Journal of Applied Biological Sciences 8 (2): 55-61, 2014
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

Electrophoretic Protein Separation Reveals the Possible Antibiofilm Mechanism of *Chromolaena odorata* Chloroform Extract Against *Pseudomonas aeruginosa*: A Preliminary Investigation

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Received: May 22, 2014
Accepted: June 26, 2014

Abstract

Chromolaena odorata (L.) King & H. Rob. is a perennial ubiquitary aromatic herb common in South East Asia, West Africa and parts of Australia. Recently, *C. odorata* leaf extract has been demonstrated to exhibit antibiofilm activity against *Pseudomonas aeruginosa*. However, the molecular mechanism underlying this biological activity has not been elucidated. To address this issue, the cytoplasmic protein expression of *P. aeruginosa* biofilm following treatment with *C. odorata* chloroform extract (COCE) was investigated. Based on SDS polyacrylamide gel electrophoresis, the treatment of COCE resulted in changes in the profile of cytoplasmic proteins of *P. aeruginosa* biofilm under aerobic and anaerobic conditions. Densitometric analysis between 400 and 750 nm successfully revealed a number of differentially expressed cytoplasmic proteins (under aerobic condition: 23 kDa, 35 kDa and 46 kDa; under anaerobic condition: 45 kDa) in *P. aeruginosa* biofilm which indicated the antibiofilm effects of COCE. We suggest that the metabolically important cytoplasmic proteins in *P. aeruginosa* biofilm may be involved in the antibiofilm mode of action of COCE. Nevertheless, the identity of these cytoplasmic proteins remains to be further investigated.

Keywords: *Chromolaena odorata; Pseudomonas aeruginosa*; antibiofilm; protein expression

INTRODUCTION

Microbial biofilm is a group of cells attached to either biotic or abiotic surface and is well known to cause a wide spectrum of infectious diseases worldwide [1]. It has been frequently shown to contribute to antimicrobial resistance in several diseases such as gonorrhea, pneumonia and meningitis. One of the common bacteria that tend to switch to the biofilm growth mode during antimicrobial regime is *Pseudomonas aeruginosa*, an anaerobic facultative microorganism [2]. According to Kiser et al. (2010), its resistance to several drugs such as ciprofloxacin, tobramycin, ceftazidime and imipenem is the result of outer membrane protein OprM overexpression and multiple efflux regulatory gene mutations (mexR, nfxB, and mexZ) [3]. This has led to a growing interest in the last few decades for the use of natural products to combat the persistent infections caused by *P. aeruginosa*.

Chromolaena odorata (L.) King & H. Rob. is a perennial ubiquitary aromatic herb common in South East Asia, West Africa and parts of Australia. It has been demonstrated to exhibit various important biological activities such as wound healing, inflammation control and killing of microorganisms [4], [5] and [6]. We have recently reported the antibiofilm activity of *C. odorata* extracts against *P. aeruginosa* under both aerobic and anaerobic conditions [7]. The killing of biofilm-forming *P. aeruginosa* has been suggested to be mediated by alteration of extracellular polymeric substance (EPS) biochemical composition of the biofilm matrix. The effects of C. *odorata* extracts on the intracellular components of biofilm-forming *P. aeruginosa* however remain to be investigated.

Understanding the molecular mechanism of a potential antimicrobial substance is pivotal controlled in order to ensure its suitable application in treating infections. It is generally accepted that the molecular mechanisms underlying the antimicrobial effects usually involves cell membrane modification, changes in ion flux and alteration of protein expression [8]. The ability of the antimicrobial substance to regulate protein expression inside the target microorganism and the metabolic activity in cytoplasmic compartment such as tricarboxylic acid cycle (TCA cycle) adversely affects the survival of the target microorganism [9], [10]. Considering all of the above, it is possible that the antibiofilm mechanism of *C. odorata* extracts is also mediated by the changes in protein expression.

The present work is aimed at determining the effects of *C. odorata* chloroform extract (COCE) on protein expression in *P. aeruginosa* biofilm under both aerobic and anaerobic conditions. Using a combination of SDS polyacrylamide gel electrophoresis and densitometry, the differential expression of cytoplasmic proteins resulting from the treatment of COCE is clearly presented. To this point, we demonstrate that the mode of antibiofilm action by COCE is in agreement with the common molecular mechanism governing the antimicrobial effects.

MATERIALS AND METHODS

Preparation of plant extract

Fresh leaves of *Chromolaena odorata* (L.) R. M. King & H. Rob. were collected from Puncak Alam, Selangor, Malaysia (Coordinates: 3° 13' 42.30"N 101° 25' 41.65"E). The taxonomic identity of the plant was authenticated by Dr Shamsul Khamis from Universiti Putra Malaysia. The leaves were washed with water and dried in oven for 48 hours at 60^0C. The dried leaves were ground into fine powder and soaked in absolute chloroform in a ratio of 1:10 (w/v) in a water bath at 50^0C for 72 hours. The impurities were then filtered using muslin cloth. Rotary evaporator was used to remove excess solvent.

Preparation of test microorganism

P. aeruginosa ATCC 10145 was grown in nutrient broth for 24 hours. The purity of bacterial culture was determined by light microscopy whilst the bacterial growth pattern was determined spectrophotometrically at 600 nm. A 0.5 McFarland Standard was used to prepare the inoculum density of 1.5×10^8 CFU/mL in phosphate buffered saline (PBS) pH 7.4 for all assays.

Microtiter plate biofilm formation assay

The antibiofilm activity of *C. odorata* chloroform extract (COCE) was determined as described by Yahya et al. (2014) [7]. The COCE was freshly prepared at 200 mg/mL which has been shown to exhibit the greatest antibiofilm activity [7]. A volume of 3.33 ml of fresh *P. aeruginosa* inoculum was loaded into the wells of 6-microtiter plate in triplicates. Then, 1.67 ml of COCE was added into the wells and mixed thoroughly. The control wells were added with 5 ml of fresh broth. The procedure was performed in duplicates in two separate 6-microtiter plates. The 6-microtiter plates were incubated separately into aerobic and anaerobic conditions for 48 hours at 37^0C. At 48 hours post inoculation, the media containing planktonic cells in each well was discarded. All wells were then rinsed twice with PBS pH 7.4. Biofilm cells were detached by pipetting action and suspended in the PBS buffer. The biofilm suspension was then used for colony forming unit (CFU) counting and protein extraction.

Colony-forming unit counting

The biofilm suspension collected the microptiter late biofilm formation assay was diluted to a ratio of $1:10^6$

using the fresh broth. A volume of 10µL of the biofilm suspension was streaked on the sterile Muller Hilton agar. The plates were then incubated for 24 hours at 37^0C. The viable cells were counted the following day to obtain CFU/mL value. The CFU counting was performed in triplicates

Extraction of cytoplasmic proteins

Extraction of cytoplasmic proteins from *P. aeruginosa* biofilm was carried out as described by Thomas et al. (1981) with minor modification [11]. The biofilm suspension from the microtiter plate biofilm formation assay was pelleted by centrifugation at 3200 rpm for five minutes which was then suspended in ice-cold lysis buffer (50mM HEPES,1mM EDTA,0.5% Triton X-100, 5% protease inhibitor) and mixed well by vortex. The solution was incubated on ice for 30 minutes. The cell lysate was then centrifuged at 13000 rpm for 10 minutes to obtain the clear supernatant. Subsequently, the acetone solvent was added into the supernatant in a ratio of 2:1 and incubated overnight at -20°C. The mixture was then centrifuged at 13000 rpm for 15 minutes to obtain the pellet for protein determination while the supernatant was discarded.

Spectrophotometric measurement

Protein determination was conducted using Bradford assay method [12]. A volume of 1 ml Bradford reagent was added into micro centrifuge tube which was then mixed with 50 µl of bovine serum albumin (BSA) as the protein concentration standard or microbial protein and incubated at room temperature for five minutes. The protein concentration was measured within one hour using a spectrophotometer at 595 nm. The protein determination assay was carried out in triplicates.

Protein separation by polyacrylamide gel electrophoresis

12% SDS polyacrylamide gel electrophoresis was performed according to NuPAGE® electrophoresis system in order to separate the microbial proteins. The electrophoresis conditions were as follows: 100-125 mA at start, 60-80 mA at end, 200V and 35 minutes. The polyacrylamide gel was then heated in a microwave for one minute, stained with Coomassie Brilliant Blue for one minute and destained with distilled water for 10 minutes. The electrophoresis procedure was carried out in triplicates.

Densitometric analysis

This analysis was conducted using GS-800 Calibrated Densitometer in the wavelength range between 400 and 750 nm to determine optical density of electrophoretically separated protein bands. The gel image was imported into the Quantity One 1-D Analysis Software and image contrast was adjusted to ensure protein bands were clearly visible on the gel. Then, the area of each protein band was selected and background intensity was subtracted from the gel image.

RESULTS

***C. odorata* chloroform extract decreases the viability of *P. aeruginosa* biofilm**

Our previous work has revealed the antibiofilm activity of *C. odorata* extracts against *P. aeruginosa*. Based on CFU counting, it has been shown to be dependent on the oxygen level and type of plant extract. The test concentration of *C. odorata* extracts that exhibited the greatest antibiofilm activity was found to be 200 mg/ml. Therefore, the same procedure of microtiter plate biofilm assay and test concentration of 200 mg/ml was employed in order to understand the possible antibiofilm mechanism of COCE. As shown by Table 1, it was observed that the treatment of COCE decreased the viability of *P. aeruginosa* biofilm under both aerobic (control: 1.76×10^{10} CFU/mL; test: $2.43 \pm 0.21 \times 10^8$ CFU/mL) and anaerobic (control: $3.7 \pm 0.5 \times 10^{10}$ CFU/mL; test: 3.0×10^8 CFU/mL) conditions.

Table 1. Effects of *C. odorata* chloroform extract on the viability of *P. aeruginosa* biofilm cells under both aerobic and anaerobic conditions. Data are expressed as mean ± standard deviation with n = 3.

Experimental condition	Control	Test (200 mg/ml)
Aerobic	$3.7 \pm 0.5 \times 10^{10}$ CFU/mL	3.0×10^{8} CFU/mL
Anaerobic	1.76×10^{10} CFU/mL	$2.43 \pm 0.21 \times 10^{9}$ CFU/mL

***C. odorata* chloroform extract alters cytoplasmic protein profile of *P. aeruginosa* biofilm**

It is generally accepted that the antibacterial activity is mediated by the intracellular changes with regards to ion concentration, enzyme catalysis and protein expression. Analyzing protein expression for example, may provide insights into how a drug candidate modulates the intracellular targets that attribute to the therapeutic effects. Therefore, the present study employs the protein profiling approach to gain a preliminary understanding of the possible control mechanism of COCE against *P. aeruginosa* biofilm. Figure 1 shows cytoplasmic protein profiles obtained from *P. aeruginosa* biofilm suspension. It was clearly observed that the treatment of COCE resulted in changes in cytoplasmic protein expression of *P. aeruginosa* biofilm under both aerobic and anaerobic conditions. Under aerobic condition, the molecular mass of cytoplasmic proteins from the control assay ranged between 6 kDa and 139 kDa whilst the molecular mass of cytoplasmic proteins from the test assay ranged between 5 kDa and 100 kDa. Meanwhile, the ranges of molecular mass of cytoplasmic proteins under anaerobic condition were between 4 kDa and 127 kDa for control assay, and between 5 kDa and 56 kDa for test assay. The electrophoretic pattern based on the SDS polyacrylamide gel is summarized in Table 2. Under aerobic and anaerobic conditions, the changes in cytoplasmic protein expression were found to be 45.8% and 76.9% respectively (Table 3).

Treatment of COCE results in down regulation of cytoplasmic proteins in *P. aeruginosa* biofilm under both aerobic and anaerobic conditions

The quantitative analysis of SDS polyacrylamide gel often involves densitometry, a quantitative measurement of optical density of electrophoretically separated proteins. Changes in densitometric value reflect either up-regulation or down-regulation of protein expression. Based on protein electrophoresis (Figure 1), there were several *P. aeruginosa* biofilm proteins present in both control and test assays. The density of those proteins was expected to vary across the type of assays and to confirm their differential expression, the densitometric analysis was conducted. Figure 2 denotes the selected protein bands and their corresponding densitometric values. It was observed that the treatment with COCE caused changes in densitometric values of several protein bands. Under aerobic condition, the treatment of COCE down regulated the proteins of 23 kDa, 35 kDa and 46 kDa (Figure 2a) while under anaerobic condition, only protein 45 kDa was down-regulated by COCE treatment (Figure 2b). As listed in Table 4, the down-regulation of those proteins under aerobic condition ranged from 84.84% to 93.39% (or from 0.066 to 0.152 fold decrease) whereas the down regulation of protein 45 kDa under anaerobic condition was noted to be 93.19% (or 0.068 fold decrease).

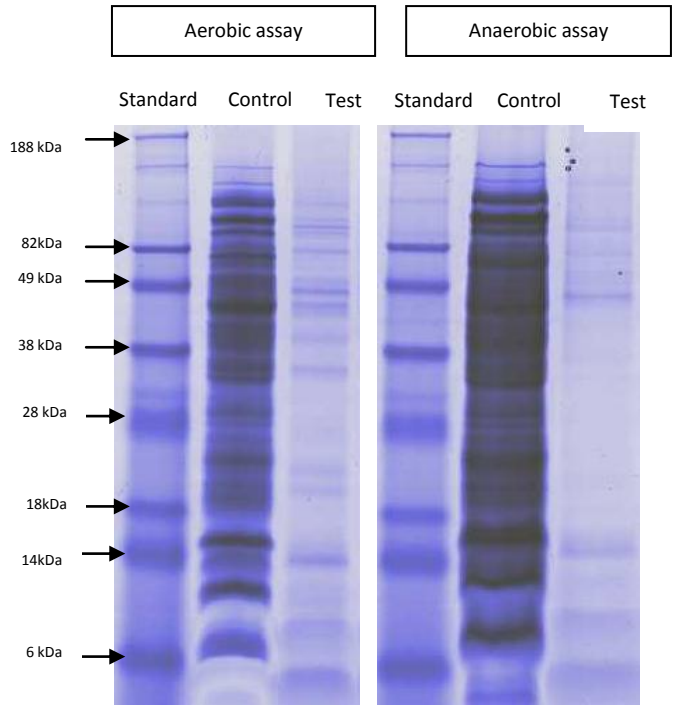


Figure 1. Cytoplasmic protein profiling of *P. aeruginosa* biofilm cells upon treatment with COCE under both aerobic and anaerobic conditions. The total amount of protein samples loaded into each well of the 12% polyacrylamide gel was 50 mg/ml. The polyacrylamide gel was stained with Coomassie Brilliant Blue and the protein samples were run alongside SeeBlue® Plus2 Pre-stained Protein Standard (4 – 250 kDa).

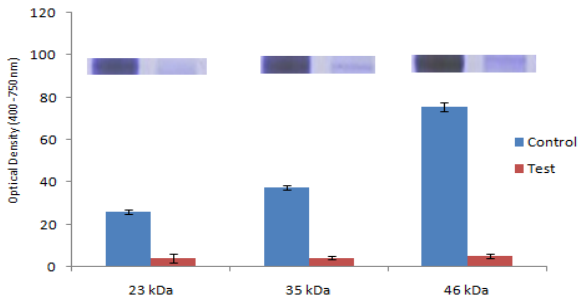
Table 2. Electrophoretic pattern of cytoplasmic proteins of *P. aeruginosa* biofilm upon treatment with COCE under both aerobic and anaerobic conditions. Grey color indicates the cytoplasmic proteins which are present in both control and test assays.

Molecular Weight (kDa)	**Protein Bands**			
	Aerobic		Anaerobic	
	Control	Test (200 mg/ml)	Control	Test (200 mg/ml)
139	+	-	-	-
127	-	-	+	-
119	+	-	-	-
110	-	-	+	-
107	+	-	-	-
100	-	+	-	-
99	+	-	-	-
91	-	-	+	-
87	+	-	-	-
86	-	-	+	-
85	-	+	-	-
84	+	-	-	-
77	-	-	+	-
75	-	+	-	-
74	+	-	-	-
73	-	-	+	-
71	+	-	-	-
68	-	-	+	-
64	+	-	-	-
62	-	+	-	-
61	-	-	+	-
58	-	-	+	-
57	+	-	-	-
56	-	-	-	+
52	-	-	+	-
51	-	+	-	-
49	-	-	+	-
48	-	+	-	-
46	+	+	-	-
45	-	-	+	+
41	-	-	+	-
40	+	-	-	-
39	+	-	+	-
37	-	-	+	-
35	+	+	-	-
34	+	-	-	-
33	-	-	+	-
31	+	-	+	-
29	-	-	+	-
28	-	-	+	-
27	+	-	-	-
25	+	-	-	-
23	+	+	-	-
21	-	-	+	-
20	-	+	-	-
19	-	-	+	-
18	-	-	+	-
15	+	-	-	-
13	-	+	-	+
11	-	-	+	-
10	+	-	+	-
8	-	+	-	-
7	-	-	-	+
6	+	-	-	-
5	-	+	-	-
5	-	-	-	+
4	-	-	+	-

Table 3. Summary of electophoretic pattern of cytoplasmic proteins from the control and test assays. Changes between the patterns are also expressed in percentages.

Experimental condition	Number of protein bands		Changes (%)
	Control	Test (200 mg/ml)	
Aerobic	24	13	-45.8
Anaerobic	26	6	-76.9

a)



b)

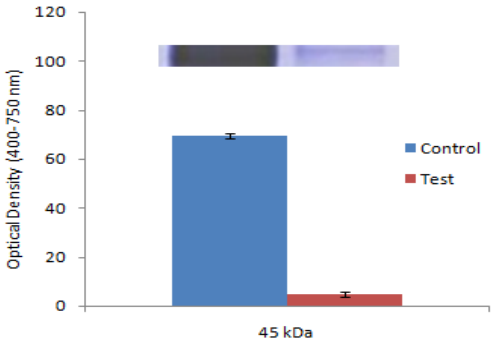


Figure 2. Differentially expressed cytoplasmic proteins in *P. aeruginosa* biofilm under aerobic (a) and anaerobic (b) conditions following COCE treatment. The densitometric measurements were performed in the wavelength range between 400 and 750 nm, with n=3.

Table 4. Analysis of cytoplasmic protein density upon treatment with COCE under both aerobic and anaerobic conditions.

Conditions	Molecular mass (kDa)	Control	Test (200 mg/ml)	Percentage of change	Fold change
Aerobic	23	26.052 ± 0.031	3.95 ± 0.002	- 84.84	- 0.152
	35	37.463 ± 0.054	4.253 ± 0.003	- 88.65	- 0.113
	46	75.437 ± 0.09	4.988 ± 0.004	- 93.39	- 0.066
Anaerobic	45	69.492 ± 0.059	4.734 ± 0.003	- 93.19	- 0.068

DISCUSSION

The choice of protein extraction method is crucial to obtain the sample of interest for further protein analysis. For many centuries, the organic solvent such as acetone has been used in precipitation of the soluble proteins [13]. This is due to the possibility that the acetone solvent which has the molecular formula of C_3H_6O decreases the hydration layer by displacing water from the protein surface. In turn, the soluble proteins can aggregate by attractive electrostatic and dipole forces. In the context of our study, the use of acetone solvent successfully precipitated the cytoplasmic proteins of *P. aeruginosa* biofilm and that was confirmed by the spectrophotometric measurement and Bradford assay. We believe that the changes in the electrophoretic pattern and densitometric values observed in our study reflect the altered protein expression in cytoplasm of *P. aeruginosa* biofilm.

The analysis of protein expression following drug treatment has received a great attention in the infectious disease investigations. In 2007, Stevens et al. studied the effects of several antibiotics on virulence-associated proteins in Methicillin-resistant *Staphylococcus aureus* (MRSA) [14]. They reported that clindamycin and linezolid antibiotics markedly suppressed the expression of Panton-Valentine Leukocidin proteins in MRSA at 48 hour post antibiotics administration. Meanwhile, relationship between altered bacterial protein expression and the plant extract treatment has been reported by Brighenti et al. (2008), whereby *Psidium cattleianum* leaf extract was tested on the viability of Streptococcus mutans and their protein expression [15]. It was demonstrated that the aqueous extract [1.6% (v/v)] of *P. cattleianum* reduced the expression of proteins involved in general metabolism, glycolysis and lactic acid production whereby the abundance of 24 spots was significantly decreased compared to control ($p < 0.05$). In conjunction with those studies, the effects of COCE on cytoplasmic protein expression (aerobic: 23 kDa, 35 kDa and 46 kDa; anaerobic: 45 kDa) in *P. aeruginosa* biofilm may suggest the down regulation of metabolically important proteins for the survival of *P. aeruginosa* biofilm.

P. aeruginosa can survive under both aerobic and anaerobic conditions [16]. In the oxygen-limiting condition, *P. aeruginosa* tends to attach on the surface, forms the biofilm community, undergoes the phenotypic shift and restrict the protein synthesis [16], [17]. In addition, Abde-Elaah *et al.* (2006) reported the down regulation of plant proteins in oxygen-limiting condition [18]. On the other hand, the antibiofilm activity of medicinal plants has been shown to be dependent on the oxygen level [19]. Collectively, it is likely that the oxygen level plays some role in both normal condition of *P. aeruginosa* biofilm and antibiofilm treatment. Consistent with the data from CFU counting, the electrophoretic patterns of cytoplasmic proteins (Table 2 and Table 3) from aerobic assays were noticeably different from those of anaerobic assays. This may explain the variation of antibiofilm mode of action by COCE under different experimental conditions.

Currently, most protein analyses on the biological samples have been combined with tandem mass spectrometry (MS/MS), a high performance analytical method which relies on the fragmentation of gaseous ionic particles during several stages of mass analysis. As described by Lorenzen *et al.* (2007), optimization of the tandem mass spectrometry could be carried out by varying the gas pressure inside the hexapole collision cell and type of collision gasses [20]. These variations would affect ion transmission and gas-phase dissociation efficiency. From the perspective of medical research, this experimental strategy provides an advantage in the discovery of protein identity with respect to disease biomarkers and novel drug targets [21]. Prior to protein identification by tandem mass spectrometry (MS/MS), the protein mixtures are usually separated by two properties (isoeletric point and molecular mass) by two dimensional SDS polyacrylamide gel electrophoresis. In future, our preliminary work on antibiofilm mechanism of COCE will be validated by a combination of two dimensional SDS polyacrylamide gel electrophoresis and tandem mass spectrometry in order to identify the differentially expressed cytoplasmic proteins in *P. aeruginosa* biofilm following the COCE treatment.

Various studies have revealed the effects of antibiotics on posttranslational modifications inside the target living cells. Basically the posttranslational modification is a metabolically important chemical process that happens on the newly synthesized proteins which involves various mechanisms such as addition of functional groups (glycosyl and phosphate groups), covalent linkage to other proteins (ubiquitination) and structural changes (proteolytic cleavage). A study by Hagiwara et al. (1988) demonstrated that the neomycin antibiotics inhibited 12-O-tetradecanoyl-phorbol-13-acetate-induced phosphorylation of 88 K Da protein and protein kinase C activity in *Potorous tridactylis* epithelial kidney (PtK2) cells and rabbit kidney cells respectively [22]. The inhibition of those cell signaling processes contributed to nephrotoxic potential. In addition, the cell wall-targeting antibiotics such as such as vancomycin and ceftriaxone, had been found to inhibit the kinase activity of serum thymidine kinase 1 (Stk1) which in turn decreased cysteine (Cys)-phosphorylation of staphylococcal accessary regulator A (SarA) and MarR family global transcriptional regulator A (MgrA) [23]. This resulted in the reduction of staphylococcal virulence. In the context of analysis of posttranslational medications, SDS polyacrylamide gel is commonly combined with Western blotting and tandem mass spectrometry (MS/MS) [24]. For example, the phosphorylated proteins are detected by antiphosphoamino acid antibodies on the SDS polyacrylamide gel which are then subjected to tryptic digestion and characterization by the tandem mass spectrometry (MS/MS) [25]. Although the intensive literature search have not reveal any scientific report on the influence of antibiofilm agents on the posttranslational modification process in the biofilm cells, based on the preliminary data presented herein (Table 1 and Figure 1), it is expected that the COCE treatment could also manipulate some posttranslational modification process in *P. aeruginosa* biofilm as part of its antibiofilm action.

CONCLUSION

We have demonstrated the effects of COCE on cytoplasmic protein expression of *P. aeruginosa* biofilm under aerobic and anaerobic conditions. It is strongly believed that the altered cytoplasmic protein expression reflect the inhibitory effects of COCE against *P. aeruginosa* biofilm. The findings from this study have provided a preliminary understanding on how the COCE could possibly control *P. aeruginosa* biofilm. Considering the variation in the electrophoretic patterns, the antibioflm mechanism of COCE seems to vary between aerobic and anaerobic conditions. The identity of differentially expressed cytoplasmic proteins in *P. aeruginosa* biofilm following COCE treatment remains to be further investigated.

Acknowledgement

This study was supported by Research Management Institute, UiTM and Malaysian Ministry of Higher Education under research grants, 600-RMI/DANA 5/3/RIF (54/2012) and 600-RMI/RAGS 5/3 (11/2012) respectively.

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