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Full Length Research Paper

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

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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 *Picarpium 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, *Picarpium trichosanthis*, antibacterial activity, antioxidant activity.

INTRODUCTION

All over the world, multiple resistances in human pathogenic microorganisms, especially A*cenetobacter baumanni*, *Pseudomonas aerugisosa* 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. baumanni* 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-

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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).

Pericarpium trichosanthis (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 linolenic 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. baumanni* 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 acoori Graminei*, *Bulbus iphigeniae Indicae*, *Rhizoma cyperi*, *Phellodendron amurense Rupr*, *Rheum palmatum L*, *Forsythia suspense*, *Omphalia lapidescens Schroet*, *Bulbus fritillariae Thunbergii*, Radix *aucklandiae*, *Radix clematidis chinensis*, *Radix Salviae Miltiorrhizae*, *Fructus psoraleae*, *Radix sanguisorbae*, *Flos lonicerae*, *Caulis akebiae,* Fructus *Carpesii Abrotanoides, Fructus xanthii, Rhizoma Polygoni cuspidati, Fructus cnidii,* Radix *stemonae,* Herba *Leonuri, Flos magnoliae*, *Radix notoginseng, Fructus gardeniae,* Herba *houtuyniae,* 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 scrophulariae, Rhizoma Coptidis,* Herba *Artemisiae Scopariae,* Radix *scutellariae,* Radix *pulsatillae, Folium Isatidis*, Herba *Senecionis 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℃. 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℃. The dried extracts were stored at 4℃ 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 (10^5 CFU/mL) according to the standard protocol described by the National Committee of Clinical Laboratory Standards (NCCLS, 2002). The potency of antibiotics (30 ul 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 ul per disc) without any test antibiotics served as a control and no inhibition was observed. Tetracycline (15 mg/L, 30 ul) 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℃ 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 ul) 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 x 10^6 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 x 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 x 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_2O/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.

Spectrophotometer determination of antimicrobial components

The active fraction of extract was dissolved in methanol (100 µg/ mL) and identified by GC-MS (GC, Agilent 6890N; MS, Agilent 5970; DB-5 column, 60 m × 250 µm × 0.25 µm). The initial condition for GC-MS analysis was set for 5 min at 50□□ then 50 ~ 150□, 5 %/min and then increase for the next 5 min and finally 150 ~ 210°C, 5 °C /min and further increase for another 5 min. The mobile phase was He with flow rate at 1.0 µm/mi. For comparative studies, experiments were also conducted in a similar manner using 4-hydroxybenzoic acid and isovanillic acid as a standard.

Antioxidation activity

Antioxidation activity was tested by DPPH (2, 2-diphenyl-1-picrylhidrazyl) 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:

$$I\ \% = (1 - (B - A)/ C) \times 100\%$$

Where B is the absorbance of the tested sample (10000, 5000, 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.

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.

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

Table 1. The resistance phenotype among 76 clinical *Acinetobacter baumannii* isolates.

Phenotype	Clinical isolates (n = 76)	Selected strains[#] (n = 27)	Resistance profile[*]
I	4	A1, A2	Amp, 20
II	2	A3	Amp 20^r
III	1	A4	Amp 30^r, Sm^r, Km^r
IV	1	A5	Amp 40^r
V	1	A6	Sm^r
VI	1	A7	Sm^r, Km^r, Gm^r
VII	1	A8	Km^r, Gm^r
VIII	1	A9	Amp 20^r, Km^r
IX	1	A10	Amp 20^r Km^r Gm^r
X	6	A11, A12	Amp 50^r
XI	5	A13, A14	Amp 20^r, Sm^r, Km^r, Gm^r
XII	2	A15	Amp 50^r, Sm^r
XIII	6	A16, A17	Amp 30^r, Sm^r, Km^r, Gm^r
XIV	1	A18	Amp 40^r, Sm^r, Km^r
XV	1	A19	Amp 40^r, Km^r, Gm^r
XVI	2	A20	Amp 40^r, Sm^r, Km^r, Gm^r
XVII	1	A21	Amp 50^r, Sm^r, Km^r
XVIII	6	A22, A23	Amp 50^r, Km^r, Gm^r
XIX	33	A24, A25, A26, A27	Amp 50^r, Sm^r, Km^r, Gm^r

[#]: The strains were selected for bioactivity test.

*Ampicillim; Amp, Gentamycin; Gm, Streptomycin; Sm, Kanamycin; Km, Tetracycline; Tc. Amp 20: 20 mg/ml, Amp 30: 30 mg/ml, Amp 40: 40 mg/ml, Amp 50: 50 mg/ml. Gm, Sm, Km: 15 mg/ml. r; resistant.

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 posses 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. aerugisosa* 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 Miltiorrhizae*, *P. Cremastrae 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 forced 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-

Table 2. Antimicrobial activity of extracts of various herbs against the tested microorganism*.

Traditional Chinese medicines	Inhibition zone (mm) [a]	Inhibition (%) [b]
Pericarpium Trichosanthis	13.8 ± 2.7	100.0
Rhizoma Coptidis	9.4 ± 1.2	65.5
Cortex Moutan	12.3 ± 4.5	89.7
Radix Isatidis	11.1 ± 3.0	24.1
Herba Euphorbiae Humifusae	10.4 ± 1.9	79.3
Herba Artemisiae Scopariae	10.6 ± 1.9	65.5
Murraya paniculata	10.8 ± 0.8	17.2
Ramulus Cinnamomi	20.9 ± 4.8	89.7
Radix Sophorae Tonkinensis	12.2 ± 2.0	89.7
Rhizoma Acori Tatarinowii	10.4 ± 1.4	100.0
Herba Plantaginis	9.2 ± 0.8	79.3
Pseudobulbus Cremastrae seu Pleiones	11.1 ± 3.2	100.0
Semen Arecae	9.7 ± 1.4	37.9
Fructus Mume	19.5 ± 1.7	100.0
Radix Clematidis	11.8 ± 1.3	13.8
Radix Cynanchi Paniculati	10.3 ± 0.6	48.3
Radix Sophorae Flavescentis	10.6 ± 1.8	31.0
Radix Acanthopanacis Senticosi	9.4 ± 0.7	93.1
Lonicera japonica Thunb.	9.2 ± 0.7	48.3
Rhizoma Cyperi	10.7 ± 1.2	96.6
Cortex Phellodendri	10.1 ± 0.9	92.8
Radix Aucklandiae	10.4 ± 1.3	96.6
Radix et Rhizoma Rhei	12.9 ± 1.6	96.6
Radix Salviae Miltiorrhizae	11.4 ± 1.4	96.6
Caulis Akebiae	10.3 ± 1.4	89.7
Fructus Forsythiae	10.9 ± 1.3	100.0
Fructus Evodiae	10.5 ± 1.0	89.7
Carpesium abrotanoides L	10.2 ± 1.0	48.3
Fructus Xanthii	11.4 ± 1.7	62.1
Omphalia	9.1 ± 1.9	96.6
Bulbus Fritillariae Thunbergii	11.8 ± 2.4	100.0

*, 29 test microorganisms were used: 27 clinical drug-resistant isolates of *A. baumannii* and two reference strains; [a], Results are reported as mean of the clinical isolates (n = 20); [b], Inhibition percentage (%): inhibited microorganisms / all examined microorganisms × 100%.

Table 3. The minimal inhibition concentration (MIC) of ethanolic extracts of various herbs and standard agents against the test microorganisms*.

Ethanol extracts	MIC(mg/ml)	Ethanol extracts	MIC (mg/ml)
Pericarpium Trichosanthis	11.2 ± 2.1	*Radix Salviae Miltiorrhizae*	11.2 ± 2.1
Radix Sophorae Tonkinensis	7.8 ± 0.8	*Pseudobulbus Cremastrae seu Pleiones*	15.5 ± 1.0
Cortex Moutan	1.9 ± 0.3	*Ramulus Cinnamomi*	1.6 ± 0.3
Fructus Forsythiae	16.6 ± 1.2	*Fructus Mume*	1.3 ± 0.2
Omphalia	17.4 ± 1.2	*Bulbus Fritillariae Thunbergii*	10.3 ± 0.8
4-hydroxybenzoic acid	0.75 ± 0.1	Isovanillic acid	> 30

*, 29 test microorganisms were used: 27 clinical drug-resistant isolates of *A. baumannii* and two reference strains.

methanol (100 µg/ ml) and identified by GC-MS. The major chemicals identified by the spectrum profile are shown in Table 5. Based on the major components and reference to the literature reports, 4-Hydroxybenzoic acid

Table 4. The minimal inhibition concentration (MIC) of *Pericarpium Trichosnthis* extracts against the *Acinetobacter baumannii* strains.

Test strains	Crude extracts MIC (mg/ml)	EA fractions MIC (mg/ml)
A1	16	1.5
A2	19	2.0
A3	11	2.0
A4	13	2.0
A5	12	2.0
A6	13	2.0
A7	20	1.5
A8	19	1.5
A9	20	1.5
A10	13	2.0
A11	12	1.5
A12	12	2.0
A13	12	2.0
A14	12	2.0
A15	11	2.0
A16	18	2.0
A17	14	2.0
A18	13	2.0
A19	14	2.0
A20	13	2.0
A21	14	2.0
A22	12	2.0
A23	13	2.0
A24	13	2.0
A25	13	2.0
A26	11	2.0
A27	11	2.0
A.b ATCC 19606	13	1.5
S.a ATCC 6538P	14	1.5
MEAN ± S.D.	13.8 ± 2.7	1.9 ± 0.2

Table 5. Major constituents of the EA fractions of *Pericarpium Trichosnthis* by GC-MS analysis.

Compounds	RT* (min)	Percentage# (%)
Malic acid	16.868	32.55
3-Hydroxy-4-methoxybenzoic acid (Isovanillic acid)	23.601	6.51
4-Hydroxybenzoic acid	24.767	18.60
n-Hexadecanoic acid	29.373	14.88
9, 12, 15-Octadecatrienoic acid, methyl ester	35.658	11.16
8,11-Eicosadienoic acid, methyl ester	36.298	6.50

*, RT indicates the retention time on the column in minutes; #, Percentage (%) indicates relative area (peak area relative to the total peak area).

has been proved to possess antibacterial activity (Cho et al., 1998). Thus, 4-Hydroxybenzoic acid and its analogous, 3-Hydroxy-4-methoxybenzoic acid (Isovanillic acid), were selected as the reference standards for the antibacterial assay. The results showed that 4-Hydroxybenzoic acid revealed antibacterial activity against the *A. baumannii* isolates with MIC value of 750 μg/ml while Isovanillic acid showed no antibacterial activity against

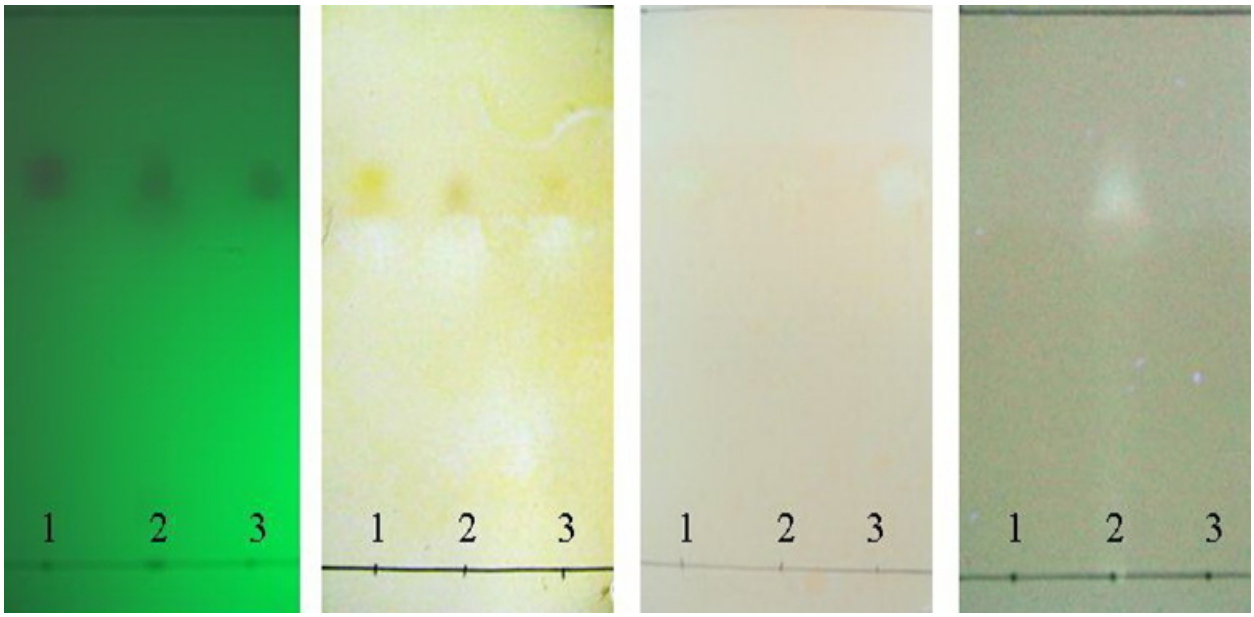

Figure 1. Chromatograms of the EA fraction of *Pericarpium Trichosnthis* developed in H_2O/Methanol (1:9; v:v) solvent. (1)4-Hydroxybenzoic acid, (2)EA fraction of *Pericarpium Trichosnthis*, (3) Isovanillic acid. (A) TLC chromatogram visualized under ultraviolet light (254 nm). (B) TLC chromatogram visualized after $FeCl_3$ spreaded. (C) TLC chromatogram visualized after treated with Dragendorff reagent. (D) TLC chromatogram visualized under ultraviolet light (365 nm) after treated with H_2SO_4.

the test strains (Table 3).

Thin-layer chromatography (TLC) analysis

2 µl of EA fraction (50 mg/ml), 4-Hydroxybenzoic acid (5 mg/ml) and Isovanillic acid (5 mg/ml) were subjected to the TLC analysis. The results are presented in Figure 1. Three of the test samples showed R_f values around 0.75 which indicated that the test samples have similar polarity (Figure 1a). Observing through UV 254 nm and 3% $FeCl_3$ developed reagent, all of the test samples appeared as color spots, this proved that the extract sample contains phenyl group (Figure 1b). Moreover, the extract sample did not appear colored after treated with Dragendorff reagent which indicated that the extract does not contain alkaloids (Figure 1c). Following 5% of H_2SO_4' spread and observed at UV 365 nm, showed that only the extract sample developed color which indicated that some other components were present in the extract except the standard benzoic acid derivatives (Figure 1d).

Time-killing curve

The strain of the clinical isolate A7 was selected for this investigation due to its more antibacterial susceptibility. The obtained results showed that when the isolate was exposed to double the MIC concentration, the EA fraction and 4-Hydroxybenzoic acid completely killed the tested microorganisms within 2 h. As shown in Figure 2, the inhibition effect lasted for more than 24 h. The partially purified components from the tested herb were considered to exhibit bactericidal activity for the test microorganism.

Antioxidant test by DPPH assay

Results of the DPPH radical-scavenging assay revealed that the EA fraction and Isovanillic acid showed substantial antioxidant activity and scavenging of DPPH radicals was concentration-dependent, with IC_{50} = 2066.2 and 647.6 ppm, respectively (Figure 3). However, 4-Hydroxybenzoic acid did not show any antioxidant activity by the DPPH assay.

Scanning electron microscopy (SEM) analysis

Treated samples of bacteria were observed by SEM to investigate any physical changes in the appearance of the cells. SEM observations confirmed the physical damage and considerable morphological alteration to the tested bacteria (A7) treated with 1/4 MIC amount of the EA fraction shown in Figure 4. These images directly illustrate the destructive effects of the extracts on the tested bacteria which showed flaccid and a loss of structural integrity, while the non-treated cells were intact and

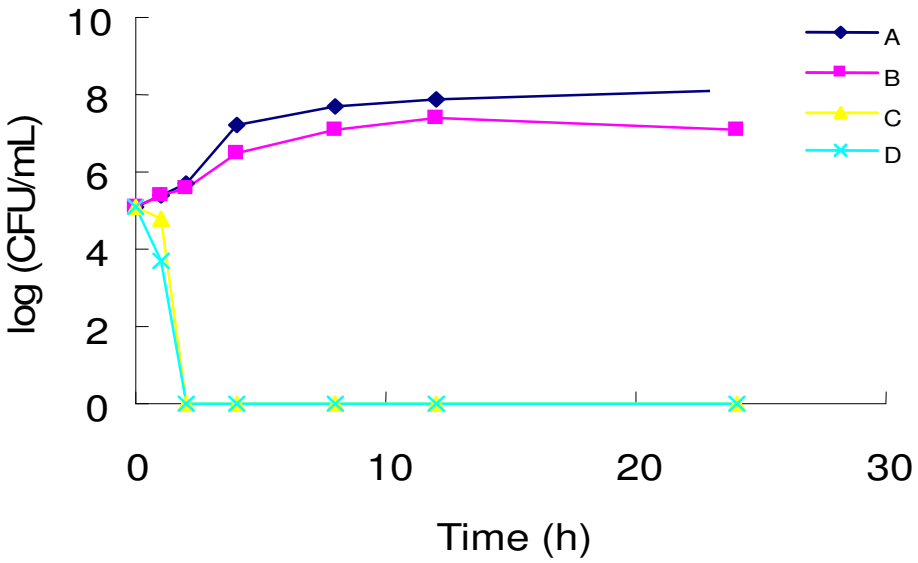

Figure 2. Bactericidal effect of the EA fraction from *Pericarpium Trichosnthis* extracts on growth of drug-resistant nosocomial strain (A7). (A) Untreated; (B) treated with DMSO solvent; (C) treated with the EA fraction; (D) treated with the standard 4-Hydroxybenzolic acid.

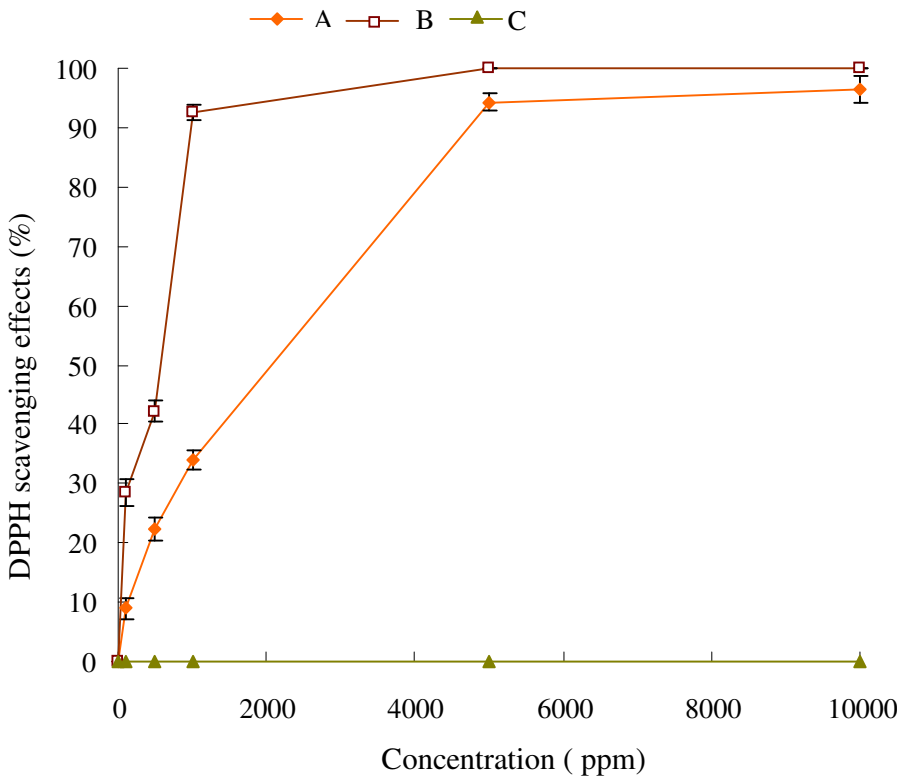

Figure 3. Antioxidant activity for free radical scavenging activity by DPPH radical. (A) the EA fraction of *Pericarpium Trichosnthis*; (B) Isovanillic acid; and (C) 4-Hydroxybenzoic acid.

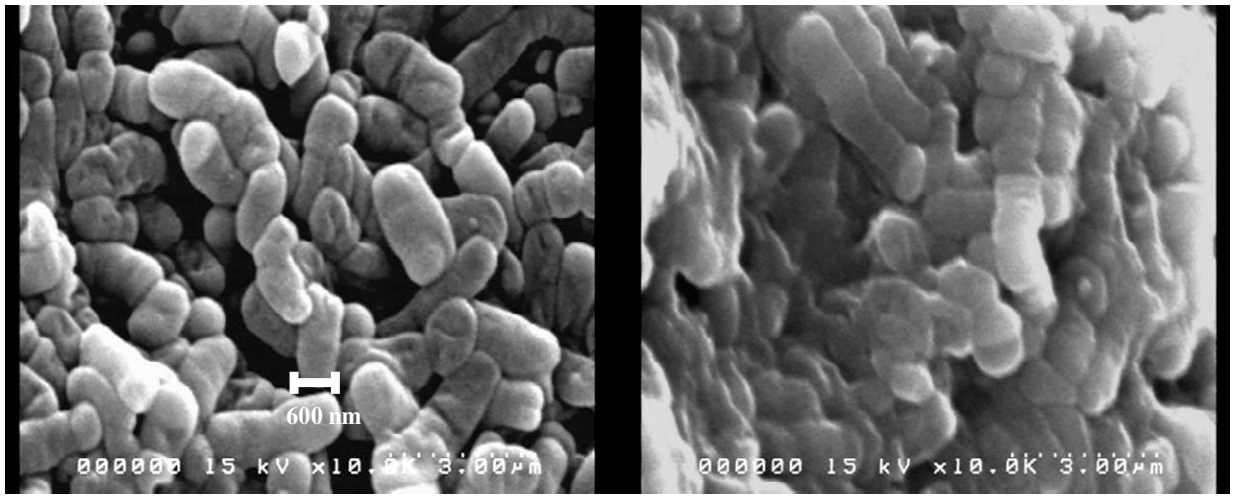

Figure 4. Scanning electron microscope observations of the pathogenic bacteria (A7) treated with the EA fraction of *Pericarpium Trichosnthis* (right) and untreated bacterial cells (left).

and showed a smooth surface.

DISCUSSION

Due to the present antibiotic, therapies are frequently accompanied by a large number of toxic side effects and development of bacterial resistance, many plant products with antibacterial or antifungal activities are turn to be more important. Therefore, the search for new antimicrobial natural products continues to draw the attention for many researchers. In the present study, we were focused on the investigation of antibacterial activities of Chinese herbal medicines against the drug-resistant *A. baumannii* isolates, which are the leading causes of nosocomial infections in long-term healthcare in Taiwan. Especially, reports on multi-drug resistant *A. baumannii* infections in hospitals have been increasing worldwide in recent years.

The ethanol extracts of *p. trichosanthis* was partial purified by partitioned extraction based on the polarity of organic solvents; the antimicrobial activity was shown in the ethyl acetate fraction, this represents that the active compounds possess mild low polarity. Following the MIC determination, the EA fraction revealed more antimicrobial activity than the crude extracts about 5 - 7 times. The results demonstrated that the active compounds can be purified and can increase their antimicrobial efficiency.

According to the results obtained from the GC-MS analysis, the major compounds, 4-Hydroxybenzoic acid and 3-Hydroxy-4-methoxybenzoic acid (Isovanillic acid), were selected for further bioactivity analysis. The bioassays showed that 4-Hydroxybenzoic acid possesses antimicrobial activity with MIC values ranging from 0.75 - 1.0 mg/mL which is 1.5-fold of the herbal extract. Due to the content of 4-Hydroxybenzoic acid in the herbal extracts is only 20%, we presume that might present other substances for the antimicrobial activity.

In the determination of antioxidant activity, the crude extracts of *p. trichosanthis* showed an IC_{50} at 2066 ppm and the major component was determined to be Isovanillic acid. As reported in the previous literature (Sang *et al*., 2002), Isovanillic acid possessed IC_{50} at 500 ppm that is similar as our results (Isovanillic acid standard IC_{50} at 648 ppm) in the DPPH radical-scavenging assay.

According to the time-killing curve, the active components was revealed as a bactericide agent which was able to kill the test bacterium completely within 2 h. Regarding to the antibacterial mechanism, by way of SEM morphology, the active components might interacts on the cell membrane or membrane proteins, this destroy the cell wall structure and result in cell death.

Conclusion

In this study, we intended to develop a broad-spectrum antibacterial agent; some Chinese medicines were studied to screen the antimicrobial activity against the clinical antibiotic pathogens. The ethanol extracts obtained from *P. trichosanthis* showed having the potential to be developed as a broad-spectrum antibacterial agent. Following the bioassay including MIC determina-tion, resistance test, SEM observation and DPPH radical-scavenging assay, this study demonstrated that *P. trichosanthis* possessed *in vitro* antibacterial and antioxidant properties against the clinical antibiotic pathogens. The major components, 4-hydroxybenzoic and isovanillic acid, were evidenced to provide the major contribution for the antibacterial and antioxidant activity, respectively. Even the components, 4- hydroxybenzoic and isovanillic acid, have been shown in the literature to exert an antibacterial effect, the presence of these compounds in *P. trichosanthis* has been extensively investigated for the

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.

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