



Isolation and identification of anti-methicillin resistant *Staphylococcus aureus* compounds from *Phyllanthus columnaris* stem bark

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ABSTRACT

Aim: *Phyllanthus columnaris* Müll.Arg. was found to possess anti-methicillin resistant *Staphylococcus aureus* (anti-MRSA) activities. This study aimed at isolating, identifying and evaluating the active compounds from the stem bark of *Phyllanthus columnaris* Müll.Arg. against MRSA.

Methodology and results: Stem bark extracts (methanol, acetone and aqueous) of *Phyllanthus columnaris* were subjected to anti-MRSA screening by disc diffusion method. MIC and MBC tests were carried out to compare the lowest concentration to inhibit and kill the sixteen MRSA tested among the three extracts. TLC bioautography were performed to detect the bioactive compounds. Isolation of the two active compounds was performed by means of preparative TLC. Morphological and ultra-structure alterations of the MRSA treated with bioactive compounds after 24 h were revealed by scanning and transmission electron microscopy. Both methanol and acetone extracts exhibited good anti-MRSA activity with the lowest minimum inhibitory concentration (MIC) value for both extracts were 0.78 mg/mL and the lowest minimum bactericidal concentration (MBC) were 1.56 mg/mL. Bioassay-guided chromatography by bioautography revealed two active anti-MRSA compounds from both tannin-free methanol and acetone extracts and characterized as stigmaterol and lupeol by nuclear magnetic resonance (NMR) spectral data. Scanning and transmission electron microscopy of MRSA treated with stigmaterol and lupeol showed cell wall disruption, release of cytoplasmic compounds and decreased in cellular volume.

Conclusion, significance and impact of study: Results obtained herein, may suggest that the stem bark of *Phyllanthus columnaris* possess anti-MRSA and the two of the active compounds isolated were stigmaterol and lupeol. Their anti-MRSA effects up to the morphological and ultra-structure studies were not reported earlier.

Keywords: *Phyllanthus columnaris*, anti-MRSA activity, TLC bioautography, SEM, TEM

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is commonly responsible for both hospital- and community-acquired infections (Hiramatsu *et al.*, 2002; Gastmeier, 2010). MRSA can cause various spectra of clinical diseases ranging from benign superficial skin infections to severe life-threatening conditions such as bacteraemia, endocarditis, pneumonia, abscesses, and soft or bone-tissue infections (Falcone *et al.*, 2009). These pathogenic Gram-positive bacteria have become a major threat to health worldwide due to its high prevalence, emergence of virulent strains and resistance to almost all available antibiotics thus increasing in mortality rate (Witte, 1999; Madani, 2002; Kerttula *et al.*, 2007; Delaney *et al.*, 2008). MRSA infection cases in hospitals are rising worldwide

due to the decrease in the susceptibility of MRSA to the antibiotics vancomycin and teicoplanin (Vaudaux *et al.*, 2001; Neoh *et al.*, 2007; Weigel *et al.*, 2007).

The emergence of resistant MRSA has led to the search for new sources of anti-MRSA drugs. Fazari *et al.* (2011) showed that methanol extracts from different parts of *Phyllanthus columnaris* displayed antibacterial activity against 13 MRSA isolates. The extracts from stem bark and root bark showed the most effective antibacterial activity and had no toxicity effects on Vero cells. In another study, *P. columnaris* stem bark demonstrated good antibacterial activity against a wide range of pathogenic bacteria (Siti-Noor-Adnalizawati *et al.*, 2013a). Herein, this study aims to further explore the anti-MRSA activity from several extracts of the stem bark of this plant. We then further isolated and identified the active

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compounds of *P. columnaris*. We also evaluate the anti-MRSA activity of the active compounds.

MATERIALS AND METHODS

Collection and preparation of extracts

The stem bark of *P. columnaris* was collected from Langkawi Island, Kedah, Malaysia and authenticated by a botanist, Mr. Sani Miran. A voucher specimen (WYA 497) was deposited at the Herbarium of Universiti Kebangsaan Malaysia. The outer layer of the stem bark was removed, dried in shaded room and ground to a fine powder using a Waring mill blender. The dried powder (100 g) was soaked in 500 mL of either methanol or acetone for three days at room temperature. The extracts were then filtered through Whatman No. 3 filter paper and concentrated to dryness using rotary evaporator (Heidolph 2 Laborota 4000, Germany). For aqueous extract, 100 g of powder was boiled for 2 h in 500 mL of water. The extract was then filtered prior to freeze drying. Tannins were removed from both methanol and acetone extracts by precipitating with diethyl ether.

Microorganisms

Clinically isolated MRSA were obtained from Hospital Serdang, Malaysia (7 isolates) and from Microbiology Laboratory culture collection, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (9 isolates). All MRSA isolates were verified by standard microbiological methods through selection of growth in Mannitol Salt Agar containing 7% NaCl with 2 mg/mL of oxacillin followed by confirmation with tube coagulase test and Antibiotic Sensitivity Test.

Anti-MRSA screening

Anti-MRSA screening of methanol, acetone and aqueous extracts were done by performing disc diffusion test according to Clinical and Laboratory Standards Institute (2006). Test solution was prepared by diluting 10 μ L of methanol, acetone, or aqueous extracts at 50 mg/mL with 5% methanol in phosphate buffered saline (PBS), 5% acetone in PBS or sterile distilled water accordingly. Antibiotic discs of vancomycin 30 μ g (BBL) and rifampicin 5 μ g (BBL) were used as positive controls for MRSA. Negative controls include 10 μ L of either 5% methanol in PBS, 5% acetone in PBS and sterile distilled water. The plates were then incubated at 37 °C for 24 h. Anti-MRSA activity was evaluated by measuring the zone of growth inhibition (diameter in mm) surrounding each disc. All tests were repeated three times in order to achieve optimal fidelity of results and to obtain the mean and standard deviation (SD).

Determination of MIC and MBC

Minimum inhibition concentration (MIC) test was done in 96-well sterile microplate according to Motlhanka *et al.* (2010) with slight modification. Two fold serial dilutions of each extracts ranging from 0.78 to 100 mg/mL were prepared prior to incorporation of 100 μ L of each diluted extract into respective wells. Sterile Mueller-Hinton Broth (80 μ L) and 20 μ L of 10^7 CFU/mL MRSA were introduced into all wells. Vancomycin hydrochloride (Sigma) at concentrations ranging from 0.0625 to 4 mg/mL was included as positive control. The extract-broth mixture serve as turbidity reference, broth only as sterility control and MRSA-broth suspension as inoculums viability controls. The test was done in duplicates. Turbidity of each well was measured after incubation at 35 °C for 24 h by using microplate reader (Biorad Model 680) at 595 nm. MIC values were determined by the lowest concentration of the extract that inhibited the microorganisms' growth. For Minimum Bactericidal Concentration (MBC) test, 10 μ L of sample from all the clear wells by MIC was streaked onto nutrient agar (NA) plates and incubated at 35 °C for 24 h. The MBC values were recorded as the lowest concentration showing no growth on the plate.

Thin layer chromatography bioautography

Thin Layer Chromatography was done to separate compounds available in tannin-free methanol and acetone extracts. For separation of compounds, 2 μ L tannin-free extract at 50 mg/mL was applied to silica gel plate (TLC aluminium sheets, 5 x 5 cm, Silica Gel 60F₂₅₄, Merck) and developed using hexane:ethyl acetate (8.5:1.5). The developed TLC plates were kept dried overnight in fume hood. The plates were sterilized under a UV lamp for 30 min and used for TLC bioautography by direct, contact and immersion/agar overlay tests against all sixteen MRSA isolates.

Direct bioautography procedure was done according to Horváth *et al.* (2002) with some modifications. The plates were dipped in nutrient broth containing 10^6 CFU/mL test bacteria. The plates were incubated at 35 °C for 2 h. The plates were then dipped in 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated further at 35 °C for 24 h in humid condition.

Contact bioautography procedure was done according to Das *et al.* (2010) with some modifications. TLC plates were placed aseptically onto nutrient agar (NA) and left for 5 h in order to allow diffusion of fractions in the agar. Fractions were marked at the bottom of agar plate and TLC plates were then removed from the surface of agar with sterile tweezer. MRSA inoculum at 10^6 CFU/mL was evenly spread onto the surface of the respective NA using sterile cotton swab and were incubated at 35 °C for 24 h.

Immersion/agar overlay procedure was done according to Sharma *et al.* (2009) with some modifications. TLC plates were covered by molten agar containing MRSA and left for 10 h to allow the fractions to diffuse into the agar. The plates were then incubated at

35 °C for 24 h. After incubation, the plates were sprayed with 5 mg/mL MTT and further incubated at 35 °C for 24 h. The areas of inhibition were marked and relevant R_f values were recorded. All the above tests were run in duplicates.

Isolation and identification of anti-MRSA bioactive compounds

Bioactive tannin-free methanol and acetone extracts (25 mg each) were further fractionated by preparative thin layer chromatography (P-TLC; 20x20 cm glass plates covered with Silica 60, F254; 0.5 mm Merck 7749) using hexane:ethyl acetate (8.5:1.5). Reference bands for the fractions were determined in parallel on another P-TLC (10x20 cm) which was detected under UV-light (at A_{254} and A_{365}). Further detection of bands was done by spraying of TLC plates with cerium (IV) sulphate solution (Bilia *et al.*, 1996; Rodriguez *et al.*, 2008). Active compounds 1 and 2 on the P-TLC were separately removed by scraping off the corresponding bands according to their R_f values and washed five times with acetone. Both compounds were subjected to TLC to check for quality and purity. Purified compounds 1 and 2 were analysed using nuclear magnetic resonance (NMR) (JEOL ECM-400) spectral data.

Scanning Electron Microscopy

MRSA which were treated separately with stigmasterol and lupeol (with their respective MIC value, 12.5 mg/mL each) and untreated (as control) were inoculated on brain heart infusion agar after 24 h incubation at 37 °C. After 24 h incubation at 37 °C, the bacteria culture agar were sliced into 1 cm³ and fixed with 2% glutaraldehyde for 24 h at 4 °C. After fixation, samples were washed with 0.1 M phosphate buffer. Subsequently, the samples were dehydrated in graded ethanol, critical-point dried in CO₂, mounted onto stubs, coated with gold and observed on a Carl Zeiss LEO 1450VP scanning electron microscope.

Transmission Electron Microscopy

MRSA with or without addition of stigmasterol or lupeol (with their respective MIC value, 12.5 mg/mL each) were harvested by centrifugation and washed once with phosphate buffer saline, pH 7.4. MRSA cells were prepared for transmission electron microscopy (TEM) by primary fixation in 2% glutaraldehyde for at least 24 h at 4 °C and post-fixation in 1% osmium tetroxide for 2 h at room temperature. The samples were dehydrated in graded ethanol, embedded in epoxy resin, polymerizing, sectioning and staining with uranyl acetate followed by Reynolds' stain. The ultrathin sections were viewed and photographed using a Philips CM12 transmission electron microscope.

RESULTS

Table 1 shows the results in anti-MRSA screening, with MIC and MBC values of extracts against tested MRSA. Aqueous, methanol and acetone extracts from *P. columnaris* stem bark with 50 mg/mL each showed inhibitory activity against all MRSA with mean of inhibition zones ranged between 9-12, 9-12 and 9-13 mm respectively. Figure 1 is a representative picture for zone of inhibition of *P. columnaris* against selected MRSA.

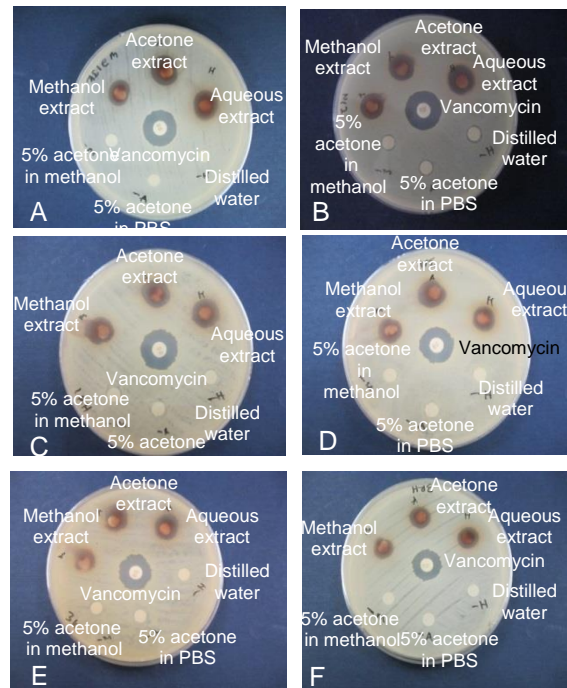


Figure 1: Zone of inhibition of extracts and controls against MRSA 21J (A), MRSA N12 (B), MRSA 1RH (C), MRSA 3AK (D), MRSA 3K (E) and MRSA 3RH (F).

Aqueous extract showed the highest MIC value (50 mg/mL) on most of the MRSA tested. Methanol extract showed lowest MIC concentration of 0.78 mg/mL against MRSA BM2 and MRSA BM3 and also acetone extract on MRSA N11, MRSA BM2 and MRSA BM3. The lowest MBC value was 1.56 mg/mL showed by methanol extract on MRSA BM2 and MRSA BM3. Acetone extract also showed the same lowest MBC value on MRSA N11, MRSA BM2 and MRSA BM3. Both methanol and acetone extracts were further investigated due to their profound anti-MRSA activity.

TLC bioautography revealed the presence of two active compounds in both tannin-free methanol and acetone extracts. These compounds were determined to exhibit inhibition areas against all sixteen MRSA tested as indicated by the R_f values 0.52 and 0.16 and agreeable in direct and contact bioautography methods but no inhibition in agar overlay. Compounds 1 (R_f 0.52) and 2

Table 1: Results for anti-MRSA assay of *P. columnaris* stem bark extracts at 50 mg/mL by disk diffusion test (zones of inhibition in mm), MIC and MBC. The diffusion test was done in triplicates and MIC/MBC in duplicates.

MRSA	Inhibition zone (mm) of control antibiotics		Methanol extract			Acetone Extract			Aqueous extract		
	Vancomycin	Rifampicin	Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)
MRSA N11	18±1.00	6±0.00	10±1.53	1.56	3.12	12±0.58	0.78	1.56	11±0.58	50.00	>50.00
MRSA N12	17±0.58	30±0.00	11±0.58	12.50	12.50	13±1.15	12.50	12.50	12±0.58	50.00	>50.00
MRSA N17	16±0.00	26.67±1.53	9±1.15	12.50	25.00	11±1.15	12.50	12.50	11±0.58	50.00	>50.00
MRSA 1RH	17±0.58	31±1.00	11±0.58	12.50	12.50	12±0.58	12.50	12.50	12±0.00	50.00	>50.00
MRSA 3AK	16±0.58	30±0.00	10±0.58	12.50	12.50	12±0.58	12.50	12.50	10±1.15	50.00	50.00
MRSA 3K	16±0.00	31.67±1.53	11±0.00	12.50	12.50	12±0.58	12.50	12.50	11±1.53	50.00	50.00
MRSA 3RH	15±0.58	25.67±1.15	11±0.58	12.50	12.50	11±0.58	12.50	12.50	11±0.00	50.00	50.00
MRSA 4J	16±0.58	25.67±1.15	9±1.15	12.50	12.50	12±0.00	12.50	12.50	11±0.58	50.00	>50.00
MRSA 21J	16±0.00	30±0.00	11±0.58	12.50	12.50	12±0.58	12.50	12.50	12±0.58	50.00	>50.00
MRSA TM	17±1.00	31.33±0.58	9±1.15	12.5	25.00	12±0.58	12.50	25.00	12±0.58	50.00	>50.00
MRSA WM1	6±0.00	35±0.00	10±1.53	3.12	6.25	10±1.15	6.25	12.5	9±1.15	50.00	>50.00
MRSA WM2	17±0.58	35.33±0.58	12±0.58	3.12	6.25	10±1.15	3.12	6.25	9±0.00	12.50	50.00
MRSA WM3	17±0.58	32.33±0.58	10±1.53	6.25	12.5	10±1.15	6.25	12.5	10±1.15	25.00	50.00
MRSA BM1	17±1.15	31.33±1.15	10±1.15	6.25	12.5	9±1.15	12.50	25.00	12±0.00	50.00	>50.00
MRSA BM2	17±0.00	33.67±0.58	10±1.53	0.78	1.56	10±1.53	0.78	1.56	12±0.58	25.00	50.00
MRSA BM3	6±0.00	30±0.00	9.67±1.53	0.78	1.56	9±1.15	0.78	1.56	12±0.58	50.00	>50.00
MRSA ATCC 43300	x	31±0.00	3	12.50	25.00	9±1.15	12.50	25.00	10±0.00	50.00	>50.00

x : Not tested

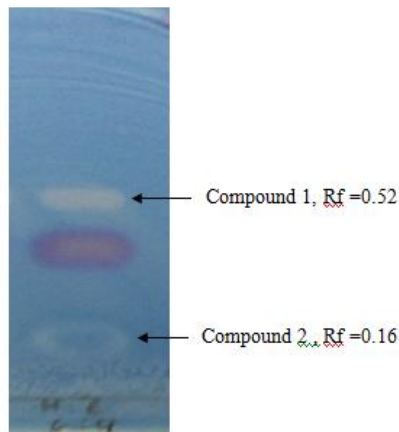


Figure 2: Representative picture of direct TLC bioautography for determination of anti-MRSA activity of compounds 1 and 2 available in tannin-free *P. columnaris* stem bark acetone extract.

(R_f 0.16) exhibited zones of inhibition on all MRSA tested. Figure 2 is a representative picture for direct TLC bioautography against MRSA.

Compounds 1 and 2 which were active on all MRSA tested were further isolated using P-TLC and analysed by NMR spectroscopy. Structural determination of the compounds by NMR enabled us to identify the two triterpenes. The molecular formulas of compounds 1 and 2 were determined as $C_{30}H_{50}O$ and $C_{29}H_{48}O$ respectively.

Compound 1: Amorphous white powder (Figure 3A). 1H NMR (Acetone- d_6 , 400 MHz): δ 3.13 (1H , dd, H-3), δ 2.45 (1H , m, H-19), δ 4.70 (1H , s, H-29a), δ 4.55 (1H , s, H-29b). ^{13}C NMR (Acetone- d_6 , 400 MHz): δ 78.4 (C-3), 151.8 (C-20), 109.9 (C-29).

Compound 2: White colourless needles (Figure 3B). 1H NMR (Acetone- d_6 , 400 MHz): δ 3.40 (1H , m, H-3), 5.32 (1H , d, H-6), 5.20 (1H , dd, H-22), 5.08 (1H , dd, H-23). ^{13}C NMR (Acetone- d_6 , 400 MHz): δ 142.4 (C-5), 139.4 (C-22), 130.2 (C-23), 121.6 (C-6), 71.8 (C-3).

Compound 1 has been identified as (3 β ,13 ξ)-lup-20(29)-en-3-ol, clerodol, monogynol B, Fagarasterol, Farganasterol or lupeol (Figure 4A) and compound 2 as stigmasterin or stigmasterol (Figure 4B).

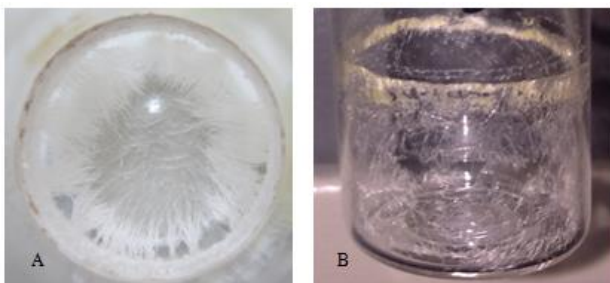


Figure 3: Physical forms of A, lupeol as amorphous white powder and B, stigmasterol as white colourless needles.

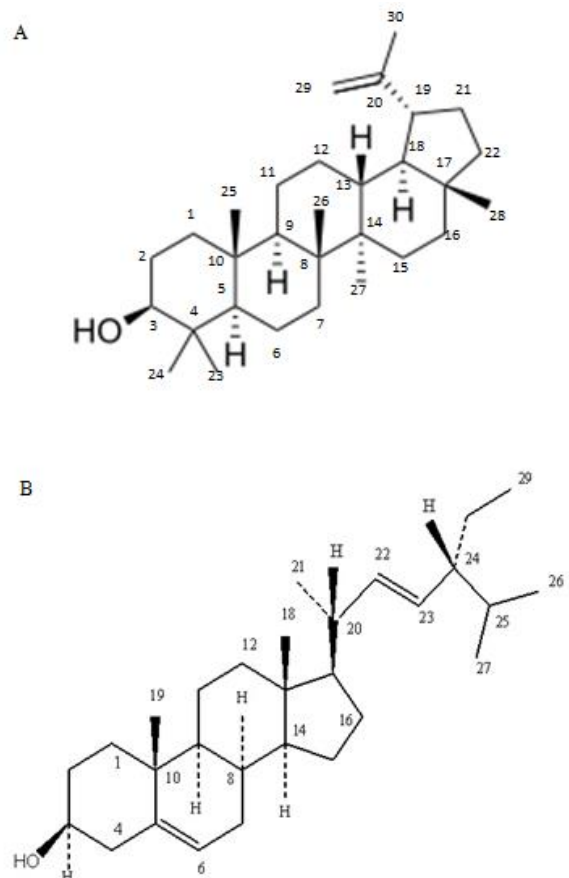


Figure 4: Chemical structure of A, lupeol and B, stigmasterol.

Scanning and transmission electron microscopy were performed for untreated and treated MRSA to determine morphological and ultra-structural changes caused by both stigmasterol and lupeol at their minimum inhibitory concentration (12.5 mg/mL) after 24 h (Figure 5). In scanning electron microscopy, untreated MRSA appeared to have a regular, smooth surface and spherical in grape-like clusters (Figure 5A). For MRSA treated with minimum inhibitory concentration of stigmasterol (Figure 5B) and lupeol (Figure 5C) at 12.5 mg/mL each, two types of cells were observed after 24 h of incubation: MRSA treated with stigmasterol showed agglomerated cells and only minor morphological alterations but MRSA treated with lupeol showed many distorted cells. These morphological features in MRSA cells may be due to the action on the cell wall followed by loss of cell volume. These findings were confirmed by transmission electron microscopy. Untreated MRSA possessed structural integrity which was confirmed by having thicker cell wall (Figure 6A and B). MRSA treated with either stigmasterol or lupeol showed disruption and damage to the cell wall, resulting in a release of cytoplasmic contents (Figure 6C and F), loss of cell walls (Figure 6E, G and H), alterations in morphology (Figure 6D and E) and decrease in cell volume (Figure 6C, D, F and G).

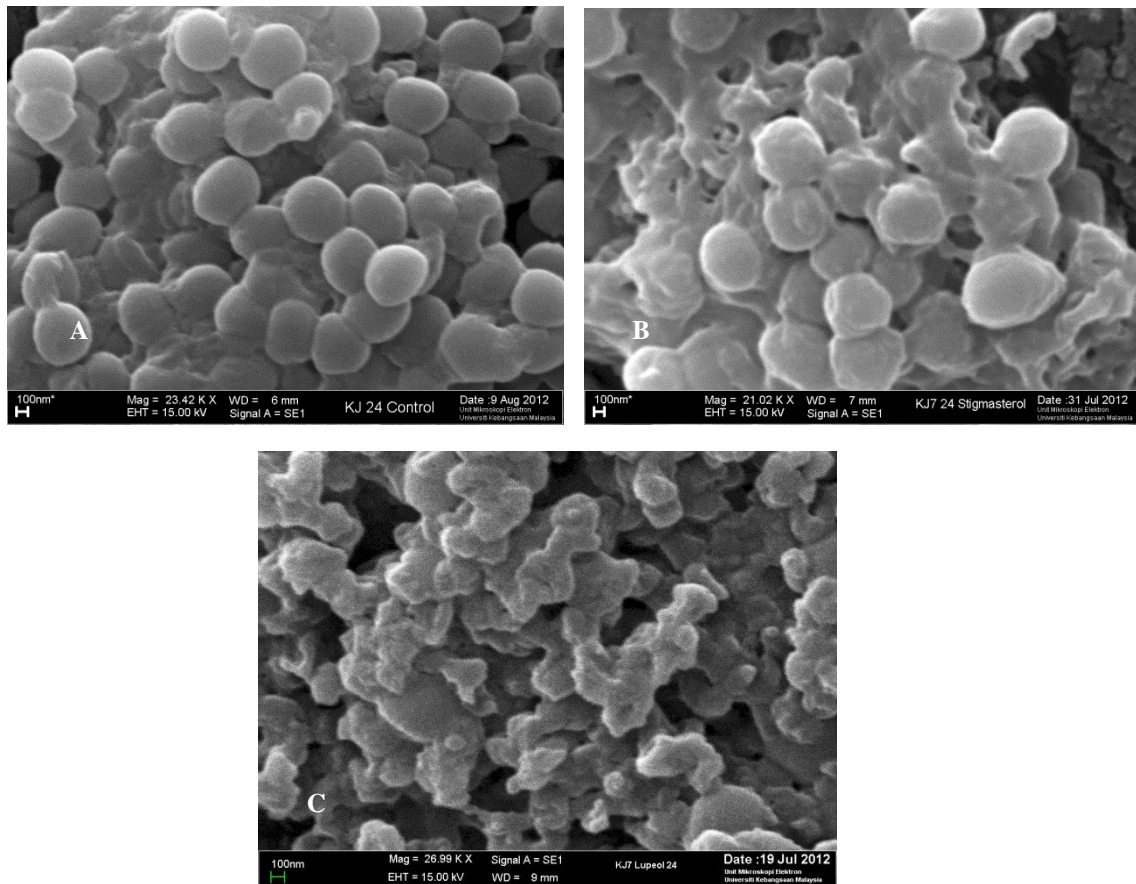


Figure 5: Scanning electron microscopy of MRSA treated with stigmasterol and lupeol obtained from *P. columnaris* for 24 h at 37 °C. A, control; B, stigmasterol at 12.5 mg/mL and C, lupeol at 12.5 mg/mL. Bars = 0.1 µm.

DISCUSSION

P. columnaris stem bark extracts showed anti-MRSA effects as indicated by disk diffusion assay, MIC and MBC tests in this study. Even though the disk diffusion test result did not show differences in the mean of the inhibition zone diameter between the three extracts, the results of MIC and MBC showed contradictory results with aqueous extract disk diffusion test. This contradiction could be due to the ability of polar active compounds in aqueous extract to diffuse further on the agar and displayed inhibition zone compared to less polar active compounds in methanol and acetone extracts which were more active but can only show inhibition zone within limited diameter due to the lesser ability to diffuse (Pauli *et al*, 2005). By performing MIC and MBC tests, the agar barrier factor that influenced the disk diffusion test was eliminated and the active compounds can directly be exposed to the tested MRSA.

Direct and contact TLC bioautography showed that both tannin-free acetone and methanol extracts have two similar active compounds that were responsible to inhibit all the MRSA isolates in the study. The observation of

antibacterial and anti-yeast activities from tannin-free methanol extracts from the same part of the plant was also reported in Siti-Noor-Adnalizawati *et al*. (2013a). Agar overlay method failed to indicate any inhibition by these two active compounds thus concluded as insensitive procedure in TLC bioautography. In a previous study by Brantner (1997) the effect of varying different parameters in antibacterial assays showed that more precise results were obtained when the bacteria were suspended in the nutrient medium which was poured over the TLC plates than when they were distributed over the solidified nutrient medium which was already on the plates. Variation of the culture medium and stains also gave different results, with para-iodonitrotetrazolium violet (INT) proved to be the most suitable detection reagent. However, there is also limitation to direct bioautography method which is only applicable to microorganisms that can grow directly on the TLC plate (Marston, 2011). In this study, we can conclude that direct bioautography method is more sensitive and suitable for the screening of anti-MRSA new compounds.

Compounds 1 and 2 which were active towards all MRSA isolates tested were identified as lupeol and

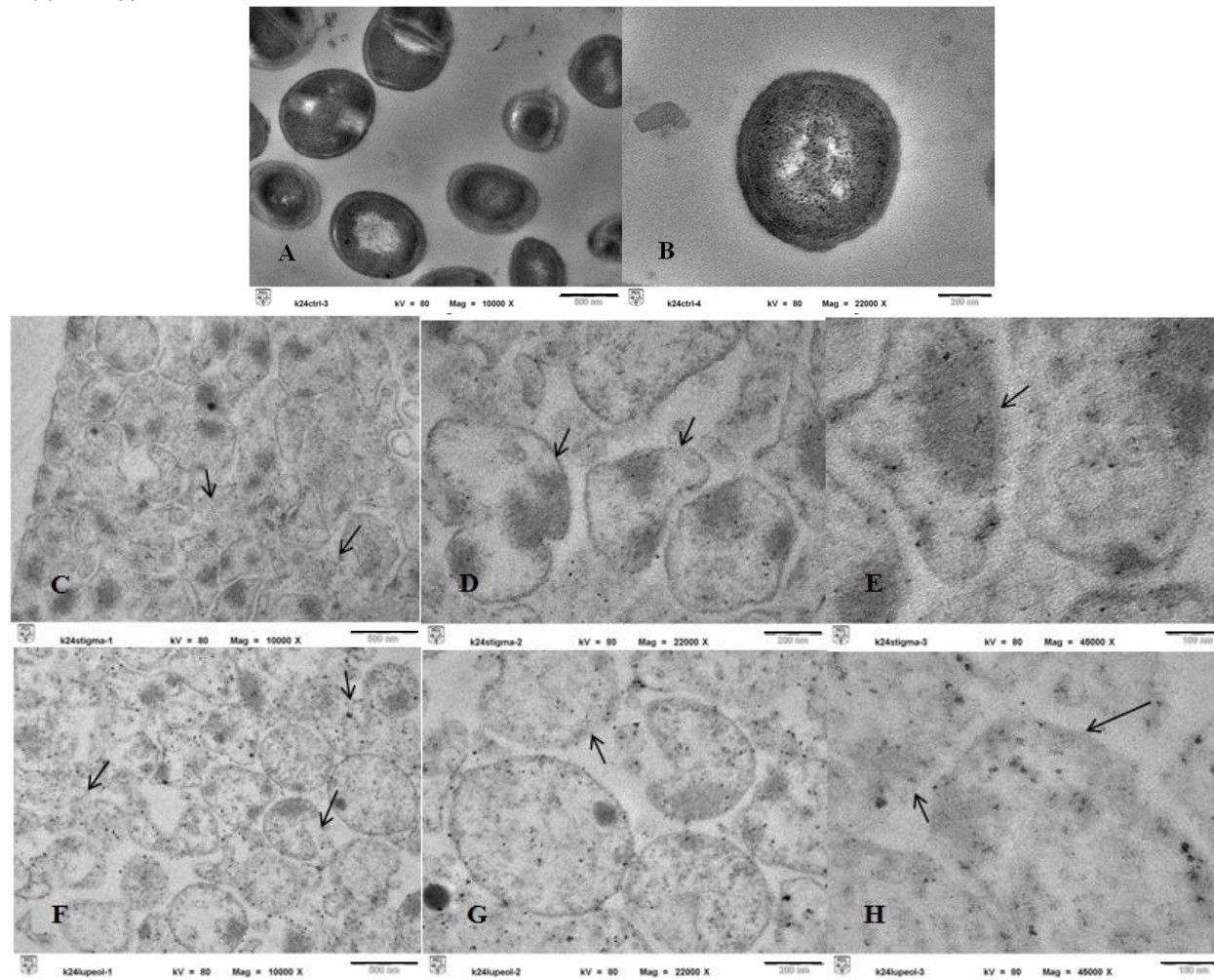


Figure 6: Transmission electron microscopy of MRSA treated with stigmasterol and lupeol obtained from *P. columnaris* for 24 h at 37 °C. A and B, control; C, D and E, stigmasterol at 12.5 mg/mL; F, G and H, lupeol at 12.5 mg/mL. Bars A, C and F = 0.5 μ m; B, D and G = 0.2 μ m; E and H = 0.1 μ m. Arrows in C and F indicates release of cytoplasmic contents; E, G and H-loss of cell walls

stigmasterol by NMR spectral data. Both compounds were also isolated by Jamal *et al.* (2009b) in ethyl acetate extract of root bark of the same plant. They also succeeded in isolating stigmasterol which is a triterpene and two more triterpenes which were also identified as taraxerone and taraxerol from the methanol extract of *P. columnaris* root bark (Jamal *et al.*, 2009a). Several studies on antibacterial activities of extracts containing lupeol and stigmasterol showed weak to moderate antimicrobial activity (Gallo and Sarachine, 2009; Parvin *et al.*, 2009; Sultana *et al.*, 2009). However, a study done by Suryati *et al.* (2011) showed contrary result with MIC values of lupeol against *E. coli*, *B. subtilis* and *S. aureus* were 150, 220 and 130 µg/mL respectively. These contradicting results in the antibacterial activity of lupeol has led to the hypothesis that different activities of this compound might be due to the ability of some bacteria to biotransform the substances yielding different metabolites that possess different activities (Eiznhamer and Xu, 2004).

Morphological and ultra-structure alterations of the MRSA treated with stigmasterol and lupeol after 24 h were revealed by scanning and transmission electron microscopy. Both compounds affect the cell wall and lead to cell wall disruption, release of cytoplasmic contents and decreased in cellular volume. Observations on crude methanolic *P. columnaris* which contain both these compounds have shown not only similar results but also causes disorganisation of cell division (Siti-Noor-Adnalizawati *et al.*, 2013b).

CONCLUSIONS

In conclusion, it is suggested that both lupeol and stigmasterol can be new source for new anti-MRSA agents with further research to be undertaken. Furthermore, the structure of both compounds differs from the currently used antibiotics. Thus, the emergence of cross-resistance is unlikely to be caused either and development of resistance to these compounds will be delayed. Studies are underway to investigate the mechanism of action of both compounds on MRSA. Future investigation will also involve *in vitro* and *in vivo* toxicity studies to elucidate cytotoxic effect of both compounds.

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