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2-D Gel Electrophoresis Map of Methicillin-Resistant *Staphylococcus aureus* Treated with *Quercus Infectoria* Gall Extract

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ABSTRACT

The widespread outbreak of Methicillin-Resistant *Staphylococcus Aureus* (MRSA) has caused clinical and epidemiological concern in hospital environment. The emergence of Vancomycin-Intermediate *S. Aureus* (VISA) and, more recently, Vancomycin-Resistant *S. Aureus* (VRSA) has further alarmed clinician and scientist worlwide. The objective of this study is to determine the optimum concentration of sample protein from MRSA after treatment with acetone extract from *Quercus infectoria* gall. Comparison of the Protein Expression Profile (PEP) between the treated MRSA and untreated strain as control was obtained using 2-dimensional gel electrophoresis. The Minimum Inhibitory Concentration (MIC) value of acetone extract of galls from *Q. infectoria* against two strains of MRSA; ATCC 33591 and PPUKM clinical isolate was determined by broth microdilution method. The MIC value of acetone extracts against both strains of MRSA was 0.3125 mg mL^{-1} compared to vancomycin (0.00195 mg mL^{-1}). The optimum concentration of MRSA protein that produced the best resolution was 100 µg. Manifold technique was observed to produce a gel with better resolution and greater number of spot compared with the strip holder technique. This study showed that there were 7 protein spots that represented the increased in the protein expression of more than 2-fold in the MRSA treated with acetone extract of galls *Q. infectoria* compared to the untreated group. This preliminary study on the PEP of *Q. infectoria* galls extract-treated MRSA may provide an insight of its antimicrobial mechanism which could lead to the identification of target protein in the future development of a new effective regimen for the treatment of MRSA infections.

Keywords: *Quercus Infectoria*, MIC, 2-D GE, Protein Expression Profile, MRSA

1. INTRODUCTION

Infectious diseases are reported to be the second leading cause of death worldwide, after heart disease and are responsible for more deaths annually than cancer (WHO, 2008). The bacteria which acquired resistance to antibiotics are a major threat even in developed countries. The wide emergence of MRSA that showed lack of susceptibility to vancomycin has caused public concern, particularly in hospitals. Clinical isolate of Vancomycin-Intermediate *S. Aureus* (VISA), was first reported in Japan (Hiramatsu *et al*., 1997).

According to Bandow *et al*. (2003), proteomic analysis is very useful in target identification and validation of a target to produce new novel antibiotic. Proteomic is the entire complement of proteins produced from the genome in a cell (Korke *et al*., 2002). In other words, proteomic encompasses all protein being expressed in the cell at a particular time (Rappsilber and Mann, 2002). Studies have shown that plants can be a rich source of anti-bacterial

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activity, anti-fungal (Yamunarani *et al*., 2005), antimicrobial (Voravuthikunchai and Kitpipit, 2005; Basri *et al*., 2012) and anti-MRSA (Vithya *et al*., 2011).

Gall from *Quercus infectoria* is a rounded abnormal growth which is found arise in young branches of oak trees deposing by *Diplolepis gallae tinctoriae* (Samuelsson, 1999). Research has shown that gall *Quercus infectoria* rich in bioactive compounds such as tannin (Haghi and Safaei, 2004), vitamin A, vitamin C, calcium, iron, fiber, protein and carbohydrate (Jalalpure *et al*., 2002). However, there has been no literature to date, that reports on the changes in protein expressed by MRSA treated by extracts from *Q. infectoria* galls. To our knowledge, this is the first report on the comparative proteomic approach to obtain an optimal protein concentration for two dimensional gel Electrophoresis (2-D GE) analysis. This preliminary study can provide important information in the study of mechanism of action of gall extract from *Q. infectoria* against MRSA. Protein from MRSA exposed to *Q. infectoria* gall extract was separated using 2-D GE and then analyzed using computer software to identify changes in differential protein expressed in the treated and control sample.

2. MATERIALS AND METHODS

2.1. Plant Materials

Q. infectoria galls used in the present study were purchased from Chow Kitt Road market in Kuala Lumpur. The specimen was deposited in Forest Research Institute Malaysia (FRIM) and was kindly identified by Dr. Mastura Mohtar with the voucher no. EZ186/93. The galls were pounded into coarse powder using pestle and mortar and blended into a dry, fine powder using an electric blender.

2.2. Preparation of Extracts

Acetone extract was prepared by adding the dried gall powder in acetone at ratio 1:5 for 24 hr at room temperature. The resulting mixture was filtered and the process was repeated by immersing the remaining residue with fresh solvent. The filtrates were then combined and concentrated under reduced pressure using a rotary evaporator. The pellet produced was finally pounded to dryness under hot air-dryer to become a powdery crude extract (Basri and Fan, 2005).

2.3. Determination of Minimum Inhibitory Concentration (MIC)

Broth serial microdilution technique was used to determine the Minimum Inhibitory Concentration (MIC) of acetone extracts of gall *Q. infectoria* and vancomycin against MRSA strains ATCC 33591 and PPUKM clinical isolate. By using the 96-microtiter well plates, 50 µL of 20 mg mL^{-1} of either the tested extract or vancomycin was transferred to the first well after addition of 50 µL Muller-Hinton (MH) broth. Then, 50 µL of solution from the first well was transferred to the next well and this process was repeated until the 10th well in which, 50 µL of the solution was removed. Subsequently, 50 µL of bacterial inoculum (10^6 bacteria/mL) was added to give a total final volume of 100 µL in each well. The well which represent positive control contained only MH broth and the antimicrobial agent whereas the negative control comprise MH broth and bacteria inoculum. Finally, the plate was incubated for 24 h at 37°C prior to addition of Triphenyl Tetrazolium Chloride (TTC) into each well for both the treated and control well. The MIC values are determined visually by the lowest concentration represented by the 1st well with no colour change which indicated absence of bacterial growth. On the other hand, the production of red color in the wells indicated bacterial growth.

2.4. Protein Extraction and Quantification

Both the ATCC 33591 and PPUKM isolates were subcultured onto MH agar and incubated at 37°C for 24 hr. The isolates were incubated in 300 mL of MH broth at 37°C and the turbidity of culture of bacteria was adjusted visually to an Optical Density (OD) of 0.08 at a wavelength of 625 nm using the spectrophotometer which corresponded to about 10^8 CFU/mL bacteria.The untreated group as control comprised 150 mL of bacteria in the beaker and 150 mL of broth. However as far as the treatment group is concerned, 25XMIC of either the acetone extract or vancomycin was mixed with bacterial turbidity adjusted to OD of 2.00 which is also 25 times that of the bacteria size in the untreated control. This is to make sure that the amount of pellet was adequate during the extraction process in order to run the 2-D gel electrophoresis. Extraction of protein samples from MRSA was performed based on modification of Thomas-Carter and Pennington (1989). Thus, a total of 300 mL of the treated culture in a conical flask was then positioned in an incubator shaking bath at 37°C for 24 hr, centrifuged at 10,000 g for 15 min at 4°C. The supernatant was discarded and the pellet were suspended in 10 mL phosphate Buffer Saline Solution (PBS). The resulting solution was then spinned again at 2,500 g for 15 min at 25°C, the pellet suspended again in 1 mL PBS and centrifuged at 9,000 g for another 15 min at 25°C. All the pellets were combined and poured into 1ml PBS to be sonicated using ultrasonic sonicator machine for 5 min every 1 min each time at 2 min intervals. Finally, the

samples were centrifuged again at 11,600 g for 10 min. Subsequently, the supernatant was taken and the protein concentration was determined by Bradford assay before the samples were stored at -80°C.

2.5. 2-D Gel Electrophoresis

The protein samples, each time at three different concentrations of 80, 100 and 120 µg, was separated based on the first dimension isoelectric point using Ettan IPGPhor 3 isoelectric focusing System (GE Healthcare Bio-Sciences, Uppsala, Sweden) following the recommendations in the manual on 24-cm Immobiline DryStrips (pH 3-10, non linear). Dry strips were initially rehydrated for 24 h with 450 µL of rehydration buffer comprising 7M urea, 2M thiourea, 4% 3-[(cholamidopropyl) dimethyl ammonio]-1-propanesulfonate, 0.5% IPG buffer (pH 3-10) and trace amounts of bromophenol blue. Then, the sample protein was solubilized with the same buffer in addition to 49 mM dithiothreitol. The dry strips were focused at a total voltage of 58.47 kVh. The focused IPG strips were left to equilibrate for 15 min in equilibration buffer (6M urea, 75mM Tris-HCl pH 8.8, 29% glycerol, 2% SDS and trace amounts of bromophenol blue) containing 1% DTT. It was then alkylated in the standard sample buffer consisting of 2.5% iodoacetamide for the following 15 min. Two-dimensional gel electrophoresis of reduced and alkylated samples was then loaded on 24 cm, 10.0% sodiumdodecyl Polyacrylamide Gel Electrophoresis (PAGE) gels on an ETTAN DALT II platform (GE Healthcare). The gels were run at 10 mA per gel in the first step and 40 mA during the second step until the bromophenol blue which is used as a tracking dye, reached the bottom of the gel.

2.6. Silver Staining

Protein spots were visualized using standard protocols as described in PlusOne Silver staining kit (GE Healthcare Bio-Sciences). The complete protocol was obeyed to analyse the analytical gels. As far as the preparative gels were concerned, the protocol was modified so that glutaraldehyde was removed from the sensitization step whereas formaldehyde was excepted from the silver reaction step (Yan *et al*., 2000). Silver-stained gels were scanned using UMAX PowerLook 1000 Imaging system where as the protein profiles were compared (Image Master Platinum software, version 6.0; GE Healthcare Bio-Sciences). Protein spots which had more than 2-fold change in relative spot volume were identified as significantly differential spots between the treated and control gels.

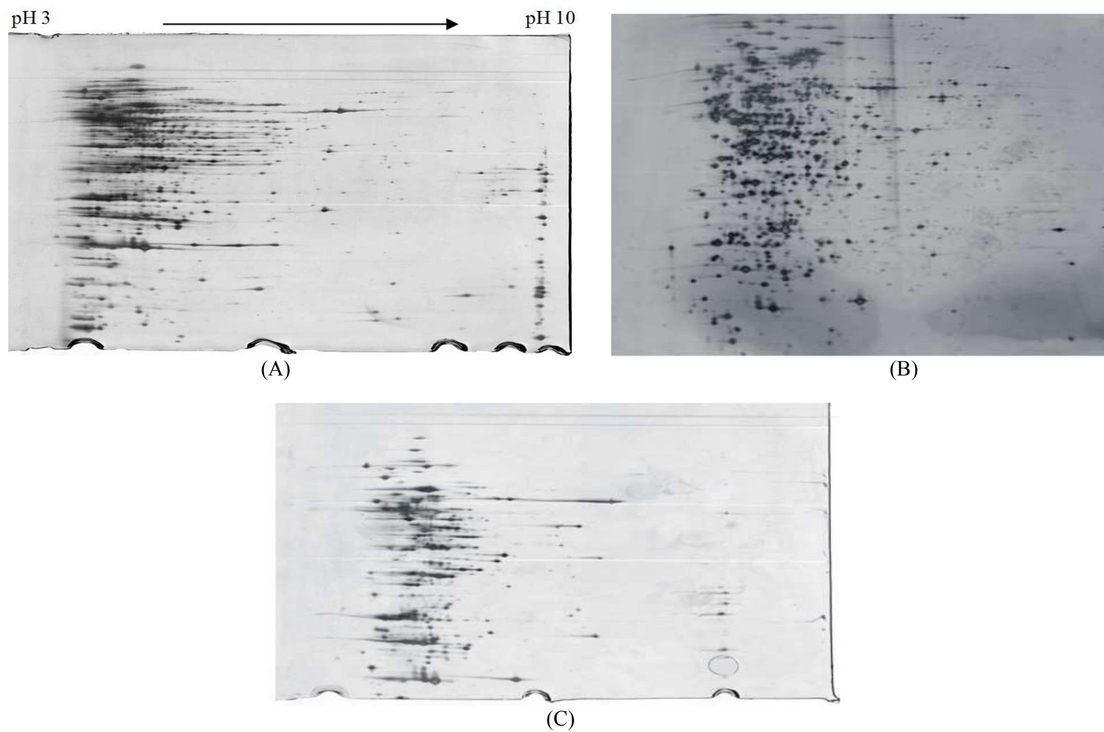
3. RESULTS

The MIC value of acetone gall extracts of *Q. infectoria* against ATCC 33591and PPUKM clinical isolate was 0.3125 mg mL^{-1}. The MIC value of vancomycin against both ATCC 33591 and PPUKM was 0.00195 mg mL^{-1}.

The protein extraction procedure was upscaled to 25 times in order to yield sufficient amount of protein in the analysis of 2-G gel electrophoresis. The concentration of protein from the untreated MRSA, vancomycin-treated MRSA and acetone extract-treated MRSA were respectively, 1,770, 15,811and 1,921 µg mL^{-1}.

The results on the comparative 2-D gel of the concentration of MRSA protein at 80, 100 and 120 µg was shown in **Fig. 1**. It was observed that 100 µg of protein sample (**Fig. 1B**), loaded in IEF yielded a quality resolution and produced more clearly defined spots compared to the protein concentration loaded at 80 µg and 120 µg. At 80 µg protein sample **(Fig. 1A)**, the spots produced in the gel was much lesser which may be due to the low abundance proteins not being detected. The 2-D GE map produced from 120 µg protein sample (**Fig. 1C**) clearly showed a reduction in the number of spots compared to that of 100 µg.

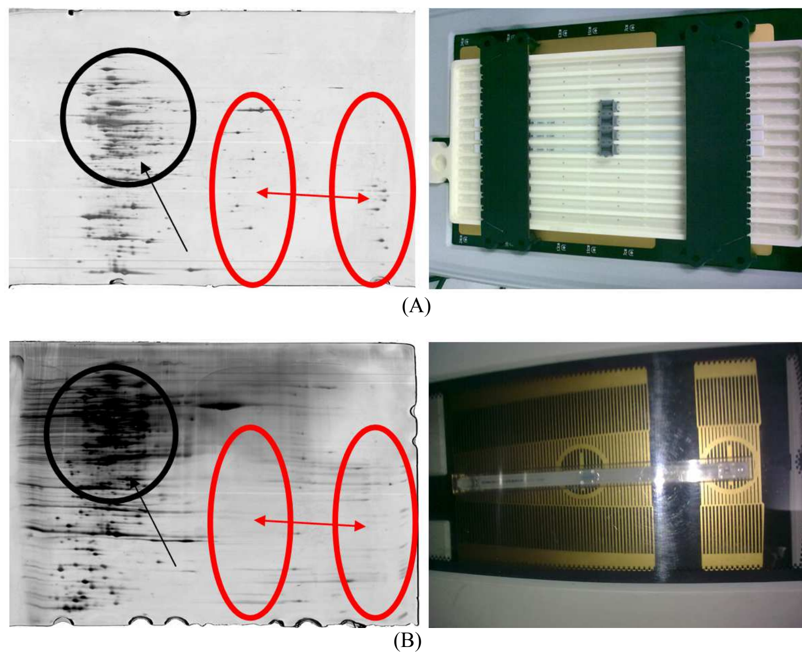
Comparison of the manifold technique and strip holder at optimum protein concentration (100 µg) showed that the former displayed a higher resolution, better reproducibility with greater number of clearer protein spots (**Fig. 2A**) in contrast to usage of strip holder technique (**Fig. 2B**). The maximum voltage of 10000V was applied in the manifold procedure whereas the strip holder run on a maximum voltage range of 8000v (EttanIPGphor Control Software User Manual 11-0034-59 Edition AB). As shown in **Fig. 3A and B**, a total of 7 protein spots in the 2-D gels were identified as significantly different between the ATCC 33591 treated with acetone extract of gall *Q. infectoria* and the untreated control group. In other words, there were 7 identified differential proteins that were expressed two-fold or higher from the control (**Fig. 4**). Cho *et al*. (2008) demonstrated that tea polyphenol can differentially induced the expression of 3 proteins in MRSA whereas Sianglum *et al*. (2011) on the other hand, reported that out of a total of 203 spots in common between the treated and untreated protein expression profiles, 18 were present only in rhodomyrtone-treated MRSA. A study of the expression of extracellular proteins by Visutti *et al*. (2011) revealed that the identification of 4 amino acid sequences from 21 protein spots from the rhodomyrtone-treated MRSA were predicted as signal peptides sequences.



(A) (B)

(C)

Fig. 1. Comparative 2-D gel map of untreated ATCC 33591at sample protein concentration of (A) 80 µg (B) 100 µg and (C) 120 µg



(A)

(B)

Fig. 2. Comparative 2-D gel map of 100 µg protein sample of ATCC 33591 using (A) manifold technique and (B) strip holder technique. The black circle showed that the intensity of horizontal streaking whereas the red circles indicate the presence or absenc of detectable spots of detectable spots

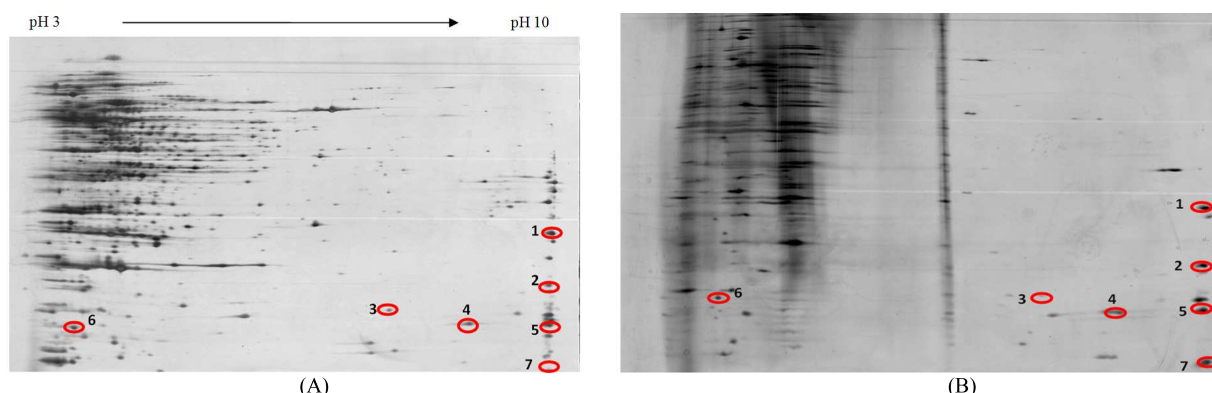


Fig. 3. Differential protein 2-D map of sample protein (100 µg) extracted from (A) untreated ATCC 33591 and (B) ATCC 33591 treated with acetone extract (1XMIC) of gall *Quercus infectoria*. The red circles correspond to the identified spots which are expressed significantly as differential proteins with greater than 2-fold increase compared to untreated (control)

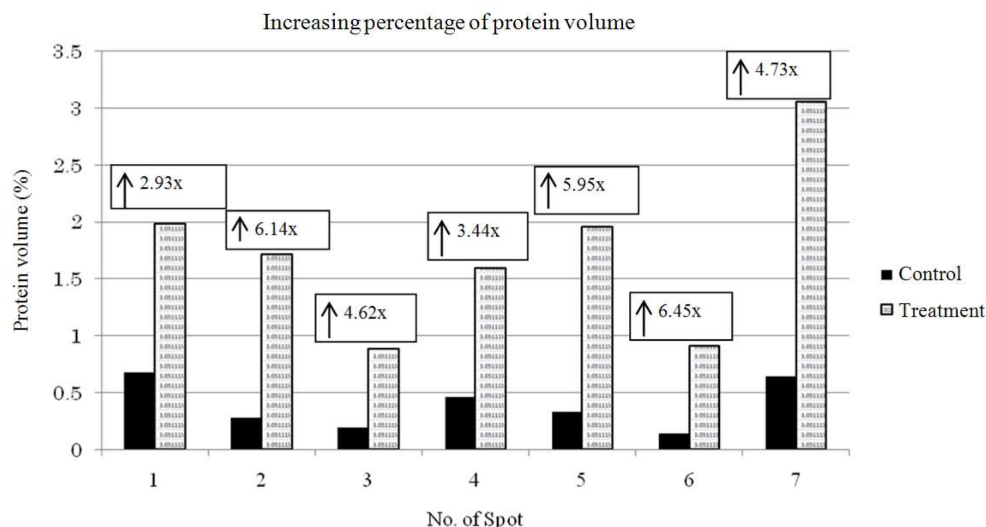


Fig. 4. Histogram showing the increase in relative spot volume (% of protein) for the seven spots determined by Image Master 2D Platinum Version 6.0. The increase (↑) in protein expression of MRSA strain ATCC 33591 in the seven spots is obtained by dividing the percentage of protein in the treatment group by untreated group (control)

4. DISCUSSION

The MIC value of acetone extract of gall *Q. infectoria* against MRSA is in accordance with Basri and Khairon (2012) which recently compared the type of interaction between *Q. infectoria* gall extracts and vancomycin using broth microdilution checkerboard assay and time-kill kinetic study. Sucilathangam *et al.* (2012) however, reported that the values of MIC for aqueous and ethanol gall extracts from *Q. infectoria* against ATCC MRSA 43300 were in the range 0.4-1.6 mg mL^{-1}. This probably indicated that the acetone extract is a better extractant than the aqueous and ethanol. According to Al-Daihan and Bhat (2012), methanol and acetone extracts were found to be more effective antimicrobial agents than the aqueous extracts. This is also supported by Eloff (1998) that acetone was more effective at extracting total phenolic compounds followed by methanol, ethanol and water. This is in accordance with Tenover *et al.* (2001) that the National Committee for Clinical Laboratory Standard (NCCLS) defined staphylococci requiring concentration of vancomycin of $\leq$0.004 mg mL^{-1} as susceptible (NCCLS, 2000). As such, the value of MIC for

vancomycin against both strains of MRSA are also in agreement with Wikler (2009) susceptibility breakpoint for vancomycin against MRSA.

Bradford assay was used to calculate the concentration of protein that was extracted from MRSA. This method is easier, faster and more sensitive compared to Lowry technique. Moreover, it has an advantage of not being affected by any interference from the common reagents and nonprotein constituents in biological samples as occured when Lowry method was employed.

Gygi *et al*. (2000) confirmed that the low abundance proteins can only be analyzed if larger starting concentration of proteins are used. The reduction in the number of spots at higher protein load was probably because the capacity of the gel to absorb protein is limited when the concentration of protein sample exceeded the solubility threshold of some proteins (Rabilloud, 2009). Solubilization of protein is very important to achieve good focusing and to get the best image of gel resolution (Marqui *et al*., 2006). It has been suggested by Zhou *et al*. (2005) that the loss of proteins during rewelling increases as the amount of protein loaded is amplified. This could well suggest that different proteins are absorbed by the gel slab at different extend, based on their molecular size, as demonstrated by Zuo and Speicher (2000) that high sample load can result in as much as 50% sample loss. Barry *et al*. (2003) claimed that in-gel rehydration procedure produced lower number of detectable spots compared to sample cup loading.

The higher the voltage, the more effective will be the separation of proteins with better resolution and save the trouble of protein saturation that could lead to inaccuracy in the comparative gel analysis if the strip holder was used. Khoudoli *et al*. (2004) reported the time taken for the IEF step was dependent on both protein loading and sample conductivity, yielding good results if conducted for a total of more than 33,000v. In addition to this, the charge of the proteins continuously becomes less as they approach their pI and to mobilize them until the last millimeters close to the pI at an optimized speed, very high electric fields must be utilized (Rabilloud and Lelong, 2011).

5. CONCLUSION

Our findings clearly demonstrated that the optimum conditions to run 2-G gel electrophoresis against MRSA treated with acetone extract from *Quercus infectoria* gall is at protein concentration sample loading of 100 µg using the manifold isoelectric focusing procedure. Changes in protein expression in acetone extract treated-MRSA indicated that 7 proteins are upregulated in the mechanism of its anti-MRSA action of the gall extract. Identification of the differential expression of these proteins is currently ongoing using MALDI-TOF MS/MS.

6. ACKNOWLEDGEMENT

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