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Proteomic Analysis of *Pseudomonas aeruginosa* Biofilm Treated with *Chromolaena odorata* Extracts

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ABSTRACT

Aim: Biofilm is the major causative factor of infectious diseases. Difficulty in combating biofilm-related diseases is typically due to persisters, heterogeneous microbial population and viscoelastic extracellular polymeric substances (EPS) matrix. Antibiofilm activities of *Chromolaena odorata* extracts have previously been demonstrated, however, the effects of its treatment on the biofilm proteome expression remains not well understood. Thus, this study was carried out to profile changes in biofilm proteome of *Pseudomonas aeruginosa* following treatment with chloroform and ethanol extracts of *C. odorata*.
Methodology and results: Biofilm was developed in 6-well microplate in the presence or absence of *C. odorata* extracts overnight at 37 °C. Whole-cell proteome analysis was carried out by combining two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Treatment with *C. odorata* extracts triggered changes in two-dimensional proteome profiles of *P. aeruginosa* biofilm under aerobic and anaerobic conditions. The differentially expressed proteins were successfully identified and were assigned to various functional categories including protein metabolism, carbohydrate metabolism, peptidoglycan metabolism, electron transport and iron transport.
Conclusion, significance and impact of study: The present study demonstrates differential proteome expression in *P. aeruginosa* biofilm following treatment with *C. odorata* extracts. This suggests that *C. odorata* extracts may target multiple biological processes to control *P. aeruginosa* biofilm. *C. odorata* extracts may be useful for development of novel antibiofilm agents.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *Chromolaena odorata*, proteomic

INTRODUCTION

Biofilm is a conglomerate of microbial community that is encapsulated by a self-produced polymeric matrix. It is capable of adhering to biotic or abiotic surface. Development of biofilm begins with attachment of microbial cells to a particular surface which then form microcolonies and three-dimensional mushroom-shaped structure (Karatan and Watnick, 2009). Over the past few centuries, the biofilm has caused a wide spectrum of problems in public health, marine and agriculture sectors. One of the important biofilm producers is *P. aeruginosa*, the causative factor of hospital-acquired infections. It is a pathogenic Gram-negative bacterium commonly found in soil, water and skin flora. In human, it frequently infects the airway, wounds, urinary tract and burns. The heterogeneity of *P. aeruginosa* biofilm has clearly been demonstrated by infinite focus microscope (IFM) (Mahat *et al.*, 2012).

C. odorata is a traditional medicinal plant that is widely used for its wound healing, antibacterial, antioxidant and anticancer properties. It contains a wide range of important phytochemicals including alkaloids, flavonoids, phenolics, saponins and tannins. This fast-growing perennial shrub is commonly found in South America, Central America, Africa and Asia countries. Its antibiofilm activity against *P. aeruginosa* has previously been reported (Yahya *et al.*, 2014), thus, highlighting the potential use of this medicinal plant to control various biofilm-associated problems such as chronic human infection, biocorrosion, poor performance of medical devices and fabric damages.

Proteomics is the large-scale analysis of protein expression of an organism that involves three major steps

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namely sample preparation, protein separation and protein identification. It allows investigation of differentially expressed proteins when comparing different types of biological samples. Indeed, there are countless applications of proteomics in the study of infectious and non-infectious diseases. Since many decades ago, many spectroscopic and proteomic studies have shed light on how the biofilm-forming bacteria survive under diverse environmental challenges (Suci *et al.*, 1994; Karatzas *et al.*, 2008; Cho and Ahn, 2014). The inhibitory effects of *C. odorata* extracts against *P. aeruginosa* biofilm has previously been reported (Yahya *et al.*, 2014). However, it remains uncertain how *C. odorata* extracts modulate the proteome expression in *P. aeruginosa* biofilm. Therefore, the present work was carried out to identify differentially expressed proteins in *P. aeruginosa* biofilm following treatment with *C. odorata* extracts.

MATERIALS AND METHODS

Plant material

The fresh leaves of *C. odorata* were collected at 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 h at 60 °C. The dried leaves were ground into fine powder and soaked into absolute ethanol or absolute chloroform in a ratio of 1:10 (w/v) in water bath at 50 °C for 72 h. The impurities were then filtered using muslin cloth. Rotary evaporator was used to remove excess solvents. For the proteomic study of *P. aerugionsa* biofilm, *C. odorata* extracts were tested at 200 mg/mL as they exhibited the highest antibiofilm activity (Yahya *et al.* 2014).

Test microorganism

P. aeruginosa ATCC 10145 was grown in LB nutrient broth (Difco Laboratories, USA) at 37 °C. Gram-staining was regularly employed to assess culture purity and colony morphology. For the purpose of each biofilm assays, the starting bacterial inoculum was adjusted to 12×10^8 CFU/mL.

Microplate biofilm assay

Biofilms were developed in 6-well microplate with and without the presence of *C. odorata* extracts. Following a 24 h incubation at 37 °C, nutrient medium was discarded and the formed biofilm fractions were rinsed twice with distilled water, suspended in 0.9% sodium chloride (NaCl) solution (Sigma, USA) and pelleted by centrifugation at 10,000 rpm for 10 min. The microplate biofilm assay procedure was performed in three replicates under both aerobic and anaerobic conditions. The anaerobic condition was developed using candle jar and a strip of paper soaked in methylene blue dye.

Protein extraction and determination

The whole-cell protein was extracted from the resulting pellet using Radio Immunoprecipitation Assay (RIPA) which consist of the following: 50 mM trisaminomethane-hydrochloric acid (Tris-HCl) pH 7.4, 150 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 2% beta-mercaptoethanol (β-ME) and protease inhibitor. Cell disruption was improved by sonication on ice at 80% amplitude, 5 cycles with 45 sec burst and incubated for 2 h on ice. The cell debris and intact cells were removed by centrifugation at 8,000 rpm, 5 min. The supernatant containing protein fraction was stored at -20 °C until further use. The concentration of protein extracted was determined using the standard Bradford assay.

Two-dimensional polyacrylamide gel electrophoresis

Protein samples were initially precipitated using 2D-Clean Up Kit. Then, the protein samples were solubilized with rehydration buffer (7 M urea, 2 M thiourea, 2% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% immobilized pH gradient (IPG) buffer, and a trace of bromophenol blue) and left overnight to rehydrate into ZOOM IPG strips (7 cm immobilized pH gradient with non-linear pH gradient range 3-10). First dimension isoelectric focusing (IEF) was performed in the ZOOM IPGRunner Mini-Cell according to the following protocol: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, 2,000 V for 1 h. The strips were subsequently equilibrated with equilibration buffer (1× NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer, NuPAGE Sample Reducing Agent, 125 mM iodoacetamide (IAA)). Gel electrophoresis was performed using 10% gels at 200 V for 45 min. Gels were stained using SYPRO Ruby overnight. Protein spots were visualized using UV illuminator (Alpha Imager HP) at 610 nm. Two-dimensional proteome maps were analysed to identify protein spots which differed significantly in intensity between control gels and test gels using Progenesis proteomic analysis software. The volume of each spot was normalized against the total volume of all spots in the gel, and the normalized values were expressed as percentage spot volume. Spots with a fold-change of at least 2.0 were excised from the gels for in-gel digestion.

Trypsin digestion

The gel plugs were washed with 50 mM ammonium carbonate (NH_4CO_3) (5 min), followed by 70% acetonitrile (ACN) (15 min) and 100% ACN (5 min) at room temperature. The liquid were discarded following each washing step. The gel plugs were then dried using Speed Vac (2,000 rpm, 4 °C, 15 min). Reduction and alkylation steps were performed by incubation with 10 mM dithiothreitol (DTT)/100 mM NH_4CO_3 for 30 min at 60 °C and with 55 mM IAA/100 mM ammonium bicarbonate (ABC) for 20 min in the dark at room temperature. Subsequently, the gel plugs were washed twice with 50%

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ACN/100 mM ABC (20 min each) and dehydrated with 100% ACN (15 min) at room temperature before overnight digestion in 25 µL of 7 ng/µL of trypsin. The digested peptides were pooled into a clean tube and dried using a vacuum centrifuge before MS analysis.

MALDI-TOF/TOF analysis

Dried peptides were dissolved in 0.1% formic acid (FA) and desalted using ZipTip C18 (Millipore, Billerica, USA), according to the manufacturer's protocol. Next, the peptides were eluted with Elution solution (0.1% trifluoroacetic acid (TFA)/50% ACN into a new microcentrifuge tube. Peptides were spotted on AnchorChip Standard Targets plate in duplicates. Matrix solubilization solution (α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN/0.1% TFA) was then spotted on each peptide spot and allowed to dry. Meanwhile, external calibrant spots solution was deposited onto calibrant anchor spots on the AnchorChip target and allowed to dry. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF) was performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA) whilst the resulting peptide mass spectra and were submitted to the Mascot search engine (Matrix science) to search against the non-redundant database of the National Center for Biotechnology Information (NCBIprot).

Bioinformatics

Identified proteins were classified according to their known biological functions by database searching using SwissProt/TrEMBL and cross-referencing with published report (Hashemi *et al.* 2019). Prediction of functional motifs and subcellular localization were carried out using ScanProsite and PSORTb respectively.

RESULTS AND DISCUSSION

Figures 1-4 show representative gels of *P. aeruginosa* biofilm proteome under aerobic and anaerobic conditions. Twelve and 18 proteins were found to be differentially expressed in the biofilm following treatment with *C. odorata* chloroform extract (COCE) under aerobic and anaerobic conditions respectively. On the other hand, seven proteins exhibited differential expression in the biofilm following treatment with *C. odorata* ethanol extract (COEE) under both aerobic and anaerobic conditions.

Differentially expressed biofilm proteins were subjected to identification by MALDI-TOF/TOF analysis (Figures 1-4). Eleven differentially expressed proteins in COCE treated biofilm under aerobic condition and nine differentially expressed proteins in COCE treated biofilm under anaerobic condition were successfully identified. On the other hand, seven differentially expressed proteins in COEE treated biofilm under both experimental conditions were successfully identified. Porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha appeared in more than one spots on a two-dimensional polyacrylamide gel.

Figures 5 and 6 show functional categories and subcellular location of identified differentially expressed proteins respectively. Approximately 56% and 67% of identified differentially expressed proteins were found to be associated with the general metabolism and cytoplasm respectively. Porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha were analyzed for functional motifs associated with the post-translational modification. They were predicted to contain functional motifs of casein kinase II phosphorylation site (PS00006), protein kinase C phosphorylation site (PS00005) and N-glycosylation site (PS00001).

Proteomics is a systematic and high-throughput approach to study protein expression of living organisms. It has become a common approach to investigate the mode of action of antimicrobial agents. The information derived from this analysis is also useful to understand how the living cells respond towards a wide range of ecological factors. In the present study, treatment with *C. odorata* extracts triggered differential proteome expression in *P. aeruginosa* biofilm. This finding is similar to Miyamoto *et al.* (2015) which demonstrated differential proteome expression in *Listeria monocytogenes* ATCC 7644 following exposure to a sub-lethal concentration of nisin.

Oxygen is reduced to water during oxidative phosphorylation in mitochondria. Changes in oxygen level would normally result in alteration in the amount reactive oxygen species and expression of proteins associated with oxidative phosphorylation. In the present study, the oxygen level was found to affect the proteome expression in both control and test biofilms. The bacterial proteomic study under aerobic and anaerobic conditions has previously been reported by Starck *et al.* (2004). They demonstrated that approximately 50 proteins in *Mycobacterium tuberculosis* were expressed only under anaerobic condition but not under aerobic condition.

Plant extraction using organic solvents is well known to be useful for various analysis of plant materials. While many previous works have demonstrated the effects of different extracting organic solvents on the phytochemical profile and biological activities of plant materials, little is known about the effects of different extracting organic solvents on protein expression. In the present work, the effects of different extracting organic solvents on the proteome profile of *P. aeruginosa* biofilm was observed. For example, spot A5-A7 were differentially expressed in the biofilm treated with COCE (Figure 1) but not in the biofilm treated with COEE (Figure 3). This result corroborates the previous work (Yahya *et al.* 2014) demonstrating differential antibiofilm activities of chloroform and ethanol extracts of *C. odorata* which are due to different phytochemical profiles.

The biofilm proteins are expected to be differentially expressed as compared to its planktonic counterpart. This underlies other physiological disparities such as metabolic rates, protection against antimicrobial attack, cell-surface interaction and cell-cell communication. In the study

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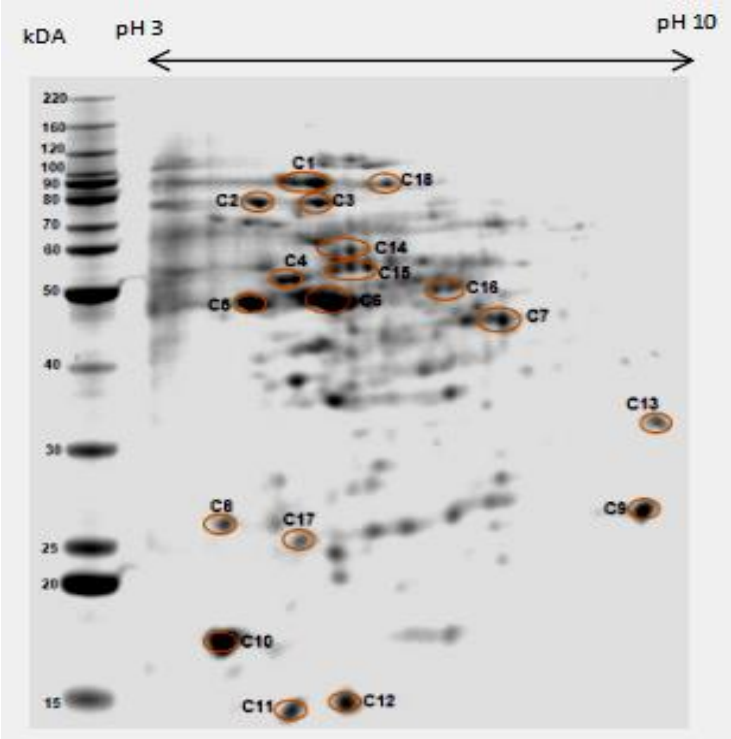

B)

Spot No.	Accession No. (UniProt)	Protein identity	Mascot score	Expression change vs control
A1	Q9HUK1	DNA toposiomerase 4 subunit A	56	↑
A2	Q9HYD5	NAD-dependent malic enzyme	58	↑
A3	Q9HVX6	Tryptophan-tRNA ligase carbomyltransferase	33	↑
A4	A5WBA1	Bifunctional protein GlmU	53	↑
A5	Q9I427	Cytochrome bo(3) ubiquinol oxidase subunit2	65	↑
A6	Q9I423	Protoheme IX farnesyltransferase 2	48	↑
A7	Q912J9	Phenazine biosynthesis protein PhzA 2	23	↑
A8	A6V753	Putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase	62	↑
A9	A6UZH4	Elongation factor Tu 1	68	↑
A10	P31961	Phosphogluconate dehydratase	64	↑
A11	Q51404	Fumarate hydratase class II	21	↑
A12	ND	-	-	↑

Figure 1: Whole-cell proteome profile of *P. aeruginosa* biofilm following treatment with *C. odorata* chloroform extract (COCE) under aerobic condition. A) Differentially expressed proteins with at least two-fold change (as indicated by red circle). Eighty µg of protein sample were focused on 13 cm, pH 3-10 non-linear IPG drystrips, followed by 12% polyacrylamide gel electrophoresis and silver staining. B) Identification of differentially expressed proteins by MALDI-TOF. ND, not detected by MS; ↑, up-regulation; ↓, down-regulation.

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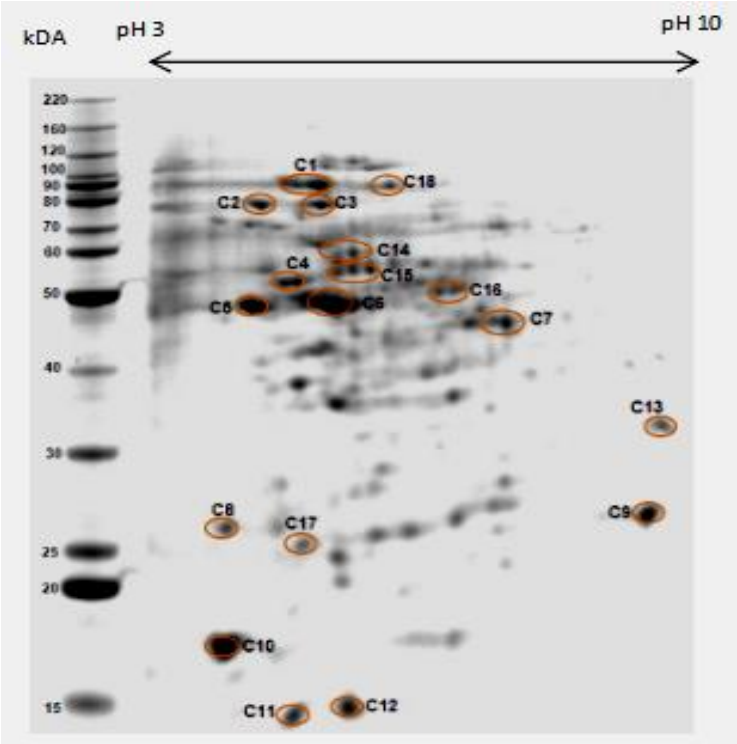

B)

Spot No.	Accession No. (UniProt)	Protein identity	Mascot score	Expression change vs control
C1	ND	-	-	↑
C2	P48632	Ferripyoverdine receptor	43	↑
C3	Q9S646	Polyphosphate kinase	40	↑
C4	Q02DF4	ATP synthase subunit beta	92	↑
C5	P32722	Porin D	58	↑
C6	ND	-	-	↑
C7	P50601	Protein TolB	68	↑
C8	ND	-	-	↑
C9	Q9I4E1	NAD-dependent protein deacylase 2	89	↑
C10	Q9HZJ8	Cell division inhibitor SulA	28	↑
C11	ND	-	-	↑
C12	ND	-	-	↑
C13	ND	-	-	↑
C14	ND	-	-	↑
C15	Q9HT18	ATP synthase subunit alpha	157	↑
C16	P57112	Soluble pyridine nucleotide transhydrogenase	26	↑
C17	ND	-	-	↑
C18	ND	-	-	↑

Figure 2: Whole-cell proteome profile of *P. aeruginosa* biofilm following treatment with *C. odorata* chloroform extract (COCE) under anerobic condition. A) Differentially expressed proteins with at least two-fold change (as indicated by red circle). Eighty µg of protein sample were focused on 13 cm, pH 3-10 non-linear IPG drystrips, followed by 12% polyacrylamide gel electrophoresis and silver staining. B) Identification of differentially expressed proteins by MALDI-TOF. ND, not detected by MS; ↑, up-regulation; ↓, down-regulation.

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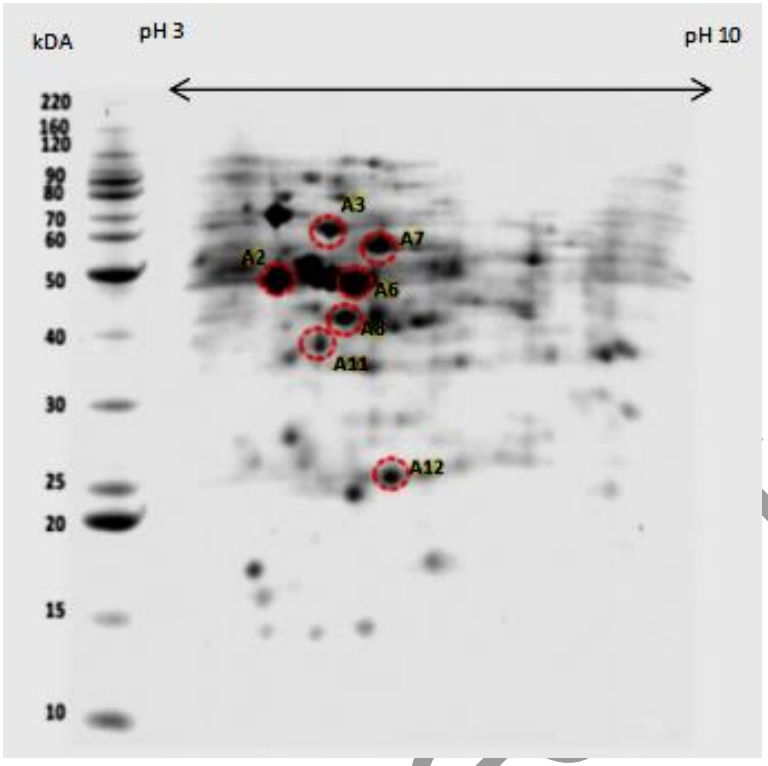

B)

Spot No.	Accession No. (UniProt)	Protein identity	Mascot score	Expression change vs control
A2	PORD_PSEAE	Porin D	235	↓
A3	CH60_PSEAE7	60kDA chaperonin	105	↑
A6	EFTU_PSEAB	Elongation factor Tu	131	↑
A7	ATPA_PSEAB	ATP synthase subunit alpha	663	↑
A8	BRAC_PSEAE	Leucine-isoleucine-valine-threonine and alanine binding protein	122	↑
A11	PORD-PSEAE	Porin D	118	↑
A12	EFTU_PSEAB	Elongation factor Tu	187	↑

Figure 3: Whole-cell proteome profile of *P. aeruginosa* biofilm following treatment with *C. odorata* ethanol extract (COEE) under aerobic condition. A) Differentially expressed proteins with at least two-fold change (as indicated by red circle). Eighty µg of protein sample were focused on 13 cm, pH 3-10 non-linear IPG drystrips, followed by 12% polyacrylamide gel electrophoresis and silver staining. B) Identification of differentially expressed proteins by MALDI-TOF. ND, not detected by MS; ↑, up-regulation; ↓, down-regulation.

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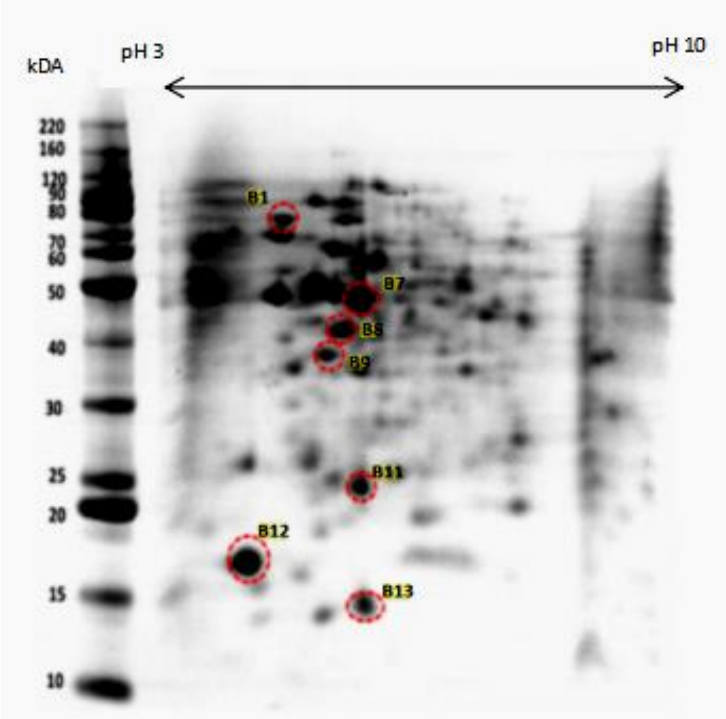

B)

Spot No.	Accession No. (UniProt)	Protein identity	Mascot score	Expression change vs control
B1	RS1_PSEAE	30s ribosomal protein	164	↓
B7	EFTU_PSEF5	Elongation factor Tu	179	↑
B8	HPPD_PSEAE	4-hydroxyphenylpyruvate dioxygenase	464	↑
B9	EFTA_PSEAE	Electron transfer flavoprotein subunit alpha	289	↑
B11	TPX_PSEAE	Probable thiol peroxidase	132	↑
B12	EFTU_PSEAB	Elongation factor Tu	229	↓
B13	EFTA_PSEAE	Electron transfer flavoprotein subunit alpha	157	↑

Figure 4: Whole-cell proteome profile of *P. aeruginosa* biofilm following treatment with *C. odorata* ethanol extract (COEE) under anerobic condition. A) Differentially expressed proteins with at least two-fold change (as indicated by red circle). Eighty µg of protein sample were focused on 13 cm, pH 3-10 non-linear IPG drystrips, followed by 12% polyacrylamide gel electrophoresis and silver staining. B) Identification of differentially expressed proteins by MALDI-TOF. ND, not detected by MS; ↑, up-regulation; ↓, down-regulation.

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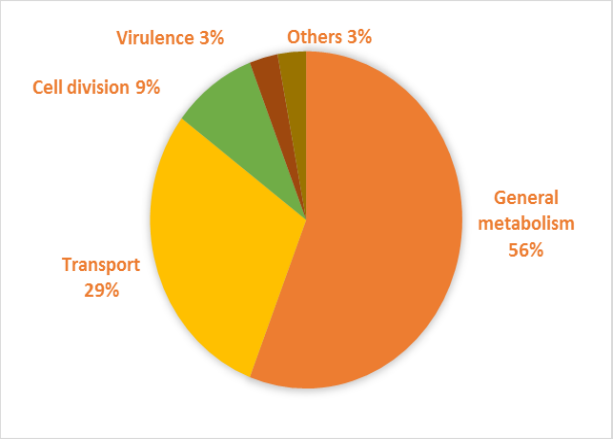

Figure 5: Functional categories of identified differentially expressed proteins in *P. aeruginosa* biofilm according to their known biological functions by database searching using SwissProt/TrEMBL.

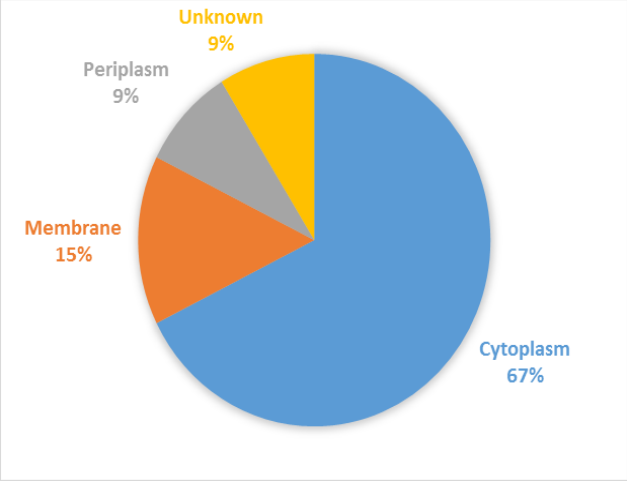

Figure 6: Subcellular locations of identified differentially expressed proteins in *P. aeruginosa* biofilm.

presented herein, the differentially expressed proteins in *P. aeruginosa* biofilm were successfully identified by MALDI-TOF/TOF. Expression of polyphosphate kinase (Rashid *et al.*, 2000), elongation factor Tu (Yahya *et al.*, 2017), ATP synthase subunit alpha (Yahya *et al.*, 2017) and fumarate dehydratase (Schlag *et al.*, 2007) in the biofilms have previously been reported.

The ATP synthase subunit alpha is a membrane-bound enzyme involved in ATP synthesis. It harnesses the energy from the proton gradient established across the inner mitochondrial membrane to drive the ATP synthesis. In the present study, expression of ATP synthase subunit alpha in *P. aeruginosa* biofilm was up-regulated in all gel spots (spot C15, Figure 2A; spot A7, Figure 3A). This overexpression of ATP synthase subunit alpha is in contrast to the reduced expression of ATP synthase subunit alpha in *Salmonella typhimurium* biofilm following treatment with antimicrobial agent dimethyl sulfoxide (Yahya *et al.*, 2017).

Porin D forms outer membrane, water filled channels that facilitates diffusion of small hydrophilic molecules. It is well known to be resistant against sodium dodecyl sulfate denaturation and is associated with peptidoglycan and lipopolysaccharide. The result from the present study revealed over expression of porin D in spot C5 (Figure 2A) and spot A11 (Figure 3A), and reduced expression of porin D in spot A2 (Figure 3A). The increased expression of porin D following treatment with *C. odorata* extracts is in parallel with Kolayli *et al.* (2004) who demonstrated an increase in expression of porin D in *P. aeruginosa* following treatment with carbapenem antibiotics.

Elongation factor Tu is known to participate in translation. It is a highly conserved protein in bacteria and facilitates the binding of aminoacyl tRNA to the ribosome. The present study showed increased expression of elongation factor Tu in spot A6 (Figure 3A), spot A12 (Figure 3A) and spot B7 (Figure 4A), and reduced expression of elongation factor Tu in spot B12 (Figure 4A). Reduced expression of elongation factor Tu in *S. typhimurium* biofilm following treatment with antimicrobial agent dimethyl sulfoxide has previously been reported by Yahya *et al.* (2017).

Failure to determine protein sequences of excised gel spots is a common problem in a wide range of proteomic analysis. This problem is commonly due to low abundance proteins and post translational modification of N termini. In the present study, appoximately 33% of excised gel spots failed to give sequences. This result supports Qi *et al.* (1996) who demonstrated the failure of determination of cell envelope protein sequences from *S. typhimurium* SL1344.

Spot visualization is a critical step in the gel-based proteomics. The use of high-sensitivity fluorescent dye such as Sypro Ruby should be helpful in selection of spots of interest for further mass spectrometry (MS) or tandem MS (MS/MS) analysis. In the present study, porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha were found to appear in more than one spots on a two-dimensional polyacrylamide gel and contain multiple functional motifs associated with the post-translational modification. The similar finding has previously been reported in other proteomics works (Yan *et al.*, 2005, Tastet *et al.*, 2006). According to Suhai (2007), the same protein may appear in different spots on a two-dimensional polyacrylamide gel due to post translational modification.

The impact of antimicrobial treatment on various biological pathways in bacteria has been well established. The varying effective concentrations of antimicrobials would normally produce differential inhibitory effects on bacterial metabolism, enzyme catalysis and proteome expression. In the present study, the majority of differentially expressed proteins in biofilm treated with *C. odorata* extracts were related to pyruvate metabolism (NAD-dependent malic enzyme, putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase), peptidoglycan

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metabolism (bifunctional protein GlmU), heme metabolism (protoheme IX farnesyltransferase 2), protein metabolism (tryptophan-tRNA ligase carbomyltransferase, Elongation factor Tu 1, 60kDA chaperonin, etc.), carbohydrate metabolism (phosphogluconate dehydratase, fumarate hydratase class II), ATP metabolism (ATP synthase subunit alpha, ATP synthase subunit beta), electron transport (cytochrome bo(3) ubiquinol oxidase subunit-2, soluble pyridine nucleotide transhydrogenase, electron transfer flavoprotein subunit alpha, etc.), iron transport (ferripyoverdine receptor) and protein transport (porin D, leucine-isoleucine-valine-threonine and alanine binding protein). According to Hamilton *et al.* (2009), metabolic reactions and biological transport are essential for biofilm development. Thus, this result suggests that altered expression of these proteins may interrupt the sequential multistages of biofilm development and support our previous finding on the antibiofilm activities of *C. odorata* extracts against *P. aeruginosa* (Yahya *et al.*, 2014).

Porins and elongation factor Tu have become the common indicators for immediate response of microbial cells towards a wide range of antimicrobial agents. Investigation of their functional motifs would be useful to understand how they possibly interact with other bacterial proteins. In the present study, porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha were predicted to contain several phosphorylations sites. This finding is in parallel with our prior suggestion that the post translational modification may cause the appearance of porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha in more than one spots on a two-dimensional polyacrylamide gel. Treweek *et al.* (2015) reported that phosphorylation alters the average size and rate of subunit exchange of oligomeric proteins while Mayer *et al.* (2015) mentioned that the majority of post translational modifications affect the isoeletric point and focusing behavior of the protein in the first dimension. Furthermore, phosphorylation of elongation factor Tu in *P. aeruginosa* has previously been reported by Alexander *et al.* (1995). They demonstrated the low ability of phosphorylated elongation factor Tu to bind amino-acyl-tRNA. Phosphostaining assay and tandem mass spectrometry may be useful to experimentally confirm the phosphorylation state of porin D, elongation factor Tu and electron transfer flavoprotein subunit alpha in P. aeruginosa biofilm. In 2017, Yahya *et al.* identified the phosphoproteins in *S. typhimurium* biofilm using both ProQ diamond phosphostaining assay and electrospray ionization-QTOF (ESI-QTOF).

CONCLUSION

The present study demonstrates the effect of *C. odorata* extracts on the proteome expression of *P. aeruginosa* biofilm. Oxygen level and different extracting organic solvents seemed to influence the outcome in terms of protein expression. Treatment with *C. odorata* extracts may interfere with multiple biological processes in *P. aeruginosa* biofilm, making this medicinal plant species an interesting candidate for biofilm control.

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