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Contributed Paper

Phytochemical Screening and Antibacterial Activities of Tuber Extract of *Stephania pierrei* Diels

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ABSTRACT

Aqueous extract of *Stephania pierrei* tubers was studied for phytochemical screening and antibacterial activities as well as for cepharanthine concentration. The results revealed that the presence of alkaloids, triterpenoids, tannins, polyphenols, phenolic compounds of 41.49±0.66 mg GAE/g extract and cepharanthine content of 33.68 mg/g extract was found in the extract. The extract exhibited dose dependent microbial activities, moreover, the antibacterial activities were observed against Gram-positive bacteria (*Staphylococcus epidermidis* and *Staphylococcus aureus*) using disc diffusion assay. Especially, *S. epidermidis* was more sensitive to the extract than *S. aureus*. *S. epidermidis* was susceptible to the extract with the MIC and MBC of 2 and 6 mg, respectively. The ultrastructure alteration in extract-treated *S. epidermidis* cells was studied using transmission electron microscopy (TEM). The bacterial cell wall was ruptured after treated with the extract for 24 h. The characterization of functional groups was achieved by Fourier transform infrared spectroscopy (FTIR). FTIR spectra revealed changes in spectral regions corresponding to changes in fatty acid and polysaccharide on cell wall.

Keywords: antibacterial activity, cell wall disruption, Fourier transform infrared spectroscopy (FTIR), *Stephania pierrei*, transmission electron microscopy (TEM)

1. INTRODUCTION

Nowadays, antimicrobial resistance has become a major global healthcare problem. This has encouraged a search for the new antimicrobial agents with the new sources for their inhibitory activities [1]. Medicinal plants available in the world contain large varieties of substances which possess therapeutic properties that can be utilized in the treatment of human diseases. Moreover, the plants have proven to be an abundant source of biologically active compounds which are important source for the discovery of new antimicrobial agents [2]. Various researchers searched for new antimicrobial substances from crude extract as novel antimicrobial agents such as *Ficus foveolata* stems extract [1], leaves of *Ajuga iva*, *Marrubium vulgare*, *Mentha pulegium* and *Teucrium polium* [3], *Aquilaria crassna* leaf extract [4], and *Allium sativum* clove extract [5]. Therefore, medicinal

plants are an important source for the discovery of new antimicrobial agents.

The genus *Stephania* Lour (Menispermaceae) consists of 60 species distributed in tropical and subtropical Asia, tropical Africa and Oceania [6]. The plants of the genus *Stephania* (Menispermaceae) are widely distributed, and have been used in folk medicine for the treatment of various ailments such as asthma, tuberculosis, dysentery, hyperglycemia, malaria, cancer and fever. Moreover, over 150 alkaloids have been isolated from this genus together with flavonoids, lignans, steroids, terpenoids and coumarins [7]. There were several reports about bioactive compounds in the extract of the genus *Stephania*. Lv *et al.* [8] isolated six new bisbenzylisoquinoline alkaloids from tubers of *Stephania epigaea*. Moreover, they reported that cepharanthine exhibited cytotoxicity against all of cancer cell lines. Baghdikian *et al.* [9] studied the new antiplasmodial alkaloids from *Stephania rotunda.* They found that a new aporphine alkaloid named vireakine along with seven known alkaloids viz. stephanine, xylopinine, roemerine, pheudoplamatine, tetrahydropalmatine, cepharanthine and palmatine. The extract of *Stephania venosa* was reported for anti-HIV activity [10] and antiproliferative effects [11].

Stephania pierrei Diels (syn. *Stephania erecta* Craib) is a species recorded from the Indo-Chinese Peninsula. The tuber is traditionally used for the treatment of body oedema, migraine and hard disease [6]. Prawat *et al.* [12] studied the alkaloids of *S. erecta* by conventional procedures. They found that cepharanthine and homoaromoline were the two major components of the extract. However, antibacterial activities from *S. pierrei* extract have never been documented. In addition, water is universal solvent which is easy to find, economical and friendly to environment. Therefore, water was used for extraction of plant products with antimicrobial activity [13]. For this reason, the aim of this research is to study phytoconstituents and the antibacterial activities of the extract of *S. pierrei* tubers and possible antibacterial mechanism.

2. MATERIALS AND METHODS

2.1 Plant Material

The tubers of *S. pierrei* Diels were collected in Sakaeo province, Thailand. The plant was identified by a botanist, Dr. Chakkrapong Rattamanee, Faculty of Agricultural Technology, Burapha University Sakaeo Campus (BUU) and specimen of the plant has been kept at Faculty of Agricultural Technology, BUU. The voucher specimen number is AgriTech-002.

The tubers were washed 3-4 times with running tap water and cut into small pieces. The plant material was dried at 50°C in a hot air oven. *S. pierrei* extract was prepared by boiling 100 g of dried plant in 500 ml of distilled water for 30 min twice. The aqueous extract of *S. pierrei* was filtered through cotton gauze and centrifuged at 2,500×g for 10 min. Supernatant was collected and concentrated at 40°C using a rotary evaporator under reduced pressure. The residue was freeze dried in a lyophilizer. The dried extract (% yield of 15.5%, w/w) was kept at -20°C until used [4].

2.2 Phytochemical Screening

The qualitative method was used to detect various phytochemicals in order to give information regarding the nature of chemical constituents in the extract. The extract was detected in the presence of alkaloids (Mayer's reagent and Wagner's reagent), saponins (Froth test) and flavonoids (Shinoda test) according to Yadav and Agarwala [14]. Cardiac glycosides (Keller killiani test), triterpenoids (Salkowski test),

tannins (ferric chloride test) and antraquinone glycosides (Borntrager's test) were detected according to De *et al.* [15]. Polyphenols were detected according to Gundidza [16]. Three drops of a mixture of 1% ferric chloride (1 ml) and 1% potassium ferricyanide (1 ml) were added to the extract. The formation of green-blue color indicated the presence of polyphenols.

2.3 Determination of Total Phenolic Compounds

The amount of total phenolic compounds in the crude extract was measured by a method described by Prior *et al.* [17]. In brief, 100 µl of 2.5 µg/ml extract were mixed with 2 ml of 2% Na_2CO_3 and incubated for 2 min at room temperature. After incubation, 100 µl of Folin-Ciocalteu reagent (diluted in methanol 1:1 v/v) were added to the reaction mixture and incubated in the dark at room temperature for 30 min. The absorbance at 750 nm was measured using spectrophotometer. The concentration was calculated using gallic acid as a standard. The total phenolic compounds were expressed as milligrams gallic acid equivalents (GAE) per gram extract (mg GAE/g extract).

2.4 Liquid Chromatography-mass Spectrometry (LC-MS) Analysis

Cepharanthine content in the crude extract was analyzed by an Agilent Technologies 6490 Triple quadrupole LC-MS. Separation was performed on a Zorbax SB-C18 column (2.1 mm × 150 mm × 1.8 µm). Electrospray ionization (ESI) parameters were set as follows: nebulizer gas (N_2); N_2 temperature (350°C); N_2 flow rate (11 L/min); pressure (20 psi); capillary voltage (3000 V); fragmentor voltage (380 V). Sample was dissolved in methanol and injected with the volume of 10 µl comparing with cepharanthine (≥95%, HPLC grade, Sigma-Aldrich, Singapore).

2.5 Antibacterial Activities

2.5.1 Bacterial strains

Antibacterial activities of the extract were tested against four bacteria including *Staphylococcus epidermidis* TISTR 518, *Staphylococcus aureus* TISTR 1466, *Pseudomonas aeruginosa* TISTR 781 and *Escherichia coli* TISTR 780. The strains were obtained from the Thailand Institute of Scientific and Technological Research.

2.5.2 Disc diffusion assay

The antibacterial activity of the extract was determined by disc diffusion method [18]. The bacterial strains (*S. epidermidis* TISTR 518, *S. aureus* TISTR 1466, *P. aeruginosa* TISTR 781 and *E. coli* TISTR 780) were cultured in a Mueller-Hinton broth at 37°C for 18 h.

Then, 100 µl of the bacteria (10^8 CFU/ml) were spread onto the Mueller-Hinton agar plate. The extract was dissolved in dimethyl sulfoxide (DMSO) with different concentrations of 100, 200 and 300 mg/ml. Sterilized filter paper discs (6 mm in diameter) were impregnated with 20 µl of different concentrations of the extract corresponding to 2, 4 and 6 mg, respectively, and placed on the previously inoculated Mueller-Hinton agar. DMSO was used as a negative control, while vancomycin (30 µl/disc) was used as a positive control. The inoculated plates were incubated at 37°C for 24 h. All disc diffusion tests were performed in triplicate and the diameters (mm) of the inhibition zone were measured after incubation. Antibacterial activity was expressed as the mean of their diameter of inhibition zones (mm).

2.5.3 Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of the minimum inhibition concentration (MIC) against

bacteria (10^7 CFU/ml) was conducted with different concentrations of the extract (2, 4 and 6 mg) according to Kamonwannasit *et al.* [4] in Mueller-Hinton broth. MIC was defined as the lowest concentration of the agent with no visible bacterial growth. The inoculated tubes were incubated at 37°C for 24 h. The MBC determination was carried out by subculturing 100 µl from each tube from MIC assay with no visible bacterial growth onto fresh Mueller-Hinton agar plate followed by incubation at 37°C for 24 h. All experiments were done in triplicate. The MBC was defined as the lowest concentration of agent required to kill the bacteria.

2.5.4. Transmission electron microscopy

Cellular damage of bacteria was determined using transmission electron microscopy (TEM). Bacterial cells and bacterial cells treated with vancomycin and the extract were harvested after incubation at 37°C for 24 h. The cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h. The cells were washed three times with 0.05 M phosphate buffer (pH 7.2). The samples were post-fixed for 2 h in 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer (pH 7.2) at room temperature and washed in phosphate buffer. The samples were dehydrated through serial concentrations of ethanol (35%, 70%, 95% and 100%, respectively) for 15 min and embedded in Spurr's resin. The resins were cut with a diamond knife using an ultramicrotome to obtain the ultrathin sections and then set on bare copper grids. The ultrathin sections were counterstained with 2% (w/v) uranyl acetate for 3 min and then with 0.25% (w/v) lead citrate for 2 min. Finally, the specimens were examined with JEOL JEM-1230 transmission electron microscope operated at 80 kV [4].

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

Bacterial suspensions were exposed to the vancomycin or the extract and incubated at 37°C for 24 h. Bacterial cells were pelleted by centrifugation at 4,000 rpm for 15 min. Each pellet was washed twice with saline and re-suspended in distilled water. Then, the cells were deposited onto Mirr IR low e-microscope slides (Kevey slide) used as a substrate for FTIR microscope analysis. The samples were then desiccated under vacuum for several hours and stored in a desiccator to form films suitable before analysis [19].

Spectra were collected on a Bruker IR spectrometer (tensor 27) coupled to an IR microscope (Hyperion 2000) with 15× magnification. To achieve high S/N ratios, 64 scans were collected in each measurement in the range of 4000-400 cm^{-1} and resolution of 4 cm^{-1}. All spectra were processed using OPUS 6.5 software (Bruker optics) and the Unscrambler 9.7 software (Camo, Norway). Second derivative and vector normalize were manipulated to explain the differences in sample thickness, minimize baseline variation and allow visual comparison [20].

The data of the effect of variation and distribution of the components in bacterial cells during cultivation was analyzed by principle component analysis (PCA). All data analysis was carried out in the spectral range from 3000-2800 cm^{-1} to 1700-900 cm^{-1}, which covers the mixed region of lipid, protein, polysaccharide and the true fingerprint region. The spectra were extracted and normalized using OPUS 6.5 software (Germany) and generated second derivatives extended to multiplicative signal correction (EMSC) and principle component analysis using the Unscrambler 9.7 software (Camo, Norway) [21].

2.7 Statistical Analysis

All assays were performed in triplicate and the results were reported as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening, Phenolic Content and Cepharanthine Concentration

The results of phytochemical screening of the *S. pierrei* extract revealed the presence of alkaloids, triterpenoids, tannins and polyphenols (Table 1). Alkaloids are the main and common phytochemicals of the genus *Stephania* which were reported by Semwal *et al.* [7]. This result maybe indicated that the phytochemicals of the extract depended on methodology of extraction. Moreover, total phenolic content of 41.49±0.66 mg GAE/g extract was obtained. Akiyama *et al.* [22] reported that several tannins have shown antimicrobial activity against *S. aureus*. Phenolic compounds are well known as radical scavengers, metal chelators, reducing agents and hydrogen donors [23]. The presence of polyphenols implied that the plants were important natural antioxidants.

Table 1. Phytochemical screening, total phenolic compounds and cepharanthine content of *S. pierrei* extract.

Test		Results
phytochemicals	Methods	
alkaloids	Mayer's test	+
	Wagner's test	+
saponins	Froth test	-
triterpenoids	Salkowski's test	+
flavanoids	Shinoda test	-
	Ferric chloride test	-
tannins	Ferric chloride test	+
	Lead acetate test	+
cardiac glycosides	Keller killiani test	-
anthraquinone glycosides	Borntrager's test	-
polyphenol	Potassium ferricyanide test	+
total phenolic compounds	Prior *et al.* [17]	41.49±0.66 mg GAE/g extract*
cepharanthine content	LC-MS	33.68 mg/g extract

*mg GAE/g extract as milligrams gallic acid equivalents (GAE) per gram extract.

In the present work the quantitative analysis of cepharanthine content obtained from *S. pierrei* extract was evaluated. The LC-MS chromatogram of the *S. pierrei* extract was shown in Figure 1. Cepharanthine content was identified by comparing retention time and m/z values obtained by MS with the mass spectra from corresponding standard tested under the same conditions. The calibration curve obtained in Multiple Reaction Mode (MRM) was used for quantification of analyzed compounds ($y = 280.18x$, $R^2 = 0.9936$). The peak obtained at the retention time of 15.9 min belonging to cepharanthine was observed. Under the same condition, cepharanthine content of 33.68 mg/g extract was achieved.

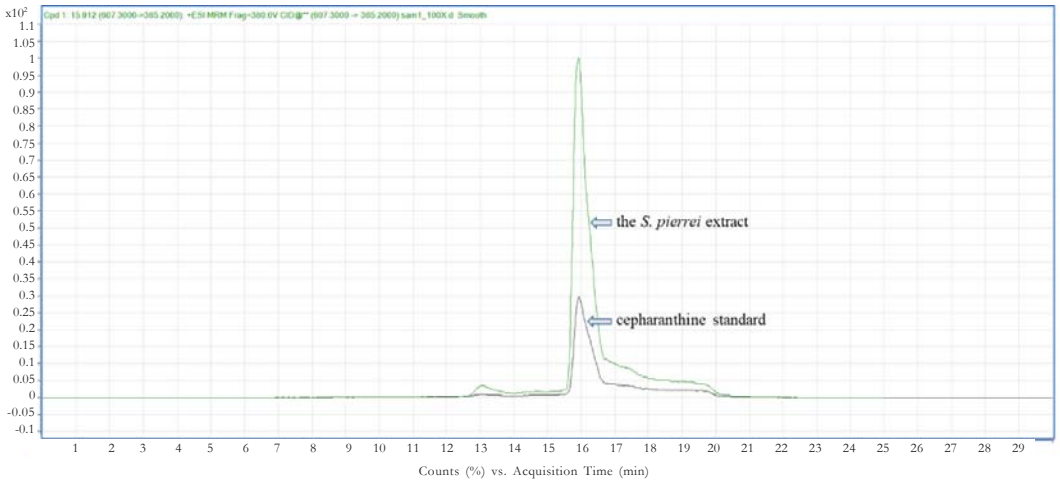

Figure 1. Liquid chromatography-mass spectrometry (LC-MS) chromatogram of cepharanthine in *S. pierrei* extract.

3.2 Antibacterial Activities

The antibacterial activities of the extract of *S. pierrei* were determined against four strains of pathogenic bacteria as shown in Table 2. The result showed that the Gram-positive bacteria (*S. epidermidis* and *S. aureus*) were inhibited by the extract, but the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were not inhibited by the extract. *S. epidermidis* was more sensitive to the extract than *S. aureus* with larger diameter of the inhibition zone. The highest antibacterial activity was found against *S. epidermidis* at 2 mg/disc. The inhibition zone of the extract was 12.16-13.50 mm, whereas vancomycin (30 µg) as control was 19.55 mm. The MIC and MBC against *S. epidermidis* were 2 and 6 mg, respectively (Table 3).

Table 2. Antibacterial activity of *S. pierrei* extract.

Microorganisms	Inhibition zone in diameter (mm)			
	The extract (mg)			Vancomycin (30 µg)
	2	4	6	
Staphylococcus epidermidis	12.16±1.16	13.16±0.98	13.50±0.54	19.55±1.90
Staphylococcus aureus	10.50±0.54	11.00±0.63	12.00±0.00	17.75±1.25
Escherichia coli	ND	ND	ND	ND
Pseudomonas aeruginosa	ND	ND	ND	ND

ND; not detected. Values are expressed as means ± standard deviation (n=3).

Table 3. MIC and MBC of *S. pierrei* extract.

Microorganisms	Concentration (mg/ml)	
	MIC	MBC
Staphylococcus epidermidis	2	6
Staphylococcus aureus	4	>10

This result indicated that the extract consisted of triterpenoids, phenolic compounds and cepharanthine which referred to antibacterial activity. Parveen *et al*. [24] reported that triterpenoids possess antibiotic, insecticidal and anthelmintic activity. Phenolic compounds such as tannins have been shown to possess antimicrobial activities [25]. Cepharanthine was referred to as analgesic, antispasmodic and bactericidal, antimalarial, antioxidant, antitumor and anti-inflammatory [26]. However, the antibacterial activity of the extract depends on the concentration of extract and strains of bacteria [3].

3.3 Transmission Electron Microscope (TEM) Observation

The effect of the extract on the possible antibacterial mechanism of *S. epidermidis* cells was studied by TEM. Vancomycin, an antibiotic which is widely known to damage bacteria cell wall of most Gram-positive bacteria, was used as control. A regular shape and cell wall of untreated *S. epidermidis* cells were found in TEM images (Figure 2A and 2D), whereas irregular shape and cell lysis were displayed on the treated cells with vancomycin (Figure 2B and 2E) and the extract (Figure 2C and 2F). This result implied that the bacterial cell wall was destroyed by the natural plant extract affecting on the metabolism of the cells as corresponding to Dong *et al.* [27].

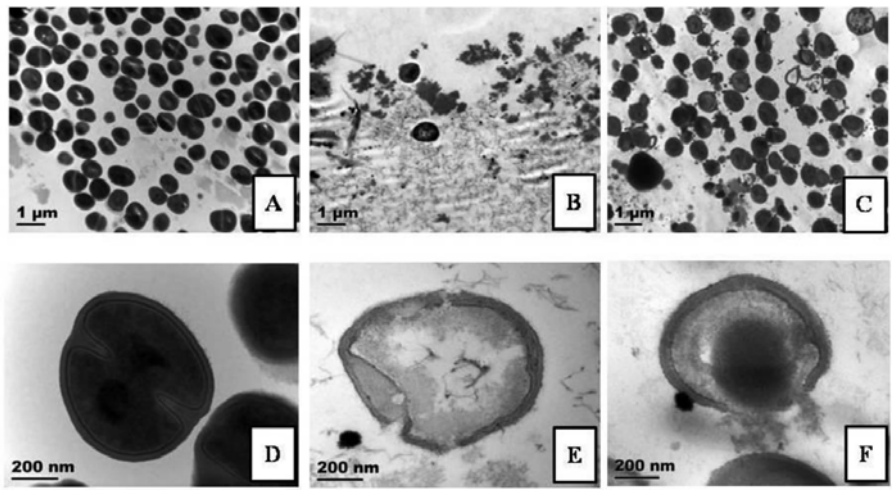

Figure 2. Transmission electron micrograph of *S. epidermidis* treated with *S. pierrei* extract. (A), (B) and (C) are overview of the untreated cells, treated with vancomycin and treated with the extract, respectively. Damaged cell wall and irregular shape were observed after 24 h of incubation with vancomycin (E) and the extract (F), compared with control (D).

3.4 FTIR Spectral Features of *S. epidermidis* Cells

Fourier transform infrared spectroscopy was used for study the structural changes of the major biological components of cells. FTIR spectra for most bacteria were reported in four different regions [5, 28-29]. Region I (3000-2800 cm^{-1}) represents fatty acid of the bacterial cell membrane with three noticeable peaks (~2960, ~2925 and ~2860 cm^{-1}). Region II (1700-1500 cm^{-1}) shows amide I and II bands of proteins and peptides with two peaks (~1650 and ~1550 cm^{-1}). Region III (1500-1200 cm^{-1}) conforms to fatty acid and proteins with three peaks (~1455, ~1400 and ~1240 cm^{-1}). Finally, region IV (1200-900 cm^{-1}) corresponds to carbohydrates and polysaccharides within microbial cell wall.

The FTIR spectra of the treated *S. epidermidis* cells with vancomycin and the extract were compared with the untreated cells. As shown in Figure 3 and 4, the changes of biochemical composition of the cells after treated with vancomycin and the extract were different from the untreated cells. The absorptions were detected in all four spectral regions that characterize the major component in the cells. In the first region, the peaks at 2962 and 2921 cm^{-1} were assigned to the C-H asymmetric stretching modes of the cell membrane fatty acids, while the peak at 2852 cm^{-1} was assigned to the symmetric stretching modes of the same group [28]. In the second region, the peaks at 1650 and 1544 cm^{-1} were assigned to the vibrations of the amide I and amide II bonds, respectively. In the third region, the peak at 1400 cm^{-1} was due to $C(CH_3)_2$ bending vibrations of proteins or carbohydrates, while the peak at 1240 cm^{-1} was attributed to the asymmetric stretching mode of phosphate groups in phospholipids or nucleic acids [28]. In the fourth region, the peaks at 1150, 1076 and 1022 cm^{-1} were assigned to the polysaccharide content in microbial cell wall. The intensity of peaks at region II (1650 and 1544 cm^{-1}) and region III (1400 and 1240 cm^{-1}) of the treated cells (vancomycin and the extract) was decreased comparing to the control. These results might be described that protein component (amide I and amide II) and phosphate group in phospholipids or nucleic acid in whole cells were decreased after treated with vancomycin and the extract leading to cell damage or cell lysis (Figure 3). In contrast, the intensity of peaks at region I (2962, 2921 and 2852 cm^{-1}) and region IV (1150, 1076 and 1022 cm^{-1}) of the treated cells was increased comparing to the control. These results might be due to production of fatty acid and polysaccharides on cell wall of bacteria in response to stressful condition [29], corresponding to the cell wall lysis in TEM images (Fig. 2E and 2F). Moen *et al.* [30] found that the amount of polysaccharides and oligosaccharides (1200-900 cm^{-1}) in bacterial cell membrane increased under non-growth survival conditions. The mechanisms for survival could be due to down regulation of most functions to save energy and to produce polysaccharides and oligosaccharides for protection against severe environments [30].

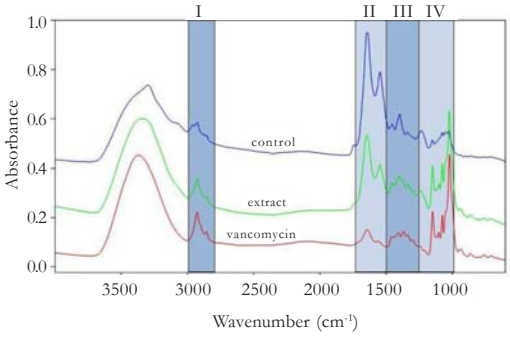

Figure 3. FTIR spectra of *S. epidermidis* cells after incubation for 24 h with the extract and vancomycin comparing to the control.

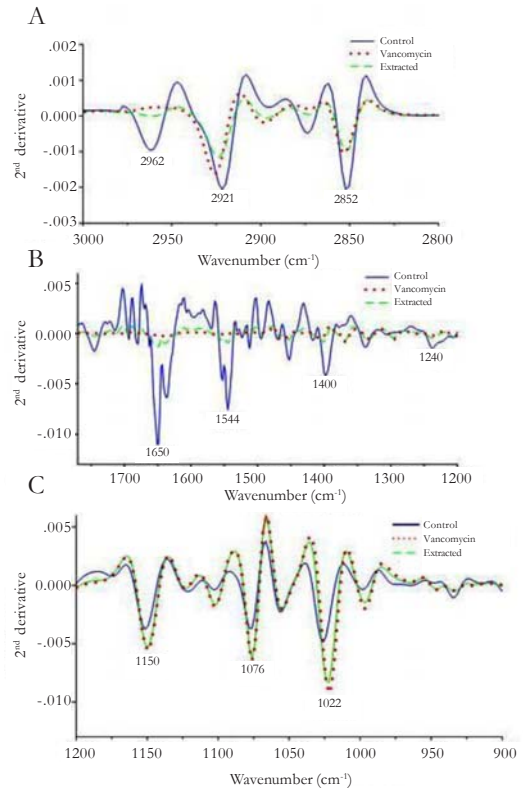

Figure 4. Representative FTIR spectra 3000-2800 cm^{-1} (A), 1700-1200 cm^{-1} (B) and 1200-900 cm^{-1} of control (heavy black line), treated with vancomycin (dot line) and treated with the extract of *S. epidermidis* (dash line).

3.5 Principal Component Analysis of Second Derivative Transformed Spectra of *S. epidermidis* Cells

PCA has been suited for analysis of the FTIR spectra, both for identification purposes and for analysis of the biochemical information in the spectra. The clusters in the data, and described similarities or differences from multivariate data sets were shown in PCA [28]. As demonstrated in Figure 5, it was found that the untreated cells as control can be easily distinguished from the treated cells (vancomycin and the extract) by the principal components. The main discriminant was the score of PC1, where the untreated cells have negative scores while the treated cells have positive scores. This result implied that the antibacterial mechanism of the extract was similar to the antibacterial mechanism of vancomycin.

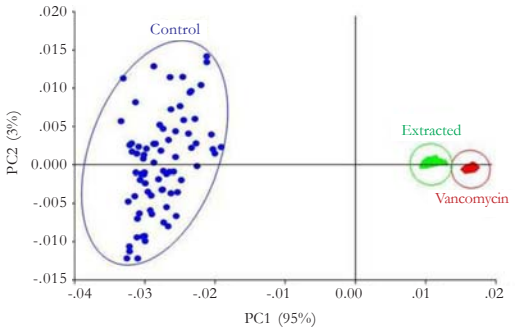

Figure 5. Principal component analysis (PCA) combination of control, treated with vancomycin and treated with the extract for *S. epidermidis*.

4. CONCLUSIONS

Phytoconstituents, total phenolics and antibacterial activities of the aqueous extract of *S. pierrei* were studied. The screening of alkaloids, triterpenoids, tannins, polyphenols and the quantity of phenolic compounds and cepharanthine in the extract were performed. The antibacterial activities of the extract depended on the bacterial strains and the concentration of the extract. *S. epidermidis* was more sensitive to the extract than *S. aureus* and Gram-negative bacteria. The antibacterial activity and mechanism of the extract were proven; therefore we suggest that *S. pierrei* might be a source for the discovery of new antibacterial agents against *S. epidermidis* and other Gram-positive bacteria.

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