Antioxidant and antibacterial properties of *Pericarpium trichosanthis* against nosocomial drug resistant strains of *Acinetobacter baumannii* in Taiwan

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Accepted 13 October, 2009

Recently, multiple resistances in human pathogenic microorganisms have developed and caused serious nosocomial infections, especially *Acinetobacter baumannii*. In this study, we aim to look for new antimicrobial substances against drug resistance strains from Chinese herbal medicines. By using the disc diffusion method, 58 ethanol extracts of Chinese herbal medicines were screened for the antimicrobial activity against 78 clinical *A. baumannii* isolates. Among the 58 plant extracts, the extracts of *Pericarpium trichosanthis* showed substantial higher broad antibacterial spectrum against the entire test organisms. The ethyl acetate (EA) fraction obtained from partition extraction revealed the antibacterial activity with MIC value at 1.9 mg/ml. The EA fraction of *P. trichosanthis* could be a bactericide based on the killing curve. The chemical components of the extracts were analyzed by GC-MS; 4-hydroxybenzoic and isovanillic acid were evidenced to provide the antibacterial and antioxidant activity, respectively. Moreover, the results obtained from SEM observation showed that the active extract might inhibit the function of outer membrane of the organisms. Overall, the extracts of *P. trichosanthis* present antibacterial and antioxidant activity indeed have the valuable to be developed into the antibiotic medicines.

Key words: Chinese herbal medicines, *Pericarpium trichosanthis*, antibacterial activity, antioxidant activity.

INTRODUCTION

All over the world, multiple resistances in human pathogenic microorganisms, especially *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, have developed and caused serious nosocomial infections (Landman et al., 2007; Liu et al., 2007). Among the nosocomial drug-resistance strains, *A. baumannii* was the most prevalence isolates in Taiwan. The recent emergence of drug-resistance strains is deeply troubling and highlights the urgent need for novel antibacterial agents.

Higher plant products with an evidence-based action against fungi and pathogenic bacteria are currently playing a growing role, since most of the currently used antibiotic therapies are frequently accompanied by a large number of toxic side effects (Rosato et al., 2007). Moreover, many research reports have demonstrated that the antibiotic therapies can sometimes develop bacterial resistance. Therefore, the search for new antimicrobial natural products continues to draw attention for many re-
searchers. Chinese medicinal herbs have been used for a long time and undoubtedly a valuable resource for new antibacterial agents (Tsai et al., 2008; Asthana et al., 2006; Natarajan et al., 2008). Chinese medicines account for a source of many natural ingredients to be developed as new antimicrobial drugs in spite of the fact that their antibacterial and antifungal action has been proven to be remarkably milder than that of commercial synthetic drugs (Tian et al., 2009; Shan et al., 2008).

Euphorbia hirta (Gua Lou Pi) is the pericarp of Trichosanthes kirilowii Maxim or Trichosanthes rosthornii Harms (Fam. Cucurbitaceae). Its traditional uses are for cough that is dry or difficulties in expectorating sputum, dyspnea, chest congestion, chest pain that is worse with pressure, yellow sputum, wheezing, dry throat, early stages of breast abscesses and clumping in the chest. The clinical application is used to treat asthmatic tracheitis, cor pulmonale asthma and coronary heart disease (Guo et al., 2006). Modern research has demonstrated that its chemical components contain a small quantity of essential oils (Chao et al., 1996; Hu et al., 2005; Xiu et al., 2004). The acid parts of essential oil are pelargonic acid, capric acid and lauric acid. The concentration of palmitic acid is the highest and linoleic acid come the second and third, respectively.

The aim of this study is to evaluate the antibacterial activities of P. trichosanthis against the clinical drug-resistance A. baumannii isolates. In this article, we report on the antibacterial activity of ethanol extracts of P. trichosanthis, accompanied by TLC and GC-MS analyses of its chemical composition and spectrophotometric quantification of the most important active principles.

**MATERIALS AND METHODS**

**Test Organisms**

A total of 78 clinical strains of A. baumannii were used in this study and isolated from patients' blood or sputum during 2003 - 2004. The samples were provided by the Chia-Yi Christian Hospital in Taiwan. Two reference strains, S. aureus ATCC 6538P and A. baumannii ATCC 19606 were purchased from the Food Industry Research and Development Institute in Taiwan.

**Plant materials**

Herbs used in this study were selected based on their usage as folk medicine, as well as indications of the presence of bioactive compounds with antimicrobial properties. 58 traditional Chinese medicinal herbs were used for the screening, including: Cortex Moutan, Pericarpium trichosanthis, Mume fructus, Ramulus cinnamomi, Radix sophorae Tonkinensis, Rhizoma acori Graminei, Bulbus iphigeniae Indicae, Rhizoma cypere, Phellodendron amurense Rupr, Rheum palmatum L., Forsythia suspense, Omphalia lapidescens Schroet, Bulbus Itriflorae Thunbergii, Radix aucklandiae, Radix clematidis chinensis, Radix Salviae Miltiorrhizae, Fructus psoraleae, Radix sanguiisorbae, Fructus Lonicerae, Caulis akebiae, Fructus Carpesii Abrotanoides, Fructus xanthii, Rhizoma Polygoni cuspidati, Fructus cnidii, Radix stemonae, Herba Leonuri, Flos magnoliae, Radix notoginseng, Fructus gardeniae, Herba houtuyiae, Semen raphani, Radix Platycodi, Semen crotonis, Rhizoma cimicifugae, Herba artemisiae, Radix glycyrrhizae, Semen Strychni, Herba Plantaginis, Murraya Paniculata Jack, Radix Isatidis, Radix Cynanchi Paniculati, Fructus Quisqualis, Radix spachilarii, Rhizoma Coptidis, Herba Artemisiae Scopariae, Radix scutellariae, Radix pulsatilae, Folium Isatidis, Herba Seneconis scandentis, Rhizoma polygonati, Melia azedarach L., Flos Chrysanthemi, Semen arecae, Radix Sophorae flavescentis, Fructus evodiae, Radix Acanthopanacis senticosi, Rhizoma cyrtomii and Herba Euphorbiae humifusae. The plants were purchased from local folk medicine dealers. The Chinese herbs were authenticated by Department of Traditional Chinese Medicine, Kaohsiung Medical University Chung-Ho Memorial Hospital, Taiwan.

**Preparation of crude extracts**

The dried herb (1 kg) was extracted with 5 L of 95% ethanol overnight by shaking in an incubator set at 200 rpm and 37°C. The ethanol extraction was repeated three times and then filtered using Whatman filter paper No. 1 to remove insoluble debris. After filtration, the ethanol extract was dried by evaporation at a temperature of 40°C. The dried extracts were stored at 4°C until the assay. The crude extracts which contained highly active, broad-spectrum components were successively partitioned with n-hexane, chloroform and ethyl acetate based on polarity; according to Chuang's method (Liu et al., 2007) with some modification. Each fraction was dried by evaporation and then subjected to the antimicrobial assay.

**Antibiotic susceptibility of the test strains**

Antibiotic susceptibility of the test strains was determined by the standard disc diffusion method. Filter paper discs (8 mm in diameter, ADVANTEC) impregnated with antibiotic solutions were placed on cation-adjusted Mueller Hinton agar plates, which were inoculated with test organisms (10⁵ CFU/mL) according to the standard protocol described by the National Committee of Clinical Laboratory Standards (NCCLS, 2002). The potency of antibiotics (30 μl per disc) is as follows: Ampicillin (Amp, 20 ~ 50 mg/L), Streptomycin (Sm, 15 mg/L), Gentamycin (Gm, 15 mg/L) and Kanamycin (Km, 15 mg/mL). Filter paper discs containing water (30 μl per disc) without any test antibiotics served as a control and no inhibition was observed. Tetracycline (15 mg/L, 30 μl) was used as the positive control for the test microorganisms. Each assay was performed in triplicate and repeated two times.

**Antibacterial activity of crude extract**

The initial screening of the alcoholic extracts for antibacterial activity was conducted by the disk diffusion analysis as previously described. The antibacterial samples were dissolved with dimethyl sulfoxide (DMSO) (1 g/ml) and tested at a concentration of 30 μg/disc. The plates were incubated at 37°C and the diameters of the inhibition zones were measured after 18 h. Filter paper discs containing DMSO without any test compounds served as a control and no inhibition was observed. Additionally, for comparative purposes, tetracycline (15 mg/L, 30 μl) was included as a reference standard. Each assay was performed in triplicate and repeated two times.

**Determination of minimum inhibitory concentration (MIC)**

The MIC of the crude extract and active fraction was determined by the agar dilution method, according to the NCCLS protocol with some modification (NCCLS, 2002). The growth media, cation adjusted Mueller-Hinton agar, was first prepared in the usual fashion and...
sterilized by autoclaving. The sterilized media were allowed to cool to 50°C and 10 ml of the molten agar was added to the test tubes, which contained different concentrations of the test drugs (herbal extracts) and the control substance (DMSO). The media and the test drugs were thoroughly mixed and poured into pre-labeled sterile Petri dishes on a level surface. The concentrations of the extracts used in this test ranged from 0.5 to 30 mg/L. The densities of the suspensions of the respective microorganisms were adjusted to $5 \times 10^5$ CFU/ml. The suspensions (100 µl) were transferred onto each plate. The plates were then incubated at 37°C for 18 h. The lowest concentration which inhibited the growth of the respective organisms was taken as the MIC. All tests were carried out in triplicate.

**Time-killing curve of the active fraction from the crude extract**

The time-killing curve was determined by Yu et al. (2005) method. The concentration of each antimicrobial agent in the cation-supplemented Mueller-Hinton broth was set at a level equal to double the MIC level of the tested strain. Inoculates of $5 \times 10^5$ CFU/ml of bacteria harvested from the colonies grown overnight was used in these experiments. Aliquots of the cultures were taken at 0, 2, 4, 8, 12 and 24 h and serially diluted in the Mueller-Hinton broth and then plated on Mueller-Hinton agar. Following 37°C and 18 h of incubation, the number of colonies was counted to determine the total viable bacteria number. A cell culture with no antimicrobial agent was assayed as the control.

**Resistance to the active fraction from the crude extract**

The tested organism (100 µl, $5 \times 10^5$ CFU/ml) was sub-cultured in sub-MIC concentration of the active fraction from the crude extract for 10 consecutive days in order to investigate their ability to develop resistance. During the 10 days, culture purity was assured by pulse field gel electrophoresis (PFGE) and the MIC of the subculture on day 11 was determined.

**Thin-layer chromatography (TLC) analysis**

TLC plates (10 x 10 cm, Merck, silica gel 60 F254) were loaded with 100 µg (2 µl of 50 mg/ml) of each active fraction of the herbal extracts. The plates were developed with H$_2$O/Methanol (1/9; v/v) as mobile phase. Spots were visualized by UV irradiations at 254 and 365 nm. Developed TLC plates were also sprayed with FeCl$_3$, Dragendorff reagents or sulfuric acid solution was used to examine the chemical components and compared with the R$_f$ of the related spots on the TLC plates.

**Scanning electron microscopy (SEM)**

The structural changes induced by the extracts on the test strains were studied using SEM as described earlier (Liang et al., 2008). For SEM analysis, the most susceptible strain (A7) was selected for the examination. The selected strain was incubated with or without 1/4 MIC amount of the active fraction extracts at 37°C for 16 h; afterwards, bacterial suspensions were spun briefly at 5000 x g at room temperature, washed in PBS and finally resuspended in 0.5 ml of the same buffer. Cells were fixed with an equal volume of 5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1.5 h. After being washed with the buffer, specimens were post-fixed for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C. After fixation, the samples were dehydrated in a graded ethanol series (30, 50, 75, 90 and 95% once) for a period of 30 min in each series. The samples were then critical-point dried in a drying apparatus (Hitachi Critical Point Dryer HCP-2) up to the critical point with CO$_2$. The fixed material was then mounted on stubs using double-sided carbon tape and coated with gold/palladium in a sputter coater system in a high-vacuum chamber. The samples were examined and digital images captured using a Leica Stereoscan (HITACHI S-2700) electron scanning microscope.

**Antioxidation activity**

Antioxidation activity was tested by DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical assay with little modification. Briefly, one ml of different concentrations of tested samples (sample extracts, 4-hydroxybenzoic acid and isovanillic acid) was mixed with one ml of 0.5 mM DPPH in ethanol. Mixtures were vigorously shaken and left for 30 min in dark. Absorbance was measured at 517 nm using ethanol as a standard. One ml of 0.5 mM DPPH diluted in 2 ml of ethanol was used as control. Inhibition of the DPPH radical was calculated using the following equation:

$$I \% = \left(1 - \frac{(B - A)}{C}\right) \times 100\%$$

Where B is the absorbance of the tested sample (10000, 1000, 500 and 100 ppm) containing tested samples with DPPH solution, A is the absorbance of the tested samples without DPPH and C is the absorbance of the control (containing all reagents except the tested samples). The IC$_{50}$ value represents the concentration of the crude extract that caused 50% inhibition.

**RESULTS**

**Antibiotic susceptibility of the test strains**

The 78 clinical isolates of *A. baumannii* were tested for their antibiotic susceptibility. According to the phenotype results obtained from the antibiotic susceptibility test, 27 of the *A. baumannii* isolates were randomly selected as the test strains for the further investigations of antibacterial activity. The selected isolates are shown in Table 1.

**Antibacterial activity of crude extracts**

For the antibacterial activity test, 27 *A. baumannii* clinical isolates and two reference strains, *S. aureus* ATCC 12600 and *K. pneumoniae* ATCC 700603 were used. The preliminary experiments showed that the MICs of the extracts were in the range of 0.5 to 10 µg/ml. The antibacterial activity was expressed as the percentage of inhibition of bacterial growth. The results indicated that the extracts have potential antibacterial activity against the test strains. The extracts showed a significant inhibitory effect on the growth of the test strains, with MIC values ranging from 0.5 to 5 µg/ml. The extracts were more effective against *A. baumannii* than *S. aureus* and *K. pneumoniae*.

**Statistical analysis**

The analysis of variance was used to compare the means or averages. The computer program M-STAT was implemented to process the data and report significant differences at $p = 0.05$.
Table 1. The resistance phenotype among 76 clinical Acinetobacter baumannii isolates.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Clinical isolates (n = 76)</th>
<th>Selected strains* (n = 27)</th>
<th>Resistance profile</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>4</td>
<td>A1, A2</td>
<td>Amp, 20</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>A3</td>
<td>Amp 20*</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>A4</td>
<td>Amp 30*, Sm*, Km*</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>A5</td>
<td>Amp 40*</td>
</tr>
<tr>
<td>V</td>
<td>1</td>
<td>A6</td>
<td>Sm*</td>
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<tr>
<td>VI</td>
<td>1</td>
<td>A7</td>
<td>Sm*, Km*, Gm*</td>
</tr>
<tr>
<td>VII</td>
<td>1</td>
<td>A8</td>
<td>Km*, Gm*</td>
</tr>
<tr>
<td>VIII</td>
<td>1</td>
<td>A9</td>
<td>Amp 20*, Km*</td>
</tr>
<tr>
<td>IX</td>
<td>1</td>
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</tr>
<tr>
<td>X</td>
<td>6</td>
<td>A11, A12</td>
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</tr>
<tr>
<td>XI</td>
<td>5</td>
<td>A13, A14</td>
<td>Amp 20*, Sm*, Km*, Gm*</td>
</tr>
<tr>
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<td>A15</td>
<td>Amp 50*, Sm*</td>
</tr>
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<td>XIII</td>
<td>6</td>
<td>A16, A17</td>
<td>Amp 30*, Sm*, Km*, Gm*</td>
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<tr>
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<td>A18</td>
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<tr>
<td>XV</td>
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<td>A19</td>
<td>Amp 40*, Km*, Gm*</td>
</tr>
<tr>
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<td>A20</td>
<td>Amp 40*, Sm*, Km*, Gm*</td>
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<tr>
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<td>A21</td>
<td>Amp 50*, Sm*, Km*</td>
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<tr>
<td>XVIII</td>
<td>6</td>
<td>A22, A23</td>
<td>Amp 50*, Km*, Gm*</td>
</tr>
<tr>
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<td>33</td>
<td>A24, A25, A26, A27</td>
<td>Amp 50*, Sm*, Km*, Gm*</td>
</tr>
</tbody>
</table>

* The strains were selected for bioactivity test.

6538P and A. baumannii ATCC 19606, were selected for the activity screening of 58 Chinese herbal extracts. The results of preliminary activity screening showed that 31 out of 58 extracts possess antibacterial activity against the test strains as shown in Table 2. In the disc diffusion assay, 10 of the active extracts revealed a broad-spectrum antibacterial activity against the 27 A. baumannii isolates and 20 P. aeruginosa isolates (previous study, Liu et al., 2007) with above 85% inhibition among the total 47 test strains. The 10 extracts, includes P. trichosanthis, R. Sophorae tonkinensis, C. moutan, F. Forsythiae, Omphalia, R. Salviae Miltiorrhiza, P. Cremastrea seu Pleiones, R. Cinnamomi, F. Mume and B. Fritillariae thunbergii, were analyzed for the minimum inhibitory concentration (MIC).

Determination of minimum inhibitory concentration (MIC)

Integrating the antibacterial activity results obtained from our previous study and this study, 10 of the Chinese herbal extracts were selected for the determination of minimum inhibitory concentration. By the agar dilution method, the results showed that the MIC values of crude extract ranges from 1 to 17 mg/mL as shown in Table 3. As a first step in our research, we intended to develop a broad-spectrum antibacterial agent, after integrating the results of disc diffusion and agar dilution assay, three of the Chinese medicines, including P. trichosanthis, F. mume and B. Fritillariae thunbergii, showed a relative higher and broader antibacterial activity against the test strains. Among the three Chinese medicines, the antibacterial properties of F. mume and B. F. Thunbergii, bioorganic acid and Peiminine, respectively, have been highly investigated and reported in the literatures (Kwon et al., 2008; Chen et al., 2006; Xue and Wang, 2007). However, for the antibacterial properties of P. trichosanthis are few studied, therefore, in the present study, we focused on the investigation of antibacterial activity of P. trichosanthis. The crude extracts of P. trichosanthis were further successively partitioned with n-hexane, chloroform and ethyl acetate based on polarity and each of the fractions obtained were subjected to the MIC determination on the 27 selected A. baumannii isolates. As shown in Table 4, the ethyl acetate fraction revealed antibacterial activity with a mean MIC value of 1.9 mg/mL which ranges from 1.5 to 2.0 mg/mL. The results showed that the different antibiotic phenotypes did not revealed substantial difference in their antibacterial susceptibility on the herbal extracts.

Spectrophotometer determination of antimicrobial components

The active fraction (EA) of extract was dissolved in me-
antibacterial and antioxidant activity. The major antibacterial components obtained from *P. trichosanthis* have great potential for application as natural antibiotics. However, further research, particularly on the interaction mechanism and cytotoxicity test is still necessary for the natural antibiotic development.

**ACKNOWLEDGEMENT**

This work was partly supported by the National Science Council in Taiwan under grant NSC98-2324-B214-001 and 98-EC-17-A-19-S2-0077.

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