeruginosa* are acidic, between pH 4 – 6, and the proteins of *E. coli* are mildly acidic, between pH 5 – 7.

3.3 Scanning electron microscopy (SEM)

SEM was adopted to observe the morphological changes to bacteria that were treated with *Lithospermi radix*. A comparison of the cells of *S. aureus* that were treated with *Lithospermi radix* to untreated cells revealed that treatment caused no apparent morphological changes but did reduce the number of cells, spreading them out (Fig. 4). The treated *E. coli* cells also exhibited no apparent morphological change (Fig. 5), maintaining a regular rod shape, but their number and length decreased. In *P. aeruginosa*, cells appeared to aggregate with multiple connected filaments and to deform upon treatment with *Lithospermi radix* (Fig. 6).

3.4 Proteomic analysis of *S. aureus* treated with *Lithospermi radix*

To identify changes to bacterial proteins that are caused by treatment with *Lithospermi radix*, a proteomic analysis of *S. aureus* was performed. Candidate proteins from decreased spots that are involved in the bactericidal mechanism of *Lithospermi radix* were selected randomly. The protein profiles of *S. aureus* before
and after treatment with *Lithospermi radix* (Fig. 1) were compared. A total of 355 and
328 protein spots were detected by 2D-GE analysis before and after this treatment,
respectively, and the match percentage was 90.93% (311 spots).

Those changes in protein expression upon treatment were detected and excisions
from gels were followed up by protein determination. Two proteins that were weakly
expressed in *S. aureus* after treatment of *Lithospermi radix* were subjected to
MALDI-TOF/TOF MS determination of proteins. They are Holliday junction
ATP-dependent DNA helicase RuvB and D-alanine:D-alanine ligase (DDI).
4. Discussion

Antibiotics are the first choice for treating bacterial infection. Different antibiotics should be used to treat different bacteria. No single antibiotics can treat a combined infection of S. aureus, E. coli and P. aeruginosa. In such cases, doctors must prescribe a combination of antibiotics to treat wound infections with these three bacteria simultaneously before the results of a wound culture have bee obtained. Sometimes, such infections not only require the use of expensive antibiotics but also increase morbidity and mortality. Additionally, a greater period of exposure is associated with greater risk of developing resistance, independently of the need for the antibiotics. As antibiotic resistance becomes more common, the need for alternative treatments increases. These involve other agents with greater antibacterial effect and lower toxicity. New antibiotics are necessary to treat microbial pathogens that are becoming increasingly resistant to available treatment. However despite a push for new antibiotic therapies, the rate of approving drugs has been declining.23

Plants contain numerous antibacterial constituents that can be used for treatment in cases of multiple bacterial infections. Of these five herbal plants in our study, Lithospermi radix (the root of Lithospermum erythrorhizon Siebold. et Zuccarinii) exhibit potent antibacterial activities. The main ingredients of Lithospermi radix includes Shikonin, Isobutylshikonin, β-Hydroxy isovaleryl shikonin, α-Meth...
yl-n-butylshikonin and quinoid. During these, Shikonin is the main ingredient of antibacterial effect. *Lithospermi radix* extract can inhibit the release of histamine and the production of inflammatory cytokine in mast cells and is utilized to treat such conditions as eczema, skin burns and frostbite.24

The images obtained by SEM demonstrate that these three bacteria undergo different morphologic changes and colony distribution upon treatment with *Lithospermi radix*, indicating the different antibacterial mechanisms to the different bacteria. Furthermore, even an individual herbal plant can have different antibacterial mechanisms against different bacteria.

The bacteria reveal different morphologic changes and colony distribution after treatment with *Lithospermi radix*. Even though the same herb plant, there are different mechanisms of antibacterial effect.

RuvB is a hexameric ATPase. The RuvB ATPase provides the energy to drive the exchange of base pairs that move the DNA branch in Holliday junction for DNA homologous recombination.25 D-alanine:D-alanine ligase (DDl) participates in bacterial cell wall biosynthesis, making it a target for the development of new antibiotics.26,27 Accordingly, RuvB and DDl may be two important targets of the bactericidal activity of *Lithospermi radix*, providing new insights into the pharmaceutical value of *Lithospermi radix* in preventing bacteria infection.
5. Conclusions

Studies on the antibacterial effect of plants are worth to be studied in the future. The observed activity validates the plants used in traditional medicine. It is noteworthy to suggest that *Lithospermi radix* may be used as an antibacterial medicine to treatment of human wound infection.

Acknowledgements

The authors would like to thank the *National Science Council of the Republic of China, Taiwan, ROC* (contract No. 98-2221-E-039-005-MY3) and the *China Medical University* (contract No. CMU99-S-44) for financially supporting this research.
References


15. Ndhlala AR, Amoo SO, Stafford GI, et al., Antimicrobial, anti-inflammatory and


Captions

Figure 1. 2D-GE analysis of total proteins in plant extracts treated *S. aureus*.

Figure 2. 2D-GE analysis of total proteins in plant extracts treated *E. coli*.

Figure 3. 2D-GE analysis of total proteins in plant extracts treated *P. aeruginosa*.

Figure 4. SEM images of *S. aureus* treated with plant extracts.

Figure 5. SEM images of *E. coli* treated with plant extracts.

Figure 6. SEM images of *P. aeruginosa* treated with plant extracts.

Table 1 The MIC and MBC of plant extracts against reference strains.

Table 2 The concentrations of plant extracts for treating bacteria in 2D-GE analysis.

Table 3 The summary of 2D-GE analysis in plant extracts treated bacteria.
Figure 1

*S. aureus* (0.25 mg/ml *Aloe barbadensis*)

*S. aureus* (6.25 µg/ml *Lithospermi Radix*)
Figure 2

E. coli

E. coli (0.8 mg/ml Aloe barbadensis)

E. coli (250 µg/ml Lithospermi Radix)
Figure 3

**P. aeruginosa**

*P. aeruginosa (0.8 mg/ml Aloe barbadensis)*

*P. aeruginosa (0.1 mg/ml Lithospermi Radix)*
Figure 6

*P. aeruginosa* + *Lithospermi radix* (0.1 mg/ml) + *Aloe barbandensis* (0.8 mg/ml)
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ND: Not Detectable
Table 2

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