In vitro Antimicrobial Mode of Action of *Cynodon dactylon (L.)* Pers. Solid Phase Extract(SPE) against Selected Pathogens

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Abstract*--Cynodon dactylon* (L.) Pers. is a type of perennial grass that possesses great medicinal values and believed able to cure many diseases and infections. This paper reports the antimicrobial mode of action of *C. dactylon* Solid Phase extract (SPE) against *Bacillus cereus, Bacillus subtilis and Staphylococcus aureus*. Mode of action study was done quantitatively via assessment on leakage of 260nm-absorbing materials, fluorometric and luminometric (ATP) assays and qualitatively via Scanning Electron Microscope observation. Assessment on membrane degradation based on 260nm-absorbing material leakage and fluorometric assay showed membrane disruption on *B. cereus* and *B. subtilis* after treated with the plant extract. SEM observation further confirmed the membrane disruption. Apart from membrane disruption, luminometric assay based on ATP quantification suggests the bacterial death was due to metabolic suppresion. Liquid Chromatography-Mass Spectrometry (LCMS) analysis revealed some possible antibacterial compounds such as Methenamine, peptides (Arg Ser Ser and Gln Arg Arg), 3-O-mycarosylerythronolide B, Triterpenoid, Cardenolide glycoside, Pandaroside, Avermectin B1a monosaccharide, Gambierol, Ginsenoside, and Halstoctacosanolide.

Keywords – *Cynodon dactylon*, antimicrobial, mode of action.

I. Introduction

OVER the years, there has been a decrease in microbial susceptibility to existing antimicrobial agents responsible for critical point drug resistance in hospitals and in communities. Different authors have reported the urgent need for new antimicrobial agents to replenish the arsenal of antimicrobial agents [1][2]. Different types of antimicrobial compounds, whether produced through chemical synthesis or naturally occurring compounds can exhibit different types of antimicrobial mode of action such as degradation of cell wall and cell membrane, inhibition of metabolic pathway, inhibition of DNA replication or through inhibition of DNA and protein synthesis [3]. Lot of studies have been discussed previously on the methods of studying the antimicrobial mode of action. Toxic effects on bacterial cell membrane structure and function have been generally used to explain the antimicrobial action in most antimicrobial agents. For this purpose, many methods have been discussed previously on the mechanism of action of those antimicrobial agents on bacterial membrane cell against many Gram positive and negative bacterial pathogens. It is important to understand the mechanism of action of particular antimicrobial agents as its become the fundamental question on developing other alternative antimicrobial agent. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which have been reported to have antimicrobial properties. Due to the structural diversity and the broad range of activity of those biologically active compounds from plant had gained so much interest from researchers to study their potential in combating pathogens. *Cynodon dactylon* is a type of perennial grass that possesses great medicinal values. It is traditionally used as a rejuvenator, for wound healing and was believed able to cure many diseases and infections [4]. Scientifically it has been reported to exhibits many important pharmacological effects [5][6]. Therefore, this study aims to investigate the antimicrobial mode of action of *C. dactylon* against some Gram positive bacterial pathogens and to identify the possible antimicrobial compounds which responsible for the action.

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II. Methodology

A. Plant Collection

Wild ecotype of the plant was collected in area of Kota Kinabalu (Lat: 6.034826, Long: 116.12316), Sabah, Malaysia. A voucher (jgobilik 1090/2011) of the collected plant was kept in School of Sustainable Agriculture (SPL), Universiti Malaysia Sabah (UMS) and a duplicate was submitted to BORH Herbarium, Institute of Tropical Biology and Conservation (ITBC), UMS for future reference.

B. Cynodon dactylon Ethanol Crude Extraction

The whole plant of *C. dactylon* was thoroughly cleaned using distilled water to remove soil and dirt and then dried

for 24-72 hours in a drying chamber at 40-50^{O}C to remove water content from the plant. To optimize and enhance the extraction yield, the dried plant was homogenized using a mechanical blender (Waring® Commercial Blender). Approximately 100g of the plant powder later was soaked into 200mL of ethanol and shaken on a platform shaker (LabCompanion^{TM}) at 150 rpm at temperature of 25 ^{O}C to obtain the plant extracts. The extracts obtained were then evaporated and concentrated under reduced pressure (768mmhg to 7mmhg) using Rota Vapor^{TM} (BUCHI) to achieve final concentration of 1g of extract per mL of solvent. The Aliquot was then kept in -20^{O}C until further use.

C. Preparation of Cynodon dactylon Solid Phase Extract (SPE)

Strata^{TM}-X 33um Polymeric Sorbent reverse phase (200mg/6mL) (Phenomenex) cartridges with 12-cartridges manifold system was used. Methanol absolute (1 mL) was used to activate the sorbent and further equilibrated with sterile deionized distilled water (1 mL). Ethanol extract of *C. dactylon* was then loaded into the cartridges and left inside the SPE sorbent matrix for few seconds up to a minute. The loaded sample was then washed with 1% methanol (1 mL). The resulted fraction yielded from wash procedure was collected and labelled as 'flush fraction'. Finally, the remaining samples inside the SPE sorbent matrix were eluted with 2mL of methanol:acetonitrile (1:1; v/v), collected and labelled as 'elute fraction'. The aliquots were taken to dryness using purified nitrogen gas. Dried aliquots were stored in -20^{O}C for further bioassays. Both flush and elute fractions were collected and tested for their respective antimicrobial activity.

D. Bacterial Pathogens

Pure cultures of three Gram positive bacterial pathogens: *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* were obtained from Queen Elizabeth Hospital (QEH), Kota Kinabalu, Malaysia. The pathogens were sub-cultured onto Nutrient Agar (NA) prior to preservation. The bacterial cultures were then preserved in 30% glycerol stock solution at -85^{O}C temperature. Prior to mode of action study, the pathogens were sub-cultured onto Muller-Hinton Agar (MHA), incubated at 37^{O}C for 24 hours then inoculums of the test pathogens were grown in Muller-Hinton Broth (MHB) and adjusted according to Mac Farland Standard to achieve approximately 1x10^{8} CFU/mL before use.

E. Assessing on the Leakage of 260nm Absorbing Material

Bacterial strains were cultured in NB and incubated at 35^{O}C for 18h. After incubation, bacteria were harvested by centrifugation at 10 000g for 10 min at 4^{O}C. The supernatant was discarded and the cells were washed twice with PBS; pH 7.4. The plant ethanol SPE flush fraction extract was added to the bacterial suspensions at 2x MIC. Meanwhile, as a control, ethanol was added to another set of bacterial suspension for the leakage control assessment. Suspensions were incubated in a water bath at 35^{O}C. Approximately 1.5 mL of bacterial samples treated with the plant extract and ethanol were removed 2 hours after incubation and centrifuged at 10 000g for 10 min at 4^{O}C. Supernatant (200µL) for each treatment was added to the wells of a 96-well plate (UV–transparent flat-bottom microplates, Corning-Costar, Fisher Scientific) and absorbance values at 260 nm were recorded using a UV spectrophotometer (Thermo Scientific). The following controls were included: a bacterial suspension in sterile PBS without the plant extract as the negative control; the plant extract incorporated into PBS and ethanol as the positive control. Where applicable, independent readings were also taken, in the presence of antibacterial agents only, to enable corrections for background contributions. The percentage of 260nm-absorbing materials leakage was calculated according to this formula:

$$\text{Leakage percentage (\%)} = \frac{\text{A260 of treated bacteria} - \text{A260 of Untreated bacteria}}{\text{A260 of ethanol treated bacteria} - \text{A260 of Untreated bacteria}} \times 100$$

F. Live/Dead BacLight Assay for Membrane Analysis

The Live⁄Dead® BacLight viability kit from Molecular Probes, Inc. (Eugene, OR, USA) was used as described previously [7]. In this assay, the SYTO-9 and propidium iodide stains compete for binding to the bacterial nucleic acid. SYTO-9 labels cells with both damaged and intact membranes, whereas propidium iodide penetrates only cells with damaged membranes. Cultures of *B. cereus*, *B. subtilis* and *S. aureus* were grown to late log phase in 30mL of NB. Twenty-five millilitres of the bacterial culture was concentrated by centrifugation at 10 000g for 10 min. The supernatant was removed and the pellet was washed once in filter-sterilized distilled water and resuspended to 1⁄10th of the original volume and then diluted 1:20 into either sterile de-ionized water (for live bacteria estimation), 70% ethanol (for dead bacteria estimation) or sterile deionized water containing test compounds at 2x MIC. Different profractions of the live and dead bacterial cells were mixed to obtain cell suspensions containing five different ratios (100 : 0, 80 : 20, 50 : 50, 20 : 80 and 0 : 100 (in %)) of live and dead cells for a data set to provide a standard regression curve. Bacterial and treatment suspensions were incubated at room temperature for 1 hour. At the end of the incubation period, the suspensions were centrifuged at 10 000g for 10 min, washed once in sterile deionized water and resuspended to achieve 1x10^{8} bacteria per mL. Volume of 100uL of each bacterial or treatment suspension was added in triplicate into separate wells of a 96-well microplate (black; Nunc). A 1x stain solution was prepared by mixing component A (3.34mmol^{-1} SYTO 9 dye) and component B (20mmol^{-1} propidium iodide) in equal profraction. To each well, 100uL of 1x stain solution was then added, and the plate was incubated in the dark for 15 min at room temperature. At the end of the incubation

period, with the excitation wavelength at 485nm, the fluorescence intensity of SYTO-9 was measured at 530nm (emission 1; Green) for each well. With the excitation wavelength still centered at 485nm, the fluorescence intensity of propidium iodide was measured at 630nm (emission 2; Red) for each well of the entire plate. The Green/Red (G/R) ratio was obtained by dividing the fluorescence intensity of the stained bacterial suspensions (F cell) at emission 1 by the fluorescence intensity at emission 2. The calculation was summarized as follow:

$$G/R \text{ ratio} = F \text{ cell, emission} 1 / F \text{ cell, emission } 2$$

The Ratio G/R was plotted versus percentage of live cells in the bacterial samples suspension.

G. Assessment on ATP activity via Bactiter™ Glo assay

The BacTiter-Glo™ Microbial Cell Viability assay is a method for determining the number of viable bacterial cells in a culture based on quantifying the ATP present, as an indicator of metabolic activity. The luminescent signal is profractional to the amount of ATP present, which is directly profractional to the number of cells in culture. An overnight culture of *B. cereus*, *B. subtilis* and *S. aureus* in NB were diluted 100x fold in fresh NB and used as inoculum. *C. dactylon* ethanol SPE flush fraction extract was used at a concentration of 2x MIC. Each well of a 96-well microtiter plate contained 100uL of the inoculum and 100uL of the plant extract. Control wells containing medium without cells were prepared to obtain a value for background luminescence. Bacterial cells without compound were used as an ATP positive control. The microtiter plate was incubated at 35^{O}C for 5 hours. Approximately 100uL of the culture was taken from each well, and mixed with the same volume of the BacTiter-Glo™ reagent in a white opaque-walled microtiter plate (Nunc). Plates were incubated for 5 minutes, and luminescence was recorded in a multi-detection microplate reader using luminometric assessment mode (Fluoroskan FL, Thermo Sc.).

H. Scanning Electron Microscope (SEM)

All bacterial pathogens of the mid-exponential growth phase were diluted with NB medium to the cell density approximately 10^8 to 10^{10} according to Mac Farland standard and further treated accordingly with 2x MIC of flush fraction of *C. dactylon* ethanol SPE extract for 2 hours at 37°C. Untreated controls were prepared in NB medium. After 2 hours of incubation, the bacterial cells were collected via centrifugation at 10,000g and then the pellet formed was washed with PBS for 3 times. Fixation was done by suspending the bacterial cells into 0.25% of glutaraldehyde solution (in PBS, pH 7.0) and then incubated at room temperature for 1 hour. After the incubation, the bacterial cells were washed with PBS for three times and then the fixed bacterial pellet was collected by centrifugation at 10,000g. Dehydrolysis of the bacterial cells were done by washing the pellets with different ethanol concentration starting with 30%, 50%, 70%, 80%, 90% and 100%, and for each ethanol treatment was incubated for 10 minutes. Incubation with 100% ethanol was done up to 1 hour. Prior to observation under SEM, the bacterial cells was mounting on carbon formvar and then coated with gold-palladium in Emitech K550x carbon coater for 1 minute. Microscopic observation was performed with a Zeiss Supra 55VP (Oberkochen, Germany) microscope. Secondary electron images were taken at low electron energies between 2 keV and 2.5 keV.

I. Mass Analysis and Compound Identification

The Liquid Chromatography-Mass Spectrometry (LCMS) analysis was carried out using an Agilent 1200 series coupled with Agilent 6200 series Quadrapole Time of Flight (Q-ToF) Mass Spectrometry (MS) Dual Electrospray Ionization (ESI) detector. Mass spectra analysis on flush fraction of *C. dactylon* SPE extract was done using the Agilent MassHunter Workstation-Qualitative analysis Software. In this software, few mass to charge ratio (m/z) peaks from a respective chromatogram were generated and the most abundant m/z was selected for generating the most probable mass for particular compound through Find by Molecular Feature algorithm. Each identified mass representing particular compounds was subjected to compound identification using online metabolites spectral database, METLIN (http://metlin.scripps.edu). The identity of compounds from the SPE fraction was identified by matching their true molecular mass with existing chemical compound databases in METLIN. Besides, METLIN, other online databases including PubChem, KEGG and HMDB were also utilized to enhance the compounds identification.

III. Result

A. Leakage of Material Absorbing at 260nm

Nucleic acid and its related compounds, such as pyrimidines and purines, absorb UV light at a wavelength of 260nm. The presence of these materials in a suspension may be used as an indicator of damage to the cell membrane caused leakage of the materials into the surrounding. Leakage was determined using ethanol as a control agent, an organic solvent that is known to cause membrane damage. Only two bacterial pathogens (*B. cereus* and *B. subtilis*) show release of material absorbing at 260nm ($P<0.05$) after treated with the flush fraction (Figure 1). The highest leakage was recorded from *B. cereus* while the lowest leakage recorded was from *S. aureus*.

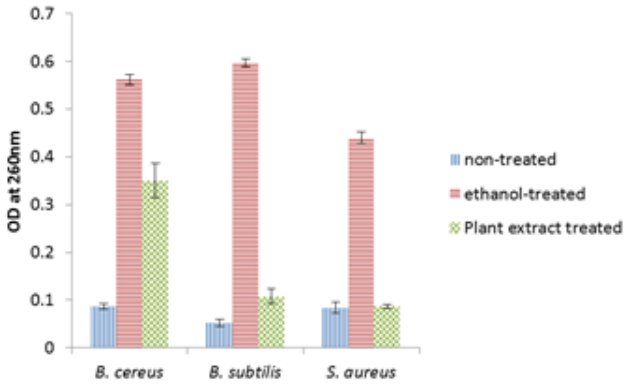

Fig. 1 OD reading for 260nm absorbing materials from each bacterial pathogen tested. Only two bacterial pathogens (*B. cereus* and *B. subtilis*) show leakage of 260nm absorbing material significantly (P<0.05) after treated with *C. dactylon* SPE flush fraction extract compared to *S. aureus*.

B. Disruption of Membrane via Live/Dead Baclight Assay

Bacterial membrane damage was further assessed using the Live⁄Dead® BacLight viability assay. The fluorescence intensities of the stained bacterial suspension at 530nm (G, green) and 630nm (R, red) represent live and dead cells, respectively. In the present study, treatment by flush fraction of *C. dactylon* ethanol SPE extract (at 2x MIC for 1 hour) on *B. cereus* and *B. subtitlis* caused damage to the bacterial cells membrane which indicated by the low fluorescence ratio with viability percentage approximately 66.38% and 82.29% respectively (Table 1). Meanwhile, the effect of the treatment on membrane integrity against *S. aureus* was not as intense as the above bacterial cells with viability percentage of 98.07%. The data obtained from Live/Dead BacLight Assay are in agreement with the observation for the leakage studies at 260nm for the tested bacterial samples.

TABLE I
EFFECT OF *C. DACTYLON* ETHANOL SPE FLUSH FRACTION EXTRACT AT 2X OF MIC ON MEMBRANE INTEGRITY OF IN TESTED BACTERIAL PATHOGENS USING BACLIGHT ASSAY

Bacterial pathogens	[a]Relative fluorescence amount	[b]Relative bacterial live percentage (%)
B. cereus	6.08±0.20	66.38
B.subtilis	7.51±0.33	82.29
S. aureus	5.67±0.09	98.07

[a]Relative fluorescence amount were presented as mean±std, obtained using Fluoroskan FL fluorometric bioassay.
[b]Relative bacterial live percentage (%) was obtained from standard regression line of the respective dead/live ratio bacterial samples.

C. Assessment on ATP Activity of Selected Bacterial Pathogens via Bactiter™ Glo Assay

The luminescence signal is proportional to the amount of ATP present, which is directly proportional to the number of metabolically active cells in the culture. The results are shown in Table 2. Treatment using the flush fraction of *C. dactylon* ethanol SPE extract on the bacterial cells resulted in reduction of ATP levels compared to the untreated (control) bacterial cells with Relative Luminescence Unit (RLU) percentage ranging from 0.11-0.27%. The data obtained suggests the flush fraction of *C. dactylon* ethanol SPE extract inhibit the growth of the tested bacterial cells not only via membrane disruption but also through other mechanism.

TABLE II
RELATIVE LUMINESCENCE ACTIVITY OF BACTERIAL PATHOGENS TREATED WITH *C. DACTYLON* ETHANOL SPE FLUSH FRACTION BASED ON RELATIVE LUMINESCENCE UNIT (RLU).

Bacterial pathogens	RLU		*Percentage (%) RLU after treatment
	Treated[a]	Control	
B. cereus	$1.10x10^4$	$1.03 x10^7$	0.11
B.subtilis	$1.56 x10^4$	$1.09 x10^7$	0.14
S. aureus	$2.80 x10^4$	$1.05 x10^7$	0.27

[a]*C. dactylon* ethanol SPE flush fraction extract treatment; Control: Relative luminescence activity of the untreated bacterial pathogens
*Percentage (%) of RLU were obtained by dividing the relative luminescence activity of the bacterial pathogens with their respective controlled (untreated bacterial pathogens)

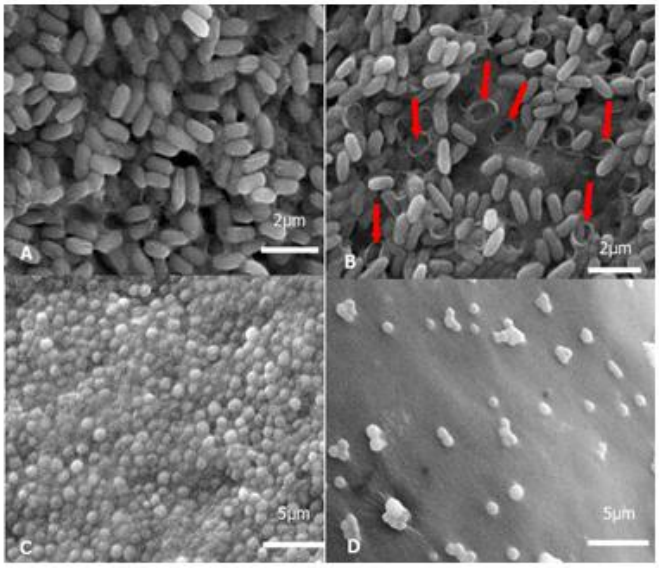

Figure 2: Effect of *C. dactylon* ethanol SPE-extract (flush fraction) on *B. subtilis* (A: Untreated; B: Treated) and *S. aureus* (C: Untreated; D: Treated) after approximately 7 hours of treatment. Note the membrane damage done by the flush fraction of *C. dactylon* ethanol SPE- extract on *B. subtilis* (red arrowed). Treatment on *S. aureus* shows reduction on the cell number but not provide any morphological evidence on membrane disruption.

D. Scanning Electron Microscope (SEM) Observation

Based on the SEM observation, it is confirmed that the flush fraction of *C. dactylon* ethanol SPE extract able to cause membrane damage as observed on *B. subtilis* (Figure 2). After treated with the extract, serious membrane disruption, indicated by large hole was produced on *B. subtilis* which most probably the causal for the bacterial death. However, no morphological evidence for membrane disruption was observed on *S. aureus* which contradict with the result obtained from leakage of 260nm absorbing material Live/Dead BacLight Assay.

E. Identification of Antimicrobial compounds

Mass spectral analysis revealed the presence of some antibacterial compounds in the fraction (Figure 3). Triterpenoid and its related compounds were known to exhibit antibacterial properties [8]. Ginsenoside was previously studied to possess antimicrobial properties against Gram positive and negative bacteria and also against fungal pathogens [9],[10] while Pandaroside had been investigated for its potential as antiprotozoal [11]. Cardenolide glycoside was previously studied to possess antimicrobial activity against soil borne and post-harvest fungi [12]. Peptide is one of the most potent classes of secondary metabolites which can exhibit very strong antimicrobial activity [13]. In the present study, two peptide compounds were identified (Gln Arg Arg and Arg Ser Ser). Same as peptide, polyketide compounds also can exhibit significant antimicrobial effect. Four polyketides were identified as mentioned in the previous section. Meanwhile, other potential antimicrobial compound which was identified based on the mass spectral analysis including methenamine. Previous work had shown that Methenamine was active against *Staphylococcus* and *Proteus* species [14].

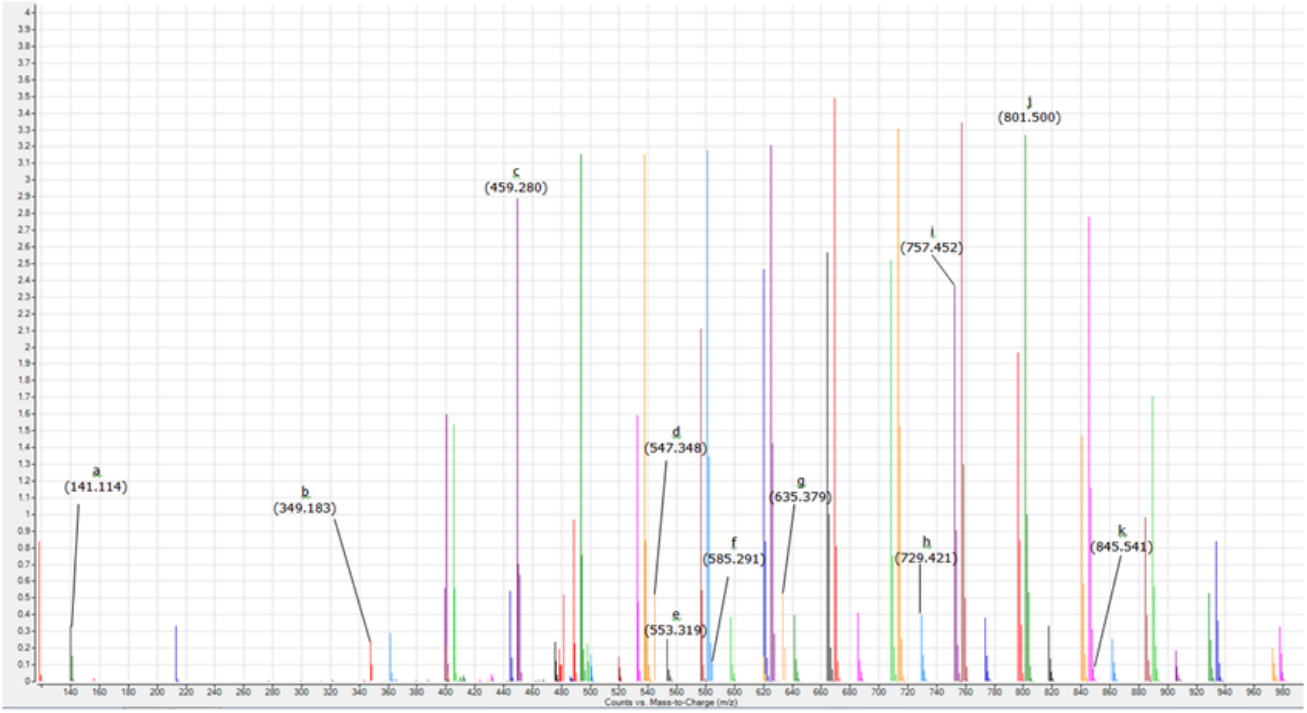

Fig. 3: Mass spectra (m/z) of some possible antibacterial compounds from flush fraction of *C. dactylon* ethanol SPE extract. a)Methenamine, b)Arg Ser Ser, c)GlnArgArg, d)3-O-mycarosylerythronolide B, e)Triterpenoid, f) Cardenolide glycoside, g)Pandaroside, h)Avermectin B1a monosaccharide, i)Gambierol, j)Ginsenoside, k) Halstoctacosanolide

IV. Discussion

Due to the diversity and abundance of natural product compounds especially in plant offer a great opportunity as an alternative medication in controlling and suppressing development of drug resistant pathogens. Previously *C. dactylon* was showed potential to be an alternative source for developing new antimicrobial agent [6]. Antimicrobial agent can exert different types of mode of action including membrane disintegration on biological cell membrane with other mode of action. In the present study, a number of potential antimicrobial compounds were identified from *C. dactylon* SPE.

Steroid and terpene derivatives identified might responsible for the antibacterial properties. In this study, three steroidal compounds which might responsible for antibacterial activity were identified; Ginsenosides, Pandaroside and Cardenolide glycoside. Previous author have reported the role of Kanamycin, an antibiotic combined with Ginsenoside extracted from Korean red ginseng against MRSA [9]. The study showed Ginsenoside enhanced the inhibitory effect, suggesting the synergistic effect of the compounds with the commercial antibiotic. Meanwhile, a recent study have demonstrated Ginsenoside extracted from *Panax quinquefolium*, an American ginseng can exert antimicrobial properties against *S. aureus*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa* and *C. albicans* [10]. Pandaroside, a steroidal

glycoside was also studied for its antimicrobial properties. Previous work has demonstrated the potential of pandaroside compounds as antiprotozoal agent against *Leishmania donovani* and *Trypanosoma brucei rhodesiense* [11]. Cardenolide glycoside is a cardiac glycoside which used to treat congestive heart failure and supraventricular arrhythmias due to reentry mechanisms, and to control ventricular rate in the treatment of chronic atrial fibrillation [15]. In different work, an author has demonstrated the antimicrobial properties of cardenolide glycoside against *Rhizoctonia solani*, *Fusarium oxysporium*, *Rhizopus stolonifer* and *Penicillium digitatum* [12]. Although most of the terpenes and the derivatives identified in *C. dactylon* were

not reported for antibacterial properties, one of the compounds, triterpenoid has gained much interest. Lot of studies has been published on the role of triterpenoids as antimicrobial agent. An author has described the role of triterpenoids from *Clerodendron trichotomum* as antibacterial agent against *S. aureus, E. coli* and *Helicobacter pylori* [16]. It is known that some hydrophobic compounds which mostly derived from steroids and terpenoids are known to possess capability to insert themselves into bacterial phospholipids bilayer and it has been reported that their bactericidal effects are linearly related to their hydrophobicity [17]. For example, in a study on plaunotol, which have the highest hydrophobicity compared to the other diterpene derivatives showed greatest bacterial membrane disintegration effect against *B. cereus* and *B. subtilis* [18]. Due to long or heavy C-skeleton structure, the insertion of terpenoidal compounds from the plant extract into the bacterial inner membrane causes it to become more fluid and permeable [19]. The increased permeability of the membrane by the insertion of hydrophobic area and the C-skeleton structure can allow internal contents to leak from the cell, which can cause growth inhibition or even death [20].

One of the most interesting compound classes found in this study was the peptide. In this study, two peptide compounds were identified; Gln Arg Arg and Arg Ser Ser. Lot of studies have been discussed previously on the potential of peptide as antimicrobial agent, which commonly referred as Antimicrobial Peptide (AMP). For instance, short Acyl-Lysyl Oligomer has been demonstrated against various Gram positive and negative bacteria [21]. Previous work has shown that AMP can exert membrane disintegration against *E. coli* and *S.aureus* [22]. The peptides identified in the present study might responsible to the antibacterial activity and probably fall into AMP class. It is well understood that regardless of their actual target of action, all antibacterial peptides must interact with the bacterial cytoplasmic membrane [23]. In this study, treatment by flush fraction of *C. dactylon* ethanol SPE against *S. aureus* shows great antimicrobial mode of action based on the ATP activity assessment although no promising evidence or very minimal hemolytic activity on membrane disintegration as shown by *S. aureus* was observed from 260nm absorbing material leakage and Live/Dead BacLight Assay assessment. However, based on SEM observation the number of bacterial cell has been reduced significantly after treated with the plant extract. Study conducted previously [21] on the effect antimicrobial peptide (AMP) on *S. aures* membrane cell have shown that this AMP can exert very strong antimicrobial effect on the bacteria via: 1) very low hemolytic activity by modify the membrane properties, thereby altering processes linked to membrane functions that depend on fluidity and charge distribution and 2) bacteriostatic effect, probably through direct inhibition of DNA function which is one of the major mode of action of AMP. In this study, it is suggested that the mode of action of the flush fraction of *C. dactylon* ethanol SPE extract happen in the same fashion where the plant extract altering the bacterial cell membrane and cause increase in the membrane permeability and allow other bioactive component to diffuse into the cell and disturb the cell metabolic activity. This effect is likely to be more pronounced for gram-positive bacteria due to the lack of additional permeability barriers, particularly the outer membrane of gram-negative bacteria.

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