Jurnal Teknologi

Full Paper

MEMBRANE-ACTIVE METHANOLIC CRUDE EXTRACT OF THERMOTOLERANT, *Aspergillus fumigatus* SSH01 AND ITS MODE OF ACTION AGAINST GRAM-POSITIVE PATHOGENS

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Article history
Received
21 May 2017
Received in revised form
15 August 2017
Accepted
1 November 2017

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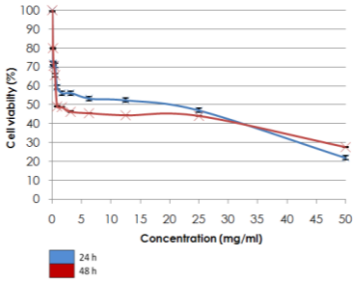
Graphical abstract



The viability of HaCat after treated with SSH01 crude extracts for 24 and 48 hours, respectively.

Abstract

This study was undertaken to investigate the antibacterial properties and the mode of actions of crude extract of *Aspergillus fumigatus* SSH01. Antibacterial properties was observed against Gram-positive pathogens and showed inhibition against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, methicillin-resistant *S. aureus* S547 (MRSA) and *Listeria monocytogenes* L10 with minimum inhibitory concentration (MIC, 0.097- 12.5 mg/ml) and minimum bactericidal concentration (MBC, 0.195 – 25 mg/ml). No surviving cells were detected after 15 h of treatment with the 2MIC of extracts for time-kill assay. Leakage of cellular contents of the treated test pathogens were identified and increased as the concentrations of the extracts increased. The study of morphological surface has shown the bacterial membrane was disrupted and caused loss of viability. This implies the antibacterial effects of *A. fumigatus* SSH01 extract may serve as the potential antibiotic.

Keywords: Antibacterial activities, disc diffusion method, minimum inhibitory concentration, minimum bactericidal concentration, scanning electron microscope

Abstrak

Kajian telah dijalankan untuk menyiasat aktiviti antibakteria dan mod tindakan ekstrak *Aspergillus fumigatus* SSH01. Aktiviti antibakteria terhadap patogen Gram-positif menunjukkan perencatan terhadap *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, rintang-methisilin *S. aureus* S547 (MRSA) dan *Listeria monocytogenes* L10 dengan kepekatan perencatan minimum (MIC, 0.097- 12.5 mg/ml) dan kepekatan bakteria minimum (MBC, 0,195-25 mg/ml). Sel mati dikesan selepas 15 jam rawatan dengan 2MIC ekstrak untuk kinetik masa-pembunuhan. Tahap kebocoran kandungan sel patogen yang diuji dikenal pasti dan meningkat selari dengan peningkatan kepekatan ekstrak. Imbasan elektron mikroskopi (SEM) menunjukkan perubahan morfologi pada permukaan membran bakteria yang menyebabkan patogen mati. Ini menunjukkan bahawa kesan anti-bakteria ekstrak *A. fumigatus* SSH01 berpotensi sebagai antibiotik.

Kata kunci: Aktiviti anti-bakteria, kaedah cakera penyebaran, kepekatan perencatan minimum, kepekatan bakteria minimum, imbasan mikroskop elektron

1.0 INTRODUCTION

For thousand of years, natural products have played such crucial roles worldwide in treating and preventing human diseases [1]. Of 23, 000 active compounds discovered from microorganisms (antimicrobial, antiviral, cytotoxic and immunosuppressive compounds), 42% are isolated from fungi while 32% by filamentous bacteria, the actinomycetes [2]. In addition, 20% of antibiotics found were contributed by fungi, 10-15% by non-filamentous bacteria while over half of it from actinomycetes and these antimicrobial agents are semi-synthetic derivatives of novel compounds (penicillin, cephalosporins, tetracycline, aminoglycosides, macrolides, ansamycins, polyenes, and glycopeptides) which are produced by chemistry or bioconversion [2]. However, fungi provide priceless secondary metabolites values in biomedical applications such as antibacterial, antifungal, larvicidal, antioxidant, molluscicidal and free-radical scavenging activities [3, 4]. It produces a great variety of unique natural products ranging from peptides, alkaloids, terpenes and polyketides [5]. *Aspergillus*, *Penicillium* and *Talaromyces* are some of the most incredible chemical factories of numerous bioactivities [6, 7, 8].

In 21st century, global healthcare issues have become one of the major problems due to antibiotic resistance microorganisms. They are capable to develop resistance against administered antibiotics within short period exposure [9]. Severe infections caused by resistant lead to complex, extended as well as costly treatments. The emergence of microorganisms called superbugs which simultaneously created multiple drug resistances (MDR) toward classes of antibiotics [10] demanded the urgent search for antimicrobial agents. The successful expressions of resistance the gene in MRSA, VRE and *Enterobacteriaceae* have dropped down the value and significances of antimicrobial agents in market [11]. In addition, the gap of discovering new antibiotic drug classes has been widened and barely introduced [12]. Most of the pharmaceutical companies have discontinued seeking for new drugs due to enormous rising of resistant and difficulty to treat nosocomial microorganisms for both Gram-positive and Gram-negative bacteria [12, 13]. A report of Antibiotic Resistance Threat in the United States, 2013 by Centers for Disease Control and Prevention (CDC) has classified a few resistance microorganisms that caused such serious threats to mankind including *Salmonella* Thypi, *Shigella*, *Streptococcus* and more. The commonly known pathogen, MRSA was estimated to cause 80 461 severe infections and 11 285 death per year. Besides, the rising of *Pseudomonas aeruginosa* as one of the multidrug resistance microorganisms which caused similar threat as MRSA contributed 6 700 serious infections and 440 deaths cases [14].

However, most of the extracted compounds tested for biological activities are from moderate temperature (mesophiles). In addition, abundant of journals published on thermotropic through decades are mainly focusing on thermostable enzymes for industrial purposes yet little on natural products. Only several early reports on antibacterial and antifungal activities from fungi demonstrated, that these thermotropic are able to release metabolites which regulate *in situ* growth behavior by fungistatic and auto-inhibition mechanisms [15, 16]. For example, *Talaromyces thermophilus* was reported to exhibit nematicidal activity [6, 7]. Besides, *Mycelia sterilia* (strain IPV F-4333) was another example of fungus that isolated from extreme environments that active when tested against filamentous fungi and yeast [17]. Later, the same metabolite was isolated from *Myriococcum albomyces* and renamed as myriocin 2 [18] was also reported to own biological properties in immunosuppressive activity [19]. Besides, *Aspergillus fumigatus* isolated from marine displayed strong antibacterial activity comparable to positive controls against Gram-positive bacteria [20]. Although there are reports regarding to thermotropic, its natural products and biological activities have been published worldwide, the understanding of it yet not so much to gain, partially-understood and fragmented. Perhaps this article could serve as pieces of knowledge of natural products, biological activities as well as mode of actions of antibacterial compounds from higher temperature inhabitants.

2.0 METHODOLOGY

2.1 Isolation and Identification of the Isolate

The strain of *Aspergillus* sp. was isolated from Sungai Serai Hot Spring, Hulu Langat, Selangor Darul Ehsan, Malaysia (GPS 3.047072 lat. and 101.700613 long.) at 40°C. It was identified macroscopically, microscopically and molecular identification. The DNA of fungal genomic was extracted from the mycelium using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc). Molecular biological procedures were conducted based on 16S DNA amplification and sequencing of the ITS region. 608 base pair internal transcribed spacer (ITS) sequence had 99% similarity to *Aspergillus fumigatus* SGE57 (JQ776545.1). The sequence data was submitted to GenBank with Accession Number KT266801.1 as *Aspergillus fumigatus* SSH01.

2.2 Preparation of 100% Methanolic Crude Extract

The methanolic extraction was conducted as described [21]. Seven mm in diameter of fresh mycelium was inoculated into 250-ml conical flasks containing 100 ml Potato Dextrose Broth (PDB). It was incubated for 9 days at 50°C under static conditions.

The liquid cultures were harvested by separating the mycelium and filtrate using Whatman No. 1 filter paper. The filtrate was extracted with methanol while the filtered mycelium was freezed at -20°C, crushed in the mortar and extracted with methanol overnight before refiltered. All filtrate were combined and evaporated to dryness using rotary evaporator at 40°C for 4 hours under reduced pressure.

2.3 Test Microbial Strains

The test microorganisms used for the screening of antimicrobial properties were grouped into three classes. Gram-negative bacteria included *Escherichia coli* ATCC 8739, *Escherichia coli* 0157: H7 E187, *Salmonella typhimurium* S836 and *Pseudomonas aeruginosa* ATCC 15442. For Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* S547 (MRSA), *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 and *Listeria monocytogenes* L10 were used. *Candida albicans* ATCC 10231 represented yeast, under the classification of fungi. *E. coli* ATCC 8739, *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633 and *Candida albicans* ATCC 10231 were obtained from Malaysian Palm Oil Board (MPOB) while the rest were purchased from Institute of Medical Research (IMR), Kuala Lumpur. The bacteria and yeast were cultivated using Tryptic Soy media and Potato Dextrose media, respectively.

2.4 Screening of Antimicrobial Properties of Crude Extract

The screening was carried out as described [22]. 0.5 McFarland-adjusted suspensions (approximately 1-2 x 10^8 CFU/ml) were spread onto the entire surface of the agar in petri dish (20 ml of agar media) and left to dry. The discs were applied. It was incubated at 37°C for 24 hours (bacteria) and 48 hours (yeast). In addition to the disc containing the extracts, positive controls (commercial antibiotics) and negative control (1% DMSO) disc were placed in each petri dish. The antibiotics used were chloramphenicol (30 ug/disc) and streptomycin (20 ug/disc) for bacteria while nystatin (100 units/disc) for yeast. Each test was done in triplicate.

2.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The analysis was done as described [23] with slight modification using resazurin powder instead of resazurin crystal [24] using microtiter plates. 0.27 g of resazurin powder was dissolved in 40 ml of distilled water, well-vortexed, and kept at 4°C for further use. Two-fold serial dilutions of the extracts (50 mg/ml) in combination of 80 µl Mueller-Hinton broth (MHB) and 10 µl of resazurin were performed. 10 µl of 0.5 McFarland-adjusted suspensions (approximately 1-2 x 10^8 CFU/ml) were added in. To avoid dehydration, moist tissue papers was placed under the plates and incubated at 37°C. The evaluation was taken at 24 hours. The well that remained dark blue at lowest concentrations after 24 hours incubation indicated no visible growth and considered as positive results. Resazurin that changed from dark blue to pinkish colour was recorded as negative results. MIC is defined as minimum concentration of the extracts that able to inhibit the bacterial growth while MBC is minimum concentration to kill the bacteria. In order to determine MBC, a loopful from dark blue wells was streaked on Mueller-Hinton agar (MHA) and incubated at 37°C for another 24 hours. The lowest concentration of the crude extract that did not show any growth on the agar was defined as MBC. Each plate had a set of controls: positive control [antibiotics (100 µg/ml) and broth] and negative control (bacterial suspension and broth). The analysis was carried out triplicates.

2.6 Time-Kill Assay

The analysis was conducted as described [25]. The crude extracts were diluted in 9 ml of MHB with the final concentration equivalent to ½MIC, MIC, and 2MIC value. For the negative control, extracts were not added but replaced with the broth. Vancomycin and penicillin G were used as positive controls. One ml of standardized bacterial suspension (approximately 1-2 x 10^8 CFU/ml) was added into the MHB that incorporated with the extracts. The broth was incubated at 37°C for 24 hours. For each 3 hours, the broths were taken and diluted by tenfold serial dilution in 0.85% saline suspension. 100 µL of each dilution was spread onto MHA plates (20 ml of agar media). The plates were incubated at 37°C for 24 h. The colonies formed in between 30 to 300 of each dilution were only counted. The assay was repeated triplicates and the mean of the reading were recorded.

2.7 Scanning Electron Microscope (SEM)

The bacterial samples in suspension were centrifuged at 3000 *xg* for 8 minutes and the supernatant was decanted. The pallet was fixed with 2.5% glutaraldehyde for 4-6 hours at 4°C. Later then, it was washed with 0.1M sodium cacodylate buffer for 3 changes of 10 minutes each. 1% osmium tetroxide was post fixed to the sample for 2 hours at 4°C. The sample was washed again with 0.1M sodium cacodylate buffer for 3 changes of 10 minutes each. During dehydration, the samples have undergone a series of different concentration (35%, 50%, 75% and 95%) of acetone of 10 minutes each. For 100% of acetone, the sample was dehydrated for 3 changes of 15 minutes each. Note that the sample was centrifuged (3000 *xg*, 8 minutes) and the supernatant was discarded each step. The cell suspension was pipetted onto aluminium foil (1 cm diameter) coated with albumin. For critical point drying, the specimen was transferred into specimen basket and dried for

about half an hour. It was then mounted onto the stub using double-sided tape or colloidal silver and coated with gold in sputter coater before viewing under scanning electron microscope (JOEL-SEM). The morphological surfaces of treated test microorganisms were observed. Vancomycin was used as the positive control.

2.8 Measurement of Cellular Content of Treated Bacteria

Leaking of cytoplasmic cellular content was determined using a spectrophotometer. The absorbance reading of 260_{nm} (for nucleic acids) and 595_{nm} (for protein) was measured after the filtrate of the bacterial cell exposed to crude extracts. The analysis was modified and conducted as described by [26, 27]. The extract was added to fresh sterile water to achieve the concentration of ½ MIC, MIC and 2MIC after 2 ml of bacterial suspension are added. Blank without inoculum and bacterial growth without crude extract were used as controls. After incubation of 30 minutes and 60 minutes, 4 ml of each different susceptible test microorganisms was removed. The samples were membrane-sterilized using 0.2 µm membrane filter into a sterile test tube. The absorbance at 260_{nm} (A_{260}) measured for nucleic acids was taken after 2 ml of filtrate was placed in cuvettes. The mean of OD_{260} was expressed as a proportion of the initial OD_{260}. In order to check the presence of DNA that leaked out, the cell-free filtrate was assessed by running 1.0 ml aliquotes of the phenol-chloroform-isoamyl alcohol (25:24:1)(v/v) concentrated on a 0.8% agarose gel. To quantify the amount of protein, the remaining filtrate was used for Bradford assay [28]. Five ml of Bradford reagent and a 100 µl of filtrate were added to a sterile test tube, vortexed and incubated for 5 minutes at room temperature before the absorbance (595_{nm}) was measured. To determine the amount of protein that corresponded to the mean absorbance values measured, the standard curve of bovine serum albumin was used. The experiment was carried out triplicates.

2.9 Rapid Analysis of Fungal Culture for Secondary Metabolites by Liquid Chromatography-Time-of-Flight Mass Spectrometry (LC/TOF-MS)

Separation of the fungal extract was performed using Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 x 150 mm, 3 µm particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was performed at 0.4 ml/min and 40°C using H2O + 0.1% Formic Acid (A) and 100% ACN (B) with 22 minutes total running time. The injection volume of sample was 1µl. The gradient started at 5% B (0-3 min); 80% B (3-10 min); 80% B (10-15 min) and 5% B (15-22 min). High-resolution mass spectrometry was carried out using a MicroTOF QIII Bruker Daltonic using an ESI negative ionization with the following settings:- capillary voltage: 4500 V; nebulizer pressure: 1.2 bars; drying gas: 8 L/min at 200°C. The mass range was at 50-1000 m/z. All the raw data was compared to the blank and the translated based on databases of NIST, MetFrag, and Medline.

2.10 *In Vitro* Cytotoxicity Assay

2.10.1 Cell Line and Culture Conditions

HaCat (human keratinocytes; normal cell line) was taken from Agro-Biotechnology Institute Malaysia (ABI), MARDI and used to investigate the cytotoxicity effect of the crude extract. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% antibiotic (penicillin-streptomycin [10,000 U/mL]). They were passaged after 80%-100% of confluency and kept at 37°C in a humidified atmosphere of 95% air and 5% $C0_2$.

2.10.2 MTT Assay

HaCat were seeded ($2x10^4$ cells/well) in 96-well plates [29]. Twenty-four hours after seeding, the growth medium was removed and treated with the 100 µL test solutions. The concentration of extracts that used in the assay was 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.097 mg/ml. After 24 and 48 hours of exposure, the viability and cytotoxicity test were performed. The MTT (5 mg/ml in phosphate-buffered saline, PBS; 150 µL) were added to each well. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% $C0_2$ for 4 hours and dimethylsulfoxide (DMSO) was added to each well to dissolve the insoluble purple formazan crystal. The absorbance was then measured at 540_{nm} with the spectrophotometer. The concentration which reduced the viability of the cells by 50% (IC_{50}) for the extracts was calculated from fitted dose-response curves. The experiments were performed in triplicate.

2.11 Statistical Analysis

All statistics were performed using SPSS (Scientific Package of Social Science) statistic version 17.0 (ANOVA) using Tukey's studentized ranged. The differences on statistical analysis of data were considered significant when the P value was < 0.05 [30].

3.0 RESULTS AND DISCUSSION

3.1 Screening of Antimicrobial Properties of 100% Methanolic Crude Extract

Table 1 shows the screening result of methanolic crude extracts at 500 µg/disc and 1000 µg/disc. *Candida albicans* ATCC 10231 was found to be resistant to both concentrations. Overall, the inhibition zones ranged from 11.00 to 18.00 mm

excluding positive controls. The inhibition zones against particular species were interpreted as weak, slight, moderate, high or strong activity following the ranges as suggested [31]. The inhibition zone of *B. subtilis* ATCC 6633 increased as the extract concentrations increased. *B. subtilis* ATCC 6633 showed moderate (11.33 mm) and strong (18.00 mm) antibacterial activities at 500 µg/disc and 1000 µg/disc, respectively. This followed by *Staphylococcus aureus* ATCC 6538 (11.67 mm), MSRA S547 (11.33 mm) and *Listeria monocytogenes* L10 (11.00 mm) with moderate antimicrobial activity.

Table 1 Screening for antimicrobial properties of SSH01

Test Strains	Inhibition zone (mm)		Positive control		N
	500 µg/disc	1000 µg/disc	C	S	
E. coli ATCC 8739	-	-	31.33 ±0.57	17.33 ±0.57	-
E. coli 0157: H7 E187	-	-	20.67 ±0.57	13.33 ±1.15	-
S. thypimurium S836	-	-	8.00 ±0.00	8.33 ±0.57	-
P. aeruginosa ATCC 15442	-	-	9.67 ±0.57	10.00 ±0.00	-
MRSA S547	-	11.33 ±0.57[a]	24.33 ±0.57	18.33 ±0.57	-
S. aureus ATCC 6538	-	11.67 ±1.15[a]	13.00 ±0.00	21.00 ±0.00	-
B. subtilis ATCC 6633	11.33 ±0.57[a]	18.00 ±1.00[c]	28.33 ±0.57	26.67 ±0.57	-
L. monocytogenes L10	-	11.00 ±1.00[a]	30.00 ±0.00	23.00 ±0.00	-
C. albicans ATCC 10231	-	-	8.67±0.57		-

Data represent mean of triplicates (diameter of paper disc included). Negative control – 1% of DMSO. Nystatin (100 unit/ disc) was used as positive control for *C. albicans* ATCC 10231. (-) represents no inhibition. C-Chloramphenicol; S-Streptomycin and N-Negative control.

3.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The data of MIC and MBC values was represented in Table 2. The concentration of tested crude extract ranged from 50 mg/ml to the lowest concentration of 0.024 mg/ml (two-fold serial dilution). The MIC values for each test microorganisms were identified by colourimetric observation. Overall, the MIC and MBC values ranged from 0.097 mg/ml to 25 mg/ml. The MIC and MBC values obtained for MRSA S547 were 0.195 and 0.780 mg/ml, respectively while for *S. aureus* ATCC 6538, the MIC and MBC values were the same (6.25 mg/ml). As for *L. monocytogenes* L10, it showed MIC value of 12.50 mg/ml and highest MBC value (25 mg/ml) was observed among of all susceptible microorganisms tested. *B. subtilis* ATCC 6633 exhibited lowest MIC value of 0.097 mg/ml and MBC value of 0.195 mg/ml. Penicillin G and vancomycin were used as positive controls (100 µg/ml) against the bacteria.

Table 2 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *A. fumigatus* SHH01 methanolic extracts (mg/ml).

Test Strains	*A. fumigatus* SSH01		Positive controls (ug/ml)	
	MIC	MBC	Penicillin	Vancomycin
MRSA S547	0.195	0.780	6.250	12.50
S. aureus ATCC 6538	6.250	6.250	3.125	12.50
L. monocytogenes L10	12.50	25.00	3.125	50.00
B. subtilis ATCC 6633	0.097	0.195	25.00	25.00

Data represent mean of triplicates.

3.3 Time-Kill Assay

Death kinetic studies are often referred to as time-kill studies and are used to determine the rate at which the antimicrobial agents kill the pathogens over the time, as well as the extent at which the activity occurs. Bactericidal activity is indicated by a 3-$\log_{10}$ reduction, the equivalent of 99.9% cell death, in the number of viable cells in the presence of the antibacterial compound. Approximately 10^8 CFU/ml of inoculum was introduced to the respective concentrations (½MIC, MIC and 2MIC) to observe the concentrations and time-dependant killing effects of the extracts against the test microorganisms as shown in Figure 1.

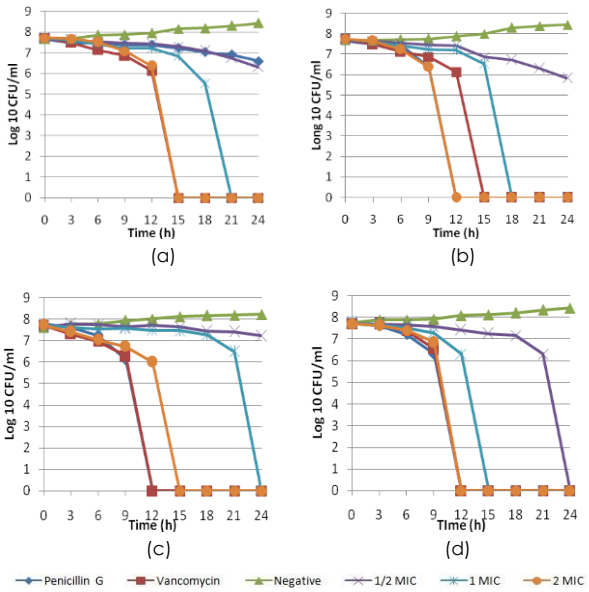


Figure 1 *In vitro* time-kill assay of (a) MRSA S547, (b) *S. aureus* ATCC 6538, (c) *L. monocytogenes* L10 and (d) *B. subtilis* ATCC 6633

½ MIC of the crude extracts was observed to give bactericidal effects against *B. subtilis* ATCC 6633 at 24 hours while bacteriostatic to the rest. However, 1MIC and 2MIC was observed to inhibit the growth and eventually wiped out the entire population of all

test pathogens at 24 and 15 hours, respectively. Overall, *B. subtilis* ATCC 6633 was demonstrated to be most susceptible to all concentration used (½MIC, MIC, and 2MIC) followed by *S. aureus* ATCC 6538, MRSA S547 and *L. monocytogenes* L10. No re-growth occurred after treated with the extracts.

3.4 Scanning Electron Microscope (SEM)

Changes in the morphology of treated cells (membrane-active mode) were revealed by scanning electron microscopy. The concentration of 2MIC was used as it able to inhibit all the susceptible pathogens growth based on time-kill assay. Deformation such irregular, striated and holes were observed on the surface of the pathogens explaining the cell wall perforation, as the intactness of the cell wall was disrupted caused by the crude extract when compared to negative controls as shown in Figure 2. *L. monocytogenes* L10 and *S. aureus* ATCC 6538 were observed to severely-disrupted compared to others. Surfaces of untreated test microorganisms were generally smooth-walled bodies compared to positive controls and treated cells. Pore formation is mainly related to the loss of viability of treated bacterial cells [32]. Such action is responsible for the rupture of bacterial membrane of vesicle containing cholesterol while leaving the membrane free from cholesterol undamaged [33].

3.5 Measurement of Cellular Content of Treated Bacteria

Table 3 shows the absorbance at 260_{nm} (A_{260}) with 5 hours interval up to 15 hours of treatments against susceptible test pathogens with three concentration of extracts (½MIC, MIC, 2MIC). It was expressed as a proportion of the initial A_{260}. The A_{260} of the cell-free filtrate of MRSA S547 and *B. subtilis* ATCC 6633 exposed to 2MIC value was observed to be higher than the control values. However, there was also increasing of A_{260} for half and one MIC against all the susceptible test microorganisms but the value was slightly increased and not exceeded the A_{260} of the positive controls throughout the periods. Table 4 shows the amount of protein present in the cell-free filtrates for 15 hours after treated with three concentrations of extracts (½MIC, MIC, 2MIC). The amount of proteins present in filtrates after exposure of cells to different concentrations of extracts was significantly different ($P<0.05$). The cell filtrates obtained after exposure of cells to the 2MIC for *S. aureus* ATCC 6538, *L. monocytogenes* L10 and *B. subtilis* ATCC 6633 contained the highest amount of proteins. The protein detected in cell-free filtrates was found to exceed the amount released by controls. The result indicated that the increasing time of exposure and higher concentration of extracts had significant ($P< 0.05$) effects on the amount of protein present in the filtrates.

Table 3 Absorbance at 260_{nm} in cell-free filtrates of test pathogens exposed to different concentrations of extracts

Test Strains	Time (h)	Crude Extract Concentrations			Positive control
		½ MIC	MIC	2MIC	
MRSA S547	0	1.11± 0.00^{a}	1.13± 0.00^{b}	1.22± 0.00^{c}	1.03± 0.01^{d}
	5	1.13± 0.01^{ab}	1.17± 0.01^{ab}	1.22± 0.00^{b}	1.08± 0.12^{a}
	10	1.17± 0.00^{a}	1.20± 0.01^{b}	1.23± 0.01^{c}	1.12± 0.01^{d}
	15	1.17± 0.01^{a}	1.22± 0.00^{b}	1.23± 0.02^{b}	1.15± 0.00^{c}
S. aureus ATCC 6538	0	1.05± 0.01^{a}	1.07± 0.00^{b}	1.08± 0.01^{c}	1.05± 0.01^{a}
	5	1.10± 0.01^{a}	1.18± 0.04^{b}	1.11± 0.00^{a}	1.22± 0.00^{c}
	10	1.16± 0.00^{a}	1.18± 0.03^{a}	1.19± 0.00^{a}	1.25± 0.01^{b}
	15	1.16± 0.00^{a}	1.19± 0.01^{a}	1.24± 0.02^{b}	1.30± 0.02^{c}
L. monocyto genes L10	0	1.06± 0.00^{a}	1.09± 0.11^{a}	1.11± 0.00^{a}	1.01± 0.00^{a}
	5	1.11± 0.00^{a}	1.11± 0.00^{a}	1.15± 0.00^{b}	1.18± 0.02^{c}
	10	1.11± 0.00^{a}	1.13± 0.01^{b}	1.55± 0.01^{c}	1.23± 0.01^{d}
	15	1.13± 0.01^{a}	1.13± 0.00^{a}	1.61± 0.00^{b}	1.25± 0.01^{c}
B. subtilis ATCC 6633	0	1.11± 0.00^{a}	1.15± 0.00^{b}	1.14± 0.00^{c}	1.03± 0.00^{d}
	5	1.14± 0.02^{ab}	1.19± 0.00^{ab}	1.22± 0.00^{b}	1.08± 0.12^{a}
	10	1.19± 0.01^{a}	1.25± 0.01^{b}	1.26± 0.00^{b}	1.11± 0.00^{c}
	15	1.19± 0.01^{a}	1.25± 0.01^{b}	1.28± 0.00^{b}	1.18± 0.03^{a}

Data are mean of triplicates ± standard deviation.$^{a-d}$ Different superscripts in each row indicate significant different ($P<0.05$).

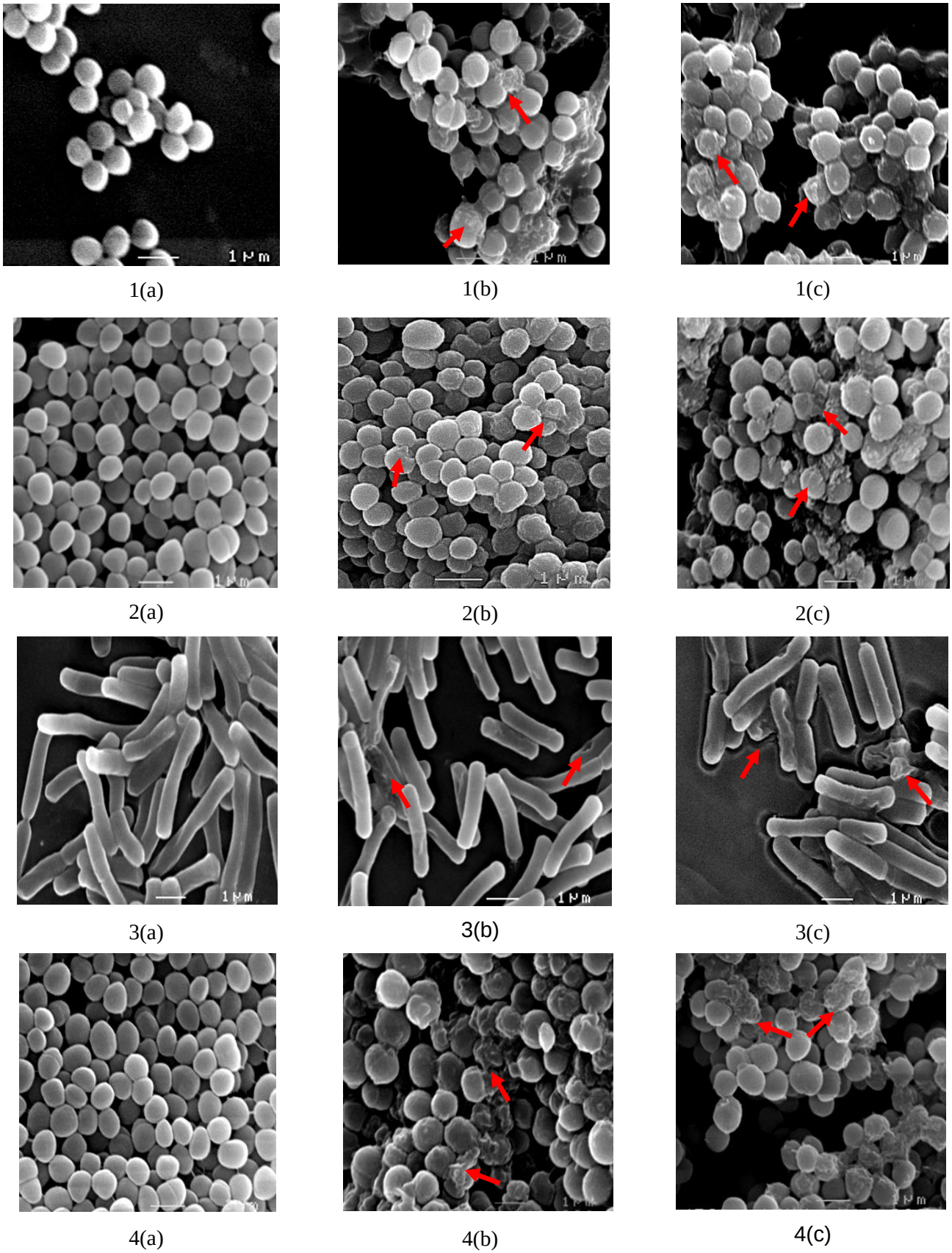


Figure 2 SEM micrograph under magnification of 10 000X. 1. MRSA S547, 2. *S. aureus* ATCC 6538, 3. *B. subtilis* ATCC 6633, 4. *L. monocytogenes* L10. All the susceptible test microorganisms were grown in the (a) free-antibiotic media and presence (b) of positive control (vancomycin) as well as (c) crude extract. Arrow points to cell collapse

Table 4 Amount of proteins (mg/ml) in cell-free filtrates of test pathogens exposed to different concentration of extracts

Test Strain	Time (h)	Crude Extract Concentrations			Positive control
		½ MIC	MIC	2MIC	
MRSA S547	0	0.663± 0.45[a]	0.831± 0.02[a]	0.903± 0.04[a]	0.663± 0.45[a]
	5	0.839± 0.01[a]	0.842± 0.03[a]	0.917± 0.03[b]	0.864± 0.01[ab]
	10	0.844± 0.00[a]	0.853± 0.01[a]	0.954± 0.03[b]	0.929± 0.01[b]
	15	0.859± 0.00[a]	0.907± 0.00[b]	0.994± 0.02[c]	1.032± 0.00[d]
S. aureus ATCC 6538	0	0.818± 0.00[a]	0.957± 0.01[b]	1.264± 0.01[c]	0.805± 0.04[a]
	5	0.828± 0.02[a]	0.959± 0.00[b]	1.300± 0.03[c]	0.847± 0.03[a]
	10	0.832± 0.03[a]	0.962± 0.08[b]	1.398± 0.00[c]	0.862± 0.02[ab]
	15	0.884± 0.00[a]	1.011± 0.03[b]	1.424± 0.00[c]	0.955± 0.01[d]
L. monocyto genes L10	0	0.892± 0.02[a]	0.915± 0.05[a]	1.052± 0.03[b]	0.928± 0.02[a]
	5	0.970± 0.01[a]	0.956± 0.02[a]	1.081± 0.04[b]	0.940± 0.05[a]
	10	0.973± 0.01[a]	0.964± 0.04[a]	1.081± 0.03[b]	0.953± 0.02[a]
	15	1.007± 0.04[a]	1.008± 0.03[a]	1.139± 0.05[b]	1.056± 0.00[a]
B. subtilis ATCC 6633	0	0.903± 0.03[a]	0.896± 0.03[a]	1.022± 0.02[b]	0.859± 0.03[a]
	5	0.942± 0.00[a]	0.956± 0.01[a]	1.088± 0.01[b]	1.003± 0.01[c]
	10	0.978± 0.00[a]	0.964± 0.01[a]	1.091± 0.01[b]	1.026± 0.03[c]
	15	0.979± 0.02[a]	1.003± 0.02[a]	1.160± 0.02[b]	1.069± 0.04[c]

Protein content was estimated by Bradford assay. Data are mean of triplicates ± standard deviation. [a-d] Different superscripts in each row indicate significant different (P<0.05).

3.6 Rapid Analysis of Fungal Culture for Secondary Metabolites by Liquid Chromatography-Time-of-Flight Mass Spectrometry (LC/TOF-MS)

Table 5 shows six metabolites that were analyzed from the fungal extract. All of the molecular weight obtained was translated based on Web Databases (Metfrag, NIST, and Metlin). However, several minor metabolites produced that were also detected from the plant instead of the fungus itself as shown in Table 6.

Table 5 Fungal metabolites detected by LC/TOF-MS

Formula	Compound	Max. *m/z*	Retention time RT [min]
$C_{16}H_{21}N_2O$	Fumigaclavine B	256.3428	1.4
$C_9H_{11}NO_3$	L-Tyrosine	181.0707	1.8
$C_6H_6O_4$	Kojic acid	142.0374	2.6
$C_{13}H_{14}O_5$	Citrinin	249.0768	9.1
$C_{26}H_{34}O_7$	Fumagillin	458.54	9.6
$C_{33}H_{44}O_8$	Helvolic acid	568.70	10.3

Table 6 Other metabolites detected by LC/TOF-MS

Formula	Compound	Max. m/z	Retention time RT [min]
$C_9H_6O_2$	Coumarin	146.0464	1.7
$C_9H_{10}N_2$	Myosmine	145.0771	8.9
$C_{24}H_{24}N_2O_{13}$	Neobetanin	548.1278	9.0

The first metabolite that was eludated (1.4 min) indicated a base peak of UV spectrum of fumigaclavine B at *m/z* 256 in LC-MS negative mode. The second eluted metabolite (1.8 min) and showed a base peak at *m/z* 181 and identified as L-Tyrosine. The compound eluted in the third position (2.6 min) indicated a characteristic of UV spectrum of kojic acid with the base peak at *m/z* 142. The next following metabolite was eludated (9.1-9.2 min) and after collision, the MS/MS peak was observed at *m/z* 249 and identified as citrinin. At 9.6 min, the 6th metabolite (fumagilin) was eludated at base peak of *m/z* 548. Finally, the last compound (10.2-10.3 min) was eluted and bombarded thus showed a UV spectrum and MS/MS peak at *m/z* 568. The metabolite was identified as helvolic acid.

3.7 *In vitro* Cytotoxicity Assay

The cytotoxicity of the crude extract was examined on skin keratinocytes due to the possibility to introduce the extract as antibacterial agents for skin diseases. After 24 hours of incubation, cell viability was determined. Different concentrations of crude extracts of SSH01 were examined *in vitro* for their cytotoxic activity against HaCat by MTT assay. The untreated cells were used as control. The result of this study revealed that the extract is cytotoxic against the cells with IC_{50} of 20 mg/ml as illustrated in Figure 3.

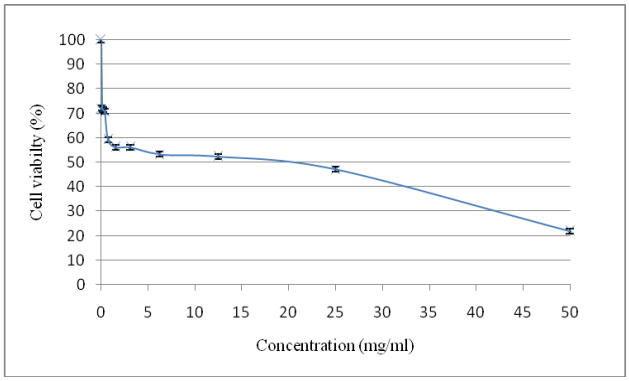


Figure 3 The viability of HaCat after treated with SSH01 crude extracts for 24 h. Data is expressed as the mean ± SD from three independent experiments

Figure 4 showed the cell viability treated with crude extracts at 48 hours. The untreated cells were used as control. The result of this study revealed that the extract is cytotoxic against the cells with IC_{50} of 0.78 mg/ml as illustrated in Figure 4.

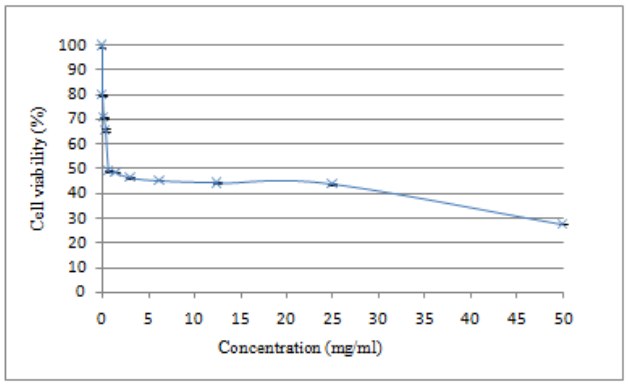


Figure 4 The viability of HaCat after treated with SSH01 crude extracts for 48 h. Data is expressed as the mean ± SD from three independent experiments

Different polarity of bioactive compounds are able to get extracted by different solvents such methanol, ethanol, water, ethyl acetate and more [34]. Methanol was chosen for this study due to its ability to extract wide variety of active compounds [35]. Many comparative studies have shown that metabolites extracted by methanol had effective antimicrobial activities compared to others [36, 37, 38]. In addition, it has the capability to extract polar compounds which have been widely reported to involve antimicrobial activities [39]. However, all tested Gram-negative bacteria and yeast in this study were resistant against the *A. fumigatus* SSH01 crude extract by showing no zone of inhibition. This may due to the effective permeability barrier located on the outer membranes that block the penetration of antimicrobial compounds [40]. Resistance could also caused by structural cytoplasmic membrane composition of efflux pumps called proteinaceous transport and multidrug resistance pumps which expel or extrude antimicrobial compounds out from the cells [41]. Yeast was also reported to have the ability to expel harmful substances (antifungal agents) by efflux pumps [42]. This study shows that the tested crude extract is effective against Gram-positive bacteria and it was implied to be targeted on the bacterial surface. The action of antimicrobial agents on the cellular membrane of the bacteria is one of the mechanisms involved in bacterial cell destruction [43, 44] which lead to total impaired to the cells [45]. The scanning electron micrograph in this study clearly denoted that the membranes of bacterial cells were crumpled, shrunken and deformities formations were remarkably shown on the cell membrane of the bacteria. This might lead to damage of the cells and caused total reduction of bacterial population as shown in time-kill assay when 2MIC was used. Failure in tolerating to different concentration of extract led to different structural membrane damage. Hence, this indicates that the methanolic crude extract is potent membrane-acting agents that caused cell lysis.

The same action was also reported by *Pestalotiopsis mangiferae*, a fungus associated with mango (*Mangifera indica)* when tested against human pathogens whereby it caused morphological alteration and destruction to bacterial cells by forming pores on the bacterial cell membranes [46]. Besides, metabolite extracted from *Fusarium proliferatum* strain BLH51 was also reported to act on cytoplasmic membrane of MRSA, caused them to aggregate, lysed and became morphologically irregular [47]. Generally, such explanation indicates that cell membrane maintains the physical integrity and viability of the cells and forms a barrier to hold the internal organelles and cytoplasm from external environment. Any disruption caused on it is threatable to the cells.

In addition, it was concluded that higher concentrations of crude extract caused irreversible damage (no re-growth) to the cytoplasmic membrane and caused bactericidal effects which were observed in time-kill assay. The data obtained by measuring optical density (OD) at 260_{nm} and 595_{nm} suggested that nucleic acids, ions, and amino acids are the cellular components that leaked out from the treated cells due to the disruption of cytoplasmic membrane [48]. A slight loss of 260_{nm} absorbing materials was observed, suggesting loss of negligible amounts of nucleic acids caused by disruption of the cytoplasmic membrane of treated cells. In addition, the leakage could also be affected by other reasons such as weakening of the cell wall or disintegration of cellular membrane by osmotic pressure when the bacterial cells were kept for several hours [49]. However, no DNA was present in the cell-free filtrates when running on the agarose gel electrophoresis. This could be due to the facts that the agarose gel electrophoresis relies on visual detection and the leaked DNA out from the cells was too little to be detected and less sensitive. In addition, detected compounds at 260_{nm} which present may not probably the DNA but RNA or other aromatic amino acids that have the ability to absorb at particular wavelength [50]. This suggestion was based on observation carried by [51] who detected 260_{nm} compounds released out from *S. aureus* MF 31 was RNA instead of DNA. The treatment at higher concentration of extract caused the loss of 260_{nm} absorbing compounds and increased amount of protein released out from the treated cells.

Several classes of metabolites were detected in the fungal methanolic crude extract and have been summarized in Table 5 and 6. Apart from *A. fumigatus*, it was reported that kojic acid was also been produced by *A. flavus*, *A. niger*, *A. ochraceous*, *A. parasiticus* and *A. oryzae* [52, 53]. It is an organic acid that is biologically produced by fungi and strikingly found in cosmetic and healthcare industries for the production of toothcare products, skin protective lotion, skin whitening cream and whitening soap [54]. However, 2 cases were reported on allergic response dermatitis to skin whitening cosmetic (kojic acid, Vitamin C derivatives, arubutin

or hydroquinone) which inhibit the tyrosine activity [55]. Polyketide L-Tyrosine was detected from non-toxigenic *A. flavus* apart from *A. fumigatus* SSH01. A wide range of valuable secondary metabolites was produced including benzylisoquinoline alkaloids (BIAs) and many polyketides whereby L-tyrosine is used as the precursor in treating Parkinson [56]. L-Tyrosine is used to transform into L-dopa by one-step oxidation reaction with the aids of enzymes (tyrosinase, tyrosine hydroxylase and tyrosine phenol-lyase) and used to treat Parkinson's disease due to depletion of dopamine in corpus striatum [57, 58]. Citrinin was reported to be found from *Penicillium citrinum* and *A. oryzae* [52]. However, it is classified as mycotoxin that produced by genera of *Aspergillus*, *Penicillium* and *Monascus* [59]. Citrinin and Ochratoxin A (also often produced by as stated) are classified as nephrotoxic to animals and have been involved in the aetiology of Balkan endemic nephropathy (BEN) to humankind [60, 61]. However, due to limited evidence of carcinogenicity in experimental animals, citrinin is not classifiable as possible human carcinogen [62]. The other organic acid, helvolic acid were also detected by LC/TOF-MS. It was previously reported to inhibit Gram-positive microorganisms including those causing gas gangrene but only to bacteriostatic effect, not bactericide [63]. Unlike the previous report, this study has shown bactericidal effects and revealed its mode of actions against Gram-positive bacteria when *A. fumigatus* was grown at higher temperature of 50°C. It is a toxin crystalline compound produced by *A. fumigatus* that posses antibacterial properties and gave bacteriostatic effect mostly against Gram-positive microorganism (*Staphylococcus aureus* and *Streptococcus pyogenes*). It was also reported to relatively non-toxic to leucocytes and tissue cultures. In addition, it was tested and applicable to be taken by ingestion, subcutaneous tissues, and peritoneal cavity and excreted out in urine [63, 64]. Besides, it caused oxidative burst to macrophages, a complete ciliostatis and rupture/damaged the epithelial cells when higher concentrations used [64]. Fumagillin, another important toxic compound was reported as an anti-tumour antibiotic which act as a potent inhibitor of angiogenesis [64]. It functions by inhibiting the proliferation of endothelial cells and *in vitro* hindered the ciliary functions of human respiratory epithelium [65]. However, their role in *in vivo* pathogenesis is still not clear yet can be postulated [64].

4.0 CONCLUSION

Aspergillus fumigatus SSH01 methanolic crude extract posses potential antibacterial properties henceforth could serve as next antibiotic specifically as membrane-active agent even though it was growing at higher than moderate temperature (mesophiles). No compounds are yet isolated in this study but it provides general depiction on purifying the extract for future study based on LC/TOF-MS results. This could serve as a platform for further study. Beneficial compounds such L-Tyrosine and fumagilin serve as other biological agents apart from focussing only on antibacterial properties. However, further studies may require in explaining the risk of toxicity (kojic acid and citrinin) of the crude extracts and its stability for the application of human use. The constituents of the extracts should be isolated, identified, characterized perhaps improvise for better potential as not only on antibacterial activity but anticancer, antibiofilm, antiviral and more.

Acknowledgement

The authors would like to express the deepest gratitude to the late Associate Professor Dr. Sieo Chin Chin for her guidance and support, and Universiti Putra Malaysia for the grant provided under the Research University Grant Scheme (RUGS) (Vot No: 9385600). The authors declared no concflict of interest.

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