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Original Research Article

Proteomic Analysis of Bacterial Expression Profiles Following Exposure to Organic Solvent Flower Extract of *Melastoma candidum* D Don (Melastomataceae)

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Abstract

Purpose: *To identify potential antibacterial protein targets following exposure to Melastoma candidum extract.*
Methods: *Plant extracts were prepared using sequential extraction method. Denaturing gel electrophoresis and MALDI TOF-TOF MS protein sequencing were used to identify differential-expressed bacterial proteins. 96-well microplate method was used to determine the minimum inhibitory concentration (MIC) values. Thin layer chromatography (TLC) bio-autobiography and gas-chromatography-mass spectrometry (GC-MS) were performed to determine the phytochemicals in the active fraction.*
Results: *Five differentially expressed bacterial proteins (four from Escherichia coli and one from Staphylococcus aureus), were identified via proteomic approach. Among the bacterial proteins identified, glutamate decarboxylase, elongation factor-Tu and α-hemolysin are especially noteworthy, as they are implicated in critical bacterial pathways pertaining to survival in acidic environment, protein translation and virulence, respectively. Additionally, we tested and reported the minimum inhibition concentrations of different M. candidum fractions and gas chromatography-mass spectrometry GC-MS analysis of the active fraction.*
Conclusion: *Glutamate decarboxylase, elongation factor-Tu and α-hemolysin represent potential antibacterial targets.*

Keywords: *Escherichia coli, Staphylococcus aureus, Melastoma candidum, Glutamate decarboxylase, Elongation factor-Tu, α-Hemolysin, Protein expression*

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INTRODUCTION

As the world is facing increasing cases of multidrug-resistant bacteria, there is an urgent need to search for novel antibacterial compounds. Besides synthetic inhibitors, potential sources of antibacterial compounds include those derived from natural products such as medicinal plants. Nonetheless, the inhibition mechanisms of many antibacterial phytochemicals remain elusive. Frequently, it is unclear which bacterial pathways or enzymes are being targeted.

In the efforts to discover potential therapeutic targets, as well as seeking for better understanding of their inhibition mechanisms, studies have been conducted to investigate the

effects of antibacterial agents on bacterial gene expressions [1,2]. For instance, using proteomic analysis methods, differentially expressed proteins have been identified in Gram-positive *Staphylococcus aureus* following exposure to silver (Ag), an active antibacterial agent incorporated in many healthcare products [3]. A similar approach has also been applied using plumbagin, an antimicrobial compound found in many medicinal plants [4]. Upon exposure to plumbagin, differentially expressed proteins from Gram-negative *Escherichia coli* have also been pinpointed.

Melastoma candidum (Melastomataceae family) is a medicinal plant distributed in the tropical and subtropical areas, including Southeast Asia. Traditionally, this plant has been used to eliminate stasis, toxin cleansing and traumatic injury treatment [5]. Studies have indicated the presence of bioactivities, such as antihypertension [6], free radical scavenging activities and monoamine oxidase inhibition activities in this plant extract [7]. Recent research works have also revealed *M. candidum*'s broad-spectrum antibacterial activities [5,8]; however, its inhibition mechanism remains unclear.

In this paper, we fractionated *M. candidum* extracts into different organic solvents and determined their corresponding minimum inhibition concentrations [9]. In our proteomic work, we identified and reported five differentially expressed bacterial proteins (four from *E. coli* and one from *S. aureus*), following exposure to *M. candidum* extracts. To the best of our knowledge, this represents the first time such bacterial protein targets are being reported in literature, in conjunction with *M. candidum* treatment. Additionally, we performed GC-MS analysis to study phytochemicals present in the active fraction. Through our works, we hope to shed light on some of the possible antibacterial therapeutic targets. Further works in this direction could potentially lead to the discovery and optimization of target-specific antibacterial agents.

EXPERIMENTAL

Preparation of plant extracts

M. candidum were collected from April to June of 2012. The plant species was authenticated by Professor Dr. Hean-Chooi Ong at the Institute of Biological Sciences, University of Malaya, Malaysia. Voucher specimen (voucher no: MHR-2012-003) was deposited at Faculty of Science, Universiti Tunku Abdul Rahman. The plant materials (flowers) were dried in an oven at 40 ºC for 48 h or until constant weight was observed. Dried plant sample was then pulverized, followed by sequential extractions using hexane, ethyl acetate, acetone, methanol and distilled water. Supernatants were then filtered, concentrated and stored in -20 ºC until testing.

Bacteria strains and culture conditions

A total of seven bacterial strains (Gram-negative: *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853; Gram-positive: *M. luteus* ATCC 4698, MRSA ATCC 33591, *S. aureus* ATCC 6538, *S. aureus* ATCC 25923,) were tested in this study. Bacteria strains were cultured overnight in Luria-Bertani broth, using autoclaved conical flasks placed in a 37 ^{o}C orbital shaking incubator, with constant shaking (200 rpm). Bacterial cultures were adjusted to 0.5 - 1 McFarland standard before used in subsequent experiments.

Minimum inhibitory concentration (MIC) assay

MIC assays were performed using the published protocol with modifications [10,11]. Briefly, a final bacterial inoculum of 5×10^5 cfu/ml was prepared using Mueller-Hinton Broth (MHB) and aliquoted into a 96-well sterile microtitre plate. Plant extracts were added into the first row of wells, and serial dilutions were performed to achieve final concentrations of 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 mg/ml. The sealed plate was then incubated at 37 ºC. After 18 - 24 h, 20 µl of 0.4 mg/ml of p-iodonitrotetrazolium chloride (Fisher Scientific) was added to each well, followed by 30 min of incubation at 37 ºC. Color changes to pink were observed. The lowest sample concentration whereby no color change was observed and recorded as the MIC value. The reported MIC values represented average values of three identical replicate trials. Commercial ampicillin and kanamycin (Sigma-Aldrich) were used as positive controls. Sterility control and solvent control were also included in the MIC assays.

Isolation of bacterial protein for SDS–PAGE analysis

Bacterial cultures were treated with 2 - 6 mg/ml of filtered plant extracts, and equivalence of solvents alone were used in negative controls. Bacterial cultures were then allowed to grow at 37 ^{o}C with constant shaking (200 rpm). At the intended time intervals (5, 30, 60 and 90 min), 20

ml of cultures were collected, and bacterial cells were harvested by refrigerated centrifugation at 9000 rpm for 10 min. Bacterial proteins were then extracted from the cell pellets and culture supernatants, using bacterial protein extraction reagent (Thermo Scientific) and ammonium sulfate precipitation.

SDS-PAGE gel electrophoresis and protein sequence analysis

SDS-PAGE gel electrophoresis was performed using 12 % resolving gel [1.5 M Tris-HCl, pH 8.8 (Bio Basic Canada, Inc), 10 % SDS (Fisher Scientific), 40 % bis-acrylamide] and 4 % stacking gel [0.5 M Tris-HCl (pH 6.9), 10 % SDS, 40 % bis-acrylamide]. Before being loaded into the wells, bacterial proteins were treated with dithiothreitol (DTT) and boiled. For ease of molecular weight estimation and comparison, protein ladder (Spectra Multicolor Broad-Range Protein Ladder, Thermo Scientific) was loaded onto each gel. The SDS-PAGE gels were run using constant electric current (135 mV) until the bromophenol blue dye front reached the bottom of the gel plate. Protein gels were then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific) staining solution for an hour, followed by overnight destaining in distilled water. Differentially expressed protein bands were then excised using sterile razor blades and subjected to analysis by MALDI TOF-TOF mass spectrometry (4800 Proteomics Analyzer, AB Sciex) (Proteomics International, Perth, Australia). Spectra were analyzed using Mascot sequence matching software (Matrix Science) with Ludwig NR Database to identify the proteins of interest.

Thin layer chromatography (TLC) bioautobiography

TLC bioautobiography was performed as previously described [12], with modification. Briefly, 0.2 mg of the plant extract (methanol fraction) was spotted at 1.5 cm from the base of a TLC plate (Silica Gel 60 F254, Merck) and developed using a 9:1 methanol:chloroform mobile phase. Two duplicate TLC plates were run concurrently: Plate A was used for bioautography, while Plate B was used to extract the spots containing antibacterial compounds. Plate A was developed as described above and then placed in a sterile petri dish. Next, 0.1 ml of bacteria culture (1×10^6 cfu/ml) was mixed into 10 ml of nutrient agar, and the agar was distributed over Plate A. Once the agar solidified, Plate A was incubated at 37 ^{o}C. After 24 h of incubation, 0.2 mg/ml of p-iodonitrotetrazolium violet solution was sprayed onto the agar. Clear zone on the agar indicated phytochemical fraction with bacterial inhibitory activity. Corresponding spot was determined on Plate B, and the silica gel was extracted with a spatula. Bioactive compounds were eluted from the silica gel using methanol. After centrifugation and subsequent filtration, the supernatant was then subjected to GC-MS analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis of TLC spots

TLC spots were analyzed by gas chromatography equipped with mass spectrometry (GCMS-QP2010 Plus, Shidmadzu). The column temperature was set to 110 ^{o}C for 2 min, then increased to 200 ^{o}C (at the rate of 10 ^{o}C/min), and finally increased to 280 ^{o}C (at the rate of 5 ^{o}C/min) and held constant for 9 min. The injector temperature was set to 250 ^{o}C (split mode with the ratio adjusted to 20:1, with injection volume of 3 ul). The flow rate of the carrier gas (helium) was set to 1 ml/min, and the ion source was set to 280 ^{o}C. The total running time of the gas chromatography was 36 min. The mass spectra were obtained from the range of m/e 40 to 700. The identities of the samples were determined by comparing the mass spectra with NIST Gas Chromatography Library database.

Statistical analysis

Data were reported as mean ± standard errors, obtained from three replicate trials. Statistical analysis was performed using SAS (Version 9.2). Data were analyzed using ANOVA test, and significant differences between means were separated using Fisher's Least Significant Difference test at $p < 0.05$.

RESULTS

In our work, the *M. candidum* was fractionated into organic solvents with different polarities and tested for their inhibition activities against a total of seven bacterial strains (Table 1). Bacterial inhibition activities were observed with *M. candidum* extracts fractionated into water, methanol and acetone. No inhibition was observed with the hexane extract, whereas ethyl acetate extract was found to inhibit only one strain of bacteria tested. The strongest bacterial inhibition activity was observed with methanol extract, followed by water and acetone extracts. The strong inhibition activities of methanol extract were indicated by its low MIC values (0.31 - 5 mg/ml).

Table 1: Minimum inhibitory concentration (MIC, mg/ml) of different *M. candidum* fractions, against Gram-positive and Gram-negative bacterial strains; ampicillin and kanamycin were included as the positive controls

Bacterial strain	Minimum inhibition concentration (mg/ml)					
	Organic solvent fractionation				*Antibiotic control*	
	Ethyl acetate	*Acetone*	*Methanol*	*Hot water*	*Ampicillin*	*Kanamycin*
Gram-negative						
E. coli 25922	-	-	5.00 ± 0^{b}	12.50 ± 0^{a}	0.08 ± 0^{c}	0.04 ± 0^{d}
E. coli 35218	-	-	2.50 ± 0^{b}	8.30 ± 2.10^{a}	-	0.04 ± 0^{b}
P. aeruginosa 27853	-	-	$5.00\pm0^{a,b}$	8.30 ± 2.10^{a}	-	2.50 ± 0^{b}
Gram-positive						
M. luteus 4698	-	-	-	0.80 ± 0^{b}	1.22 ± 0^{a}	-
MRSA 33591	5.00 ± 0^{a}	3.44 ± 0^{b}	0.31 ± 0^{f}	1.56 ± 0^{c}	1.04 ± 0^{d}	0.63 ± 0^{e}
S. aureus 25923	-	-	1.25 ± 0^{b}	6.30 ± 0^{a}	0.02 ± 0^{c}	-
S. aureus 6538	-	4.17 ± 0.83^{a}	$0.63\pm0^{b,c}$	$2.30\pm0.80^{a,b}$	0.02 ± 0^{c}	-

Data are reported as mean ± SE values (n=3). Different super indexes indicate significantly different mean values within a row, determined by the Fisher's Least Significant Difference (LSD) test ($p < 0.05$)

Table 2: Summary of protein identification of differentially expressed proteins using MALDI TOF-TOF MS. Protein bands (a to e) labeled as in Figures 1 and 2

Protein band	Protein size (in aa)	No. of peptide sequences identified	No. of amino residues identified	Sequence covered (%)	Annotation
A	728	14	167	22.9	Formate C-acetyltransferase
B	476	10	113	23.7	Glutamate Decarboxylase
C	471	13	132	28.0	Tryptophanase
D	394	9	107	27.2	Elongation Factor Tu 2
E	322	7	62	19.3	α-Hemolysin

Next, by using proteomic approach, we aimed to identify differentially expressed bacterial proteins. In this effort, we focused on using two bacterial strains as the models, with representatives from Gram-negative (*E. coli* 35218) and Gram-positive (*S. aureus* 6538) bacteria. Following treatment with the *M. candidum* extract, bacterial cellular proteins were extracted and prepared using Bacterial Protein Extraction Reagents (Thermo Scientific) and ammonium sulfate precipitation. In the negative control, *M. candidum* extract was substituted with solvent alone as treatment. Purified bacterial proteins were then separated on denaturing polyacrylamide electrophoresis gels. Figure 1a showed the protein expression profile of *E. coli* at different time intervals, following exposure to *M. candidum* extract. Here, a total of four differentially expressed bacterial proteins (as indicated by letters a to d) were identified, excised and subjected to analysis by MALDI TOF-TOF mass spectrometer. The identities and characteristics of these proteins were summarized and reported in Table 2.

Additionally, in order to determine the effects of *M. candidum* extract on the bacterial secretomics profiles, we resolved to use ammonium sulfate precipitation to isolate bacterial proteins secreted into the culture mediums, upon exposure to *M.*

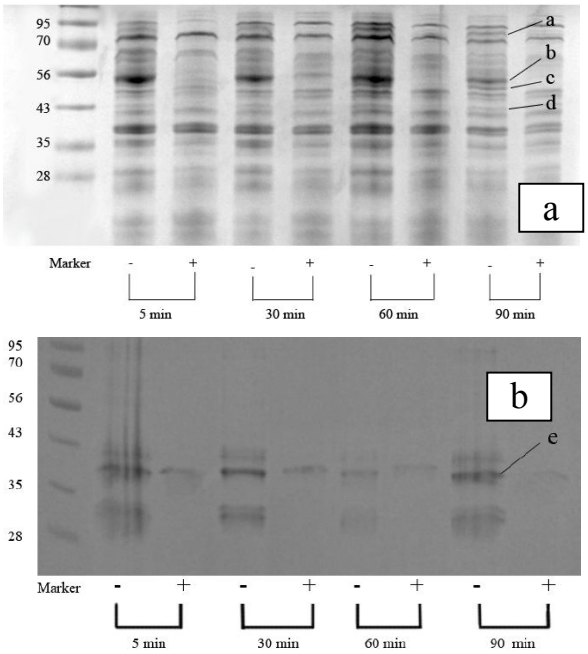

Figure 1: Protein expression profiles of (a) *E. coli* and (b) *S. aureus*, at time intervals of 5, 30, 60, 90 min, following exposure to *M. candidum* extract. (-) and (+) indicated the absence or presence of *M. candidum* extract in bacterial culture medium, respectively. Labels (a to e) indicate differentially expressed protein bands selected for MALDI TOF-TOF MS sequence analysis

M. candidum extract. Here, a differentially expressed protein band of approximately 35 to 43 KDa was identified from the *S. aureus* culture medium (Figure 1b). The protein was excised and subjected to sequence determination by MS. Based on protein sequencing result, the identity of this protein was determined to be α-hemolysin (a bacterial exotoxin protein)(Table 2). We also attempted, without success, to identify differentially expressed exoprotein from other bacterial strains, following exposure to *M. candidum* extract (data not shown). Lastly, we used GC-MS analysis to determine the phytochemicals present in the *M. candidum* extract. From the GC spectrum, seven major peaks were identified (Figure 2). Peaks 1 to 7 were selected and subjected to MS analysis. The identities and characteristics of these peaks were determined and summarized in Table 3.

DISCUSSION

Since the medieval age, medicinal plants have been utilized by different ethnic groups for assorted medicinal purposes, including but not limited to antibacterial applications. Recent advances in analytical science have enabled the identification of an ever expanding library of plant-derived bioactive compounds, noticeably those with antibacterial activities. These plant-derived secondary metabolites are of a diverse chemical nature, including different derivatives of phenolic acids, flavonoids, alkaloids and terpenoids compounds. Yet, relatively few studies have focused on illustrating and explaining how these medicinal plants exert their bacterial inhibitory activities. In most cases, it remains elusive which bacterial enzymes or pathways are actually affected by these antibacterial medicinal plants.

It is hoped that with more knowledge gained regarding the antibacterial mechanisms of these medicinal plants, potential therapeutic targets in bacteria could be revealed. The revelation of these novel targets could facilitate the design and enhancement of powerful antibacterial agents. This sure will be welcoming news in a

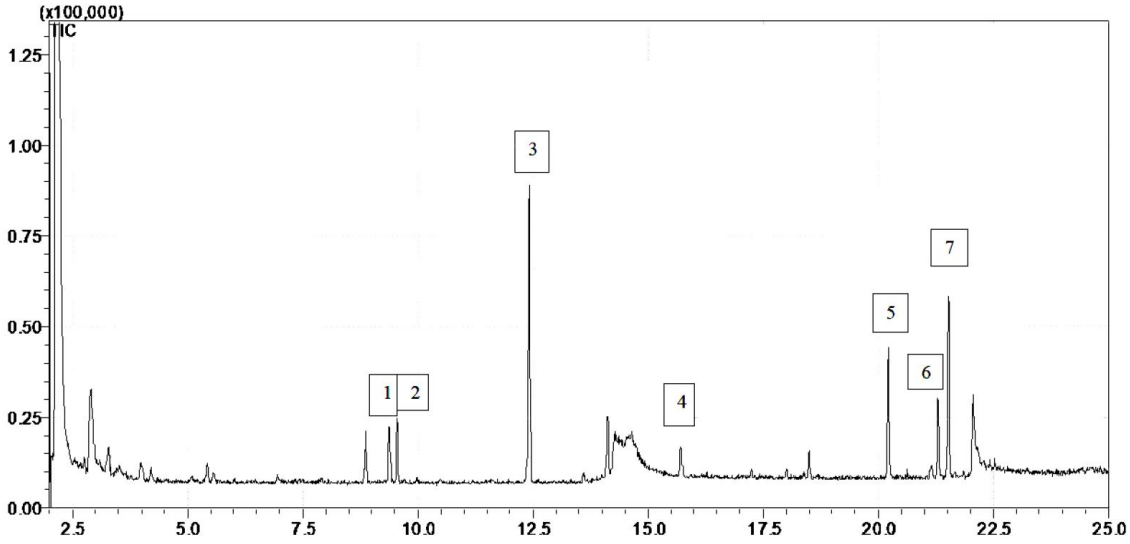

Figure 2: GC analysis of the active fraction of *M. candidum* methanol extract. Major peaks (compounds 1 to 7) were selected for further analysis with MS

Table 3: Major chemical compounds identified from the active fraction of *M. candidum* methanol extract by GC-MS

Peak no.	Retention time (min)	Name of compounds	Molecular formula	Molecular weight	Purity (%)
1	9.41	Butanoic acid, butyl ester	C8H6O2	134	80
2	9.54	2-Heptanamine, 5-methyl	C8H19N	129	80
3	12.44	Phenol, 2,4-bis(1,1-dimethylethyl)	C14H22O	206	90
4	15.73	1-Propanone, 2-amino-1-phenyl	C9H11NO	149	79
5	20.23	1-Butyl 2-(2-ethylhexyl) phthalate	C20H30O4	334	85
6	21.37	Hexadecanoic acid, 15-methyl, methyl ester	C18H36O2	284	82
7	21.54	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy, methyl ester	C18H28O3	292	79

world faced with increasing cases of multi-drug resistant bacteria.

In our study, we chose to focus on *M. candidum*, a medicinal plant with broad-spectrum antibacterial activities. In general, it is more challenging to inhibit Gram-negative bacteria, as they are protected by the presence of outer membranes [13] and efflux pumps [14]. Interestingly, the *M. candidum* was found to inhibit both Gram-negative and Gram-positive bacteria, including *E. coli, P. aeruginosa* and MRSA. Our fractionation and MIC results suggested that the antibacterial compounds in *M. candidum* extract may most likely be those of the polar family, as the strongest activities were observed with aqueous and methanol fractions.

In our GC-MS analysis of the methanol fraction, a total of seven compounds were identified. Literature search revealed that Compound 3 (phenol, dimethyl) [15] and Compound 6 (hexadecanoic acid, methyl ester) [15-17] have previously been reported with antibacterial activities. Meanwhile, a closely related derivative (hexadecanoic acid, ethyl ester), fractionated using ethanol from Brazilian *M. subsericea*, was reported with antibacterial activities in a separate study [18]. The bromo derivative of Compound 1 (butanoic acid, butyl ester) has also been tested with antibacterial activities [19]. In addition, the presence of Compound 5 (phthalate) has previously been reported in the aerial parts of *B. retusa*, [20] and *C. serrata* [21]. Both of these plant species demonstrated inhibitory activities against *E. coli, S. aureus* and *P. aeruginosa*. Interestingly, synthetic derivatives of Compound 4 (1-propanone) has previously been reported with inhibitory activities against *E. coli* and *S. aureus* [22,23].

In our proteomic analysis, a total of five differentially expressed bacterial proteins were identified. To the best of our knowledge, this represents the first time such bacterial protein targets are being reported in literature, upon exposure to *M. candidum* extract. Formate c-acetyltransferase is an enzyme involved in bacterial glucose and pyruvate metabolism [24], while glutamic acid decarboxylase (GAD) is utilized by bacteria to counteract the influx of protons from acidic environment, [25]. This feature of GAD enables the enteric bacteria such as *E. coli* to survive in the acidic environment of animal stomach. Interestingly, the expression level of elongation factor Tu (EF-Tu) was also affected by *M. candidum*. As EF-Tu interacts specifically with tRNAs during the protein elongation process, it is tempting to speculate that *M. candidum* extract may exert its inhibitory action via the bacterial protein translation pathway. This discovery is especially exciting considering that the protein translation process is critically essential to the bacteria survival, and it is highly conserved across different species of bacteria. [26]. It remains to be determined whether *M. candidum* extract affected EF-Tu in a similar mechanism as kirromycin, a commercial antibiotic targeting the bacterial EF-Tu [27,28]. Lastly, we also detected reduced α-hemolysin expression in the growth medium of *S. aureus*. α-Hemolysin is reported as a pore-forming exotoxin secreted by many pathogens, and it targets the host immune cells [29]. Multiple studies have also indicated the roles of this exotoxin in tissue barrier disruption and the subsequent lethal infection [30].

CONCLUSION

Our study has identified five bacterial proteins with reduced expression levels, upon treatment with *M. candidum* extract. Three of these bacterial proteins are especially noteworthy as potential therapeutic targets, namely GAD, EF-Tu and α-hemolysin. Inhibition of these bacterial targets may likely prove fatal to the invading bacterial strains, by rendering the bacteria vulnerable in acidic environment (human stomach), unable to replicate (by blocking the translation pathway) and reduced virulence (by repressing the expression of membrane-damaging exotoxin), respectively. Currently, it remains unclear how the bioactive compounds from *M. candidum* influenced these bacterial proteins. To complicate matters further, the question of whether one compound inhibited the bacteria single-handedly or a group of these compounds worked synergistically needs to be addressed too. It is hoped that further works in this direction could potentially lead to the discovery of effective therapeutic agents similar to that or better than kirromycin.

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