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Investigation of antibacterial mechanism and identification of bacterial protein targets mediated by antibacterial medicinal plant extracts

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ABSTRACT

In this paper, we investigated the antibacterial mechanism and potential therapeutic targets of three antibacterial medicinal plants. Upon treatment with the plant extracts, bacterial proteins were extracted and resolved using denaturing gel electrophoresis. Differentially-expressed bacterial proteins were excised from the gels and subjected to sequence analysis by MALDI TOF–TOF mass spectrometry. From our study, seven differentially expressed bacterial proteins (triacylglycerol lipase, N-acetylmuramoyl-L-alanine amidase, flagellin, outer membrane protein A, stringent starvation protein A, 30S ribosomal protein s1 and 60 kDa chaperonin) were identified. Additionally, scanning electron microscope study indicated morphological damages induced on bacterial cell surfaces. To the best of our knowledge, this represents the first time these bacterial proteins are being reported, following treatments with the antibacterial plant extracts. Further studies in this direction could lead to the detailed understanding of their inhibition mechanism and discovery of target-specific antibacterial agents.

1. Introduction

Medicinal plants are rich reservoirs of phenolic compounds, and different parts of the plants have been consumed traditionally for a variety of medicinal applications (Jaberian, Piri, & Nazari, 2013; Wong, Li, Cheng, & Chen, 2006). For the past few decades, the studies on medicinal plants have grown dramatically, and their usages have increased worldwide, partly encouraged by the great commercial potentials of plant-derived therapeutic agents (Cragg, Grothaus, & Newman, 2014). The potentials of plant-derived therapeutic agents are further encouraged by the fact that a significant number of currently available pharmaceutical drugs could be traced back to their origins in plants. Well-known examples include vinblastine (an alkaloid drug) derived originally from *Catharanthus roseus* (Madagascar periwinkle) and topotecan (a camptothecin analog) derived from *Camptotheca acuminate* (Asian happy tree) (Facchini & De Luca, 2008; Lorence & Nessler, 2004). In fact, it was estimated that nearly half of modern pharmaceutical drugs are derived from plants (Newman & Cragg, 2007). Additionally, increasing plant-based food supplements have been marketed to cater for the strong consumer demands. Example includes the cranberry supplement, which was reported to be effective in treating urinary tract infection (Caillet, Côté, Sylvain, & Lacroix, 2012; Wu, Qiu, Bushway, & Harper, 2008).

With the increasing occurrences of multi-drug resistant bacteria strains, the world is currently facing an urgent need to find new antibiotic derivatives (Gardete & Tomasz, 2014). Accumulated evidences are pointing toward the effectiveness of plant-derived compounds in inhibiting multi-drug resistant bacterial strains such as *methicillin-resistant Staphylococcus aureus* (Gyawali & Ibrahim, 2014). Previously, our laboratory and others had reported the minimum inhibitory concentrations (MIC) of three antibacterial medicinal plants, namely *Callicarpa formosana*, *Melastoma candidum*, and *Scutellaria barbata* (Wang, Hsu, & Liao, 2008; Wong, Yong, Ong, & Chaia, 2013). However, the exact bacterial inhibition mechanism of these aforementioned plant extracts remained to be elucidated. For instance, it is still unclear which bacterial pathways or enzymes are being targeted by these antibacterial plant extracts. Additionally, no information is available regarding their effects on the structural integrity of bacterial cell membranes.

In this paper, we evaluated the antibacterial mechanism of these aforementioned edible medicinal plants, using proteomic

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analysis and scanning electron microscope (SEM). Upon treatments with antibacterial plant extracts, differentially-expressed bacterial proteins were first resolved using denaturing gel electrophoresis, followed by sequence determination with MALDI TOF–TOF mass spectrometry. Additionally, we investigated on how the antibacterial plant extracts affected the bacteria physically, through viewing of the bacterial membrane surfaces with SEM. Lastly, gas chromatography–mass spectrometry (GC–MS) was also applied to investigate the phytochemical contents of the medicinal plant extracts. Through the combination of these techniques, we aim to identify potential phytochemical-mediated antibacterial therapeutic targets, along with better understanding of their mechanisms.

2. Material and methods

2.1. Preparation of plant extracts

Medicinal plants were collected around Perak state (Malaysia) from April to July of 2012. The plants species were authenticated by Professor Dr. Hean-Chooi Ong at the Institute of Biological Sciences, University of Malaya, Malaysia. These plants were dried in oven at 40 °C until constant weights were achieved. The dried plants were then pulverized using a warring blender, followed by extraction using methanol solvent. The supernatants were collected, filtered and then stored in −20 °C.

2.2. Isolation of bacterial proteins for SDS–PAGE analysis

The bacteria strains (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 6538) were cultured overnight in Luria–bertani (LB) broth using conical flasks in an orbital shaker at 37 °C and 200 rpm. Bacterial cultures were adjusted to 0.5–1 McFarland standard before used in subsequent experiments. The bacterial cultures were then treated with optimal concentrations (2–6 mg/ml) of the filtered plant extracts, and sterile water is used as the negative control. After the addition of plant extracts, the bacterial cultures were keep in an orbital shaker (37 °C, 200 rpm). At the designated time intervals, 20 ml of bacterial culture was collected and centrifuged at 9000 rpm for 10 min to separate the bacterial cell pellet from the supernatant. The pellet was then subjected to treatment with Bacterial Proteins Extraction Reagent (Thermo Scientific), and the supernatant was subjected to ammonium sulphate precipitation. The precipitated proteins were collected by centrifugation at 9000 rpm for 10 min.

2.3. SDS–PAGE gel analysis and identification of differentially expressed bacterial proteins

SDS–PAGE gel electrophoresis was carried out using 12% resolving gel [1.5 M Tris–HCl pH 8.8, 10% SDS (Fisher Scientific), 40% bis-acrylamide (Bio Basic Canada)], and 4% stacking gel [0.5 M Tris–HCl pH 6.9, 10% SDS, 40% bis-acrylamide]. The bacterial proteins were treated with dithiothreitol (DTT) and boiled, before loading into the wells. Gel electrophoresis was performed with constant electric current of 135 mV, until the bromophenol blue (BPB) reached the bottom of the gel plate. The protein gel was then stained with Coomassie Brilliant Blue R-250, and the molecular weights of the bacterial proteins were determined using commercial protein markers (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific). Differentially expressed protein bands were excised from the gel using sterile razor blades and analysed by MALDI TOF–TOF mass spectrometry (4800 Proteomics Analyzer, AB Sciex) (Proteomics International, Perth, Australia). The spectra were analysed using Mascot sequence matching software (Matrix Science) with Ludwig NR Database to identify the proteins of interest.

2.4. Investigation of morphological changes on bacteria membranes via scanning electron microscopy (SEM)

The bacteria strains were cultured overnight in an orbital shaker set at 37 °C and 200 rpm. Next, bacterial cultures were adjusted to 0.5–1 McFarland standard and treated with antibacterial plant extracts (0.6–5.0 mg/ml) or sterile water (control) for 2 h. Treated and non-treated bacteria cultures were incubated with 2.5% glutaraldehyde overnight. Following three washes with PBS, the samples were hydrated with ascending concentrations of ethanol (25% for 5 min, 50% for 10 min, 75% for 10 min and three changes of 100% ethanol for 10 min each). The samples were then dried through freeze drying. Subsequently, the dried samples were adhered onto double-sided adhesive conductive carbon tape (which was mounted on a copper stage). The samples were then coated with platinum (JEOL JFC-1600 Auto Fine Coater) before viewing by SEM.

2.5. Gas chromatography–mass spectrometry (GC–MS) analysis of TCL spots

Thin layer chromatography (TLC) was performed as previously described (Eloff, 2004). TLC spots were analysed by gas-chromatography equipped with mass spectrometry (GCMS-QP2010 Plus, Shidmadzu). Column (BPX-5, SGE Analytical Science) temperature was initially set at 110 °C and increased to 280 °C (increased at a rate of 5–10 °C/min). The flow rate of the carrier gas (helium) was set to 1 ml/min. The total running time of the gas chromatography was 36 min. The mass spectra were obtained from the range of m/e 40–700. The identities of the samples were determined by comparing the mass spectra with NIST Gas Chromatography Library Database.

3. Results and discussion

3.1. Identification of differentially expressed bacterial proteins

Through proteomic study, our objective was to identify differentially expressed bacterial proteins, upon treatment with antibacterial plant extracts. In this approach, we focused on three bacterial strains (*E. coli*, *P. aeruginosa*, *S. aureus*) treated with three medicinal plant extracts (*C. formosana*, *M. candidum*, *S. barbata*). Bacterial cellular proteins were extracted using Bacterial Protein Extraction Reagents (Thermo Scientific) and ammonium sulphate precipitation. In our results, a total of seven differentially expressed bacterial proteins were identified (Fig. 1). Differentially expressed bacterial proteins were excised and subjected to sequence analysis by mass spectrometry. The identified proteins and their characteristics were summarised and reported in Table 1.

Protein bands a, b (Fig. 1A) and c (Fig. 1B) were extracted from bacterial culture supernatants, using ammonium sulphate precipitation method. Based on MALDI TOF–TOF protein sequencing results, protein bands a to c were determined as triacylglycerol lipase, N-acetylmuramoyl-L-alanine amidase and flagellin, respectively. Protein bands d, e (Fig. 1C) and f, g (Fig. 1D) were extracted using Bacterial Protein Extraction Reagents and identified as outer membrane protein A, stringent starvation protein A, 30S ribosomal protein s1 and 60 kDa chaperonin, respectively.

Among the seven differentially-expressed proteins identified, two of them (bands f and g) are pertaining to the bacterial protein translational machinery. The 30S ribosomal protein S1 (band f) is an essential component critically involved in the translational pathway (Lafontaine & Tollervey, 2001). Interestingly, the 60 kDa chaperonin (band g) was previously reported with functional roles to stimulate proper folding of newly synthesized bacterial polypeptides, as well as to promote refolding of denatured proteins

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