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Metabolomic Investigation of *Elizabethkingia meningoseptica* Response Challenge with *Excoecaria agallocha* Leaf Extract

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Disease causes alterations in metabolism that may possibly be acknowledged as metabolic signatures and used to distinguish healthy and disease conditions.The present study was conducted for analytical platform that allows comprehensive analysis of microbial metabolomics. Different metabolites have been evaluated *in vivo* to characterize *Elizabethkingia meningoseptica* response on effects of methanol leaf extracts of *Excoecaria agallocha* using Liquid chromatography/mass spectrometry (LC/MS), utilizing a time-of-flight (TOF) mass analyzer. *In vitro* screening at different concentrations of *E. agallocha*methanol leaf extracts showed alteration in the shape of *E.meningoseptica* using scanning electron microscope (SEM). The profile of metabolites *in vivo* experiment showed 82 compounds that were initially identified.Only 75 compounds satisfy the *P-Corr*cut-off point of less than 0.05 and at least 2-fols change. In bacteria isolated from skin and kidney, initially a total of 240 metabolites were identified and only 79 metabolites satisfied the *P-Corr*cut-off point of less than 0.01 and at least 2-fold change. The result from scanning electron microscope (SEM) showed *E.meningoseptica* destroyed at concentration of 12.5 mg/mlof methanol leaf extracts of *E. agallocha.* To conclude, *E. agallocha* are diverse, produce compounds with antimicrobial activity and are a suitable sourcefor antimicrobial natural products.

Key words: *Elizabethkingia meningoseptica*, metabolites, LC/MS Q-TOF,*Excoecaria agallocha*,SEM.

Metabolites from mangrove plants are of interest for the discovery of antimicrobial compounds after increasing antimicrobial resistance of bacteria against antibiotics[1, 2, 3]. Moreover, many researchers have studied the antimicrobial activity of mangrove plant extracts on fish bacteria; [4]used methanol leaves extract of *Avicennia marina (A. marina)* and *Rhizophora apiculata (R. apiculata) leaves* against *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *Vibrio anguillarum* in marine ornamental fish.Another study by[5] used *Sonneratia caseolaris* and *Rhizophora apiculata* against fish pathogenic bacteria; *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Enterobacter brevis*, *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophila*. Furthermore, [6]used leaves extract of *Excoecaria agallocha* against fish pathogenic bacteria *Vibrio. harveyi* , *Vibrio parahaemolyticus*, *Aeromonas hydrophyla*, *Serratia spp* and *Bacillus subtilis*. Microbial

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metabolomics has received much attention in recent years mainly because it supports and complements a wide range of microbial research areas from new drug discovery efforts to metabolic engineering[7]. The term metabolomics refers to the comprehensive (qualitative and quantitative) analysis of the complete set of all low molecular weight metabolites present in and around growing cells at a given time during their growth production cycle [8]. Currently, as a result of the applications of metabolomics, there is a vast advancement in the development of new diagnostics, biomarkers, and subsequently treatments for disease. However, studying on how the metabolic profile of a complex biological system is altered in response to stresses such as disease, exposure to toxins and diet leads to a major impact on detection and control of diseases. Thus, diseased states cause alterations in metabolism that may possibly be acknowledged as metabolic signatures and used to set apart between healthy and disease conditions[9]. Moreover,metabolic signatures like these are based on the contrasting metabolite profile of different disease states and LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity [8]. On the other hand,Electron Microscopy (EM) is an important viewing technique for the study of microorganisms and there are plenty of applications of the electron microscope to the fields of biology, medicine, and material science [10]. Recently, [11]explore the potential of tea extracts on*Streptococcus mutans*using scanning electron microscopy (SEM). In the study of [12],used scanning electron microscopy (SEM) to reveal the effect of the aqueous extract of Piper betle L. leaves on *Streptococcus mutans*. In other study of[13]investigated the morphological effect of extract of *Quercus infectoria* galls on pathogenic bacteria *Escheria coli, Staphylococusaurus* and *Bacillus subtilis* using scanning electron microscope (SEM).Hence, the present study focused on investigating the potential effect of metabolites from mangrove plant *Excoecaria agallocha* which could be used to inhibit *Elizabethkingia meningoseptica* growth via LC/MS QTOF and investigate the potential effects of extract by usingscanning electron microscopy (SEM) on the surface morphology of *E. meningoseptica*.

MATERIALS AND METHODS

Chemicals and solvents utilized were of LC/MS grade except where otherwise stated. Acentonitrile (ACN) and methanol were from Merck (KgaA, Darnstadt, Germany). Ultra-pure low resistance distilled water was from Milipore (MA, USA).

Bacterial Isolation

Diseased African catfish, *Clarias gariepinus* (Burchell, 1822) were collected from a local farm culture Marang River Terengganu, Malaysia (05°12'N, 103°13'E). The fish weighed from 350 to 400 g. The fish were dissected according to[14]was performed by standard methods [15]. Shieh agar supplemented with tobramycin (1 mg ml^{-1}), enriched Anacker and Ordal's agar (EAOA) supplemented with polymyxin B (10 U ml-1) and neomycin (5 µg ml-1) were prepared. Bacteriological swabs were aseptically taken from the skin lesions liver and kidney of catfish. Incubation was done at 28°C for 48 h. The presence of flexirubin-type pigments in bacterial colony was determined using the KOH method[16]. Shieh agar and Anacker and Ordal's agar (EAOA) were selected in isolate and maintain bacteria in present study. The pure cultures were stored in enriched Anacker and Ordal's broth (EAOB) supplemented with 20% glycerol at -80°C.

Bacterial Identification

The bacterial colony morphology was determined after 48 h of incubation at 28°C. Physiological tests temperature tolerance at 5°C, 28°C, 37°C, 42°C and salt tolerance in different concentrations 0.5 %, 1.5 %, 3 %, 6 %, 8 %, 10 %) of NaCl (w/v) were also performed according to[17, 18, 19] . The isolates were subject to hemolysis test. The isolates were further biochemically identified using BBL-CrystalTM Enteric/Non Fermenter Identification System Kit (USA), API 20E and API ZYM System (bioMerieux, France) according to the manufacturers' instructions.

16S rRNA Identification

The DNA was extracted from the isolates using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. 16S rRNA gene PCR was carried out using published universal bacterial primers (5'-GAT TAG ATA CCC TGG TAG TCC AC-3' and 5'-

CCC GGG AAC GTA TTC ACC G-3') to generate a fragment approximately 610 bp in length according to[20, 21] in a Master Cycler Personal (Eppendoft, Germany). The PCR was done with minor modification. The reaction began with an initial denaturation step of 96°C for 2 min followed by 35 cycles of 96 ˚C for 1 min, 60˚C for 1 min, and 72°C for 1 min. The reaction was terminated by an extension step of 72°C for 10 min. Negative control with sterile distilled was included in PCR. The PCR products were analyzed by 1.5% agarose gel (molecular grade) electrophoresis, stained with ethidium bromide, and viewed under ultraviolet light. The PCR products were purified using Gene MATRIX PCR/DNA Clean-Up DNA Purification Kit (EURx, Poland). The DNA sequences deduced were subject to standard basic local alignment search tool (BLAST) analysis at National Center for Biotechnology.Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=MegaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) to determine identity of the isolates.

Collection of plant materials and processing

Excoecaria agallocha leaves were collected from the mangrove rural area in Terengganu, Malaysia. The plant was identified at the Plant Taxonomy Laboratory, University Malaysia Terengganu (UMT) Malaysia. The plant leaves were washed with tap water and then with distilled water to remove epiphytes and other debris. The leaves were then air dried in a shade area for 3 weeks before being grounded to powder (15 g dry weight) and then extracted with 100 ml of methanol for 48 hours according to the methods of [5]. The extract was filtered using a filter paper. This procedure was repeated three times and the samples were pooled. The solvent was evaporated from the crude extract by a rotator evaporator (Buchi, Switzerland) and the dried extracts were stored at 4°C until further use.

Experimental animals and designs

African catfish, *Clarias gariepinus* (Burchell, 1822), weight ranging from 450-500 g, were collected alive from hatchery of Universiti Malaysia Terengganu, Malaysia. The fish were acclimatised in 20 L tank/10 fish (salinity 0.25 ± 0.5 ppt, pH 7.2 ± 0.2, temperature 26 ± 2°C; dissolved oxygen 4.4 ± 0.1 mg/L; photoperiod: light-dark cycle of 14:10 h) and were provided with continuous aeration. The fish were fed twice daily at 3% of body weight with 3-5 mm dry pelleted diet (10% carbohydrate, 20% lipid, 55% protein, 12% ash and 3% vitamin and minerals) during acclimatisation periods for 14 days. Food was withdrawn 24 h prior to the experiment. In order to determine the metabolic profile of fish and bacteria, the experimental approaches were divided into 2 groups as described in the following sections.

Determination of metabolic profiles of bacteria isolated from the fish that were fed with plant extract versus bacteria isolated from the fish that were not fed with plant extract.

Group 1 (ten fish) was fed with crude leaf extract of *Excoecaria agallocha* at concentration of 50 mg kg^{-1} for 2 weeks. Group 2 (ten fish) was fed in the absence of plant extract. The fish were placed individually in the flow chambers and were deprived of food 24 h. The fish were anaesthetised with solution containing 300 mg/ml tricainemethanesulphonate (MS-222) (Argent Chem. Lab., U.S.A.). Both Groups were injected intraperitoneal with bacteria *Elizabethkingia meningoseptica* at dose levels of $4x10^5$ CFU mL^{-1}(this dose was calculated previously from determination of Lethal dose LD_{50}). After 24 h, bacteria swab from kidney was taken for test.

Determination of metabolic profiles of bacteria isolated from skin versus bacteria isolated from kidney.

This experiment consisted of two groups of fish. Each group of fish (10 fish) was fed normally for 2 weeks. Group 1 was injected with bacteria *Elizabethkingiameningoseptica* at a dose of $4x10^5$ CFU mL^{-1} previously isolated from the infected skin of fish. Group 2 were also injected with the same bacteria at a dose levels of $4x10^5$ CFU mL^{-1} previously isolated from the infected kidney of fish. After 24 hours the bacteria were re-isolated from skin and kidney.

Sample preparation

Bacteria cell extraction

For preculture and main culture of *Elizabethkingia meningoseptica* medium, Anacker and Ordal's broth (EAOB) was used. Cultivations were carried out in duplicate at 28°C and 200 rpm on incubator rotary shaker (Innova 40 R, USA). Aerobic cultures of *E. meningoseptica* were grown

in 500 mL Anacker and Ordal's broth (EAOB). Bacterial cell count was carried out using a UV-VIS spectrophotometer (Shimadzu, Japan) with optical density (OD) at 600 nm. Subsequantly, the medium was filtered with filter-sterilized Whatman No 1 filter paper (Whatman, China) and centrifuged at 11,000 rpm for 15 minutes at 4˚C. The supernatant was removed and the cell pellet was washed with cold 0.6% (w/v) NaCI two times. The cell pellet was transferred into the tube pre-immersed in liquid nitrogen for 1 minute, and subsequently resuspended in chloroform: methanol 1:2 (v/v). Glass beads 1:1 (v/v) were then added according to the volume of the cells and sonicated for 5 minutes with 20 sec pulse on and 20 sec pulse off. Then 2 mL of chloroform: water (1:1) (v/v) was added into the tube and centrifuged at 11,000 rpm for 5 minutes at 4˚C. The upper layer was transferred into different 1.5 mL centrifuge tubes, dried by vacuum dryers, mixed with 30 ¼l mobile phase (95%:5%, v/v), vortexed and centrifuged at 11000 rpm, 10 minutes, 4˚C. Finally, 20 ¼l supernatant was transferred into auto sample vial to be injected into LC/MS QTOF [22].

LC/MS Q-TOF analysis

Analysis of sample was performed using a 1200 Rapid Resolution Series (Agilent Technologies, Santa Clara, CA, USA), which comprised of a binary pump, degasser, well plate autosamplerwith thermostat and thermostat column compartment along with 6520 QTOF mass-spectrometer with a dual-ESI source. The column compartment was a Zorbax Eclipse XDB-C18 with 1.8 ¼m particle size and 4.6x50 mm column dimensions (Agilent Technologies, CA, USA) with the temperature maintained at 4˚C. Samples were analyzed in positive mode with the mobile phase (A) consisted of 0.1% (v/v) formic acid in water and solvent (B) was 0.1% (v/v) of formic acid in ACN. The flow rate was 0.25 mL/min and the injection volume was 2 ¼L. A linear gradient was developed over 18 minutes from 5% to 95% of mobile phase (B). The total run time was 30 minutes for each run. ESI source settings were 4000 V for V Cap, 65 V for skimmer and 125 V for fragment. The nebulizer was set at 45 psig, the flow rate of nitrogen drying gas was set at 12 L/min and drying gas temperature was maintained at 35˚C. The machine was tuned regularly to adjust TOF and quadrupole parameters in order to achieve the desired signal intensity and resolution. Calibration was also done preceding each run. The reference mass correction (< 2 ppm) was done online at each run with two reference masses (121.050873 and 922.009798) selected for correction of low and high masses.

Data processing

Data were collected using Aglient Mass Hunter Workstation Data Acquistion software and processed using Agilent Mass Hunter Qualitative Analysis software. Compound identification, statistical analysis and visualization were performed using Agilent Mass Professional Profiler (MPP) coupled with METLIN Personal Metabolite Database. For all set comparisons, the parameters used were as follows based on manufacture's instruction.

i. Total entities detected were filtered for compounds that were present in at least 2 samples.

ii. Only entities that appeared in at least 50% of samples in at least one condition were retained.

iii. The p-Corr (correction) with the lowest number of compounds expected by chance was chosen instead of p-value.

iv. The cut off point for the fold-changes analysis was set to entities with at least 2-fold changes (FC e" 2.0) filtering in abundance.

The entities list was subsequently submitted to ID Browser for the identification of the entities. The identified compounds were finally reanalyzed using MPP by various statistical tests, clustering analysis and unsupervised principal component analysis (PCA).

Samples grouping for biological interpretations

In order to determine the potential metabolites that were differentially presence in various experimental samples, the samples were grouped for comparison as shown below:

i)Bacteria isolated from fish that were fed with plant extract versus bacteria isolated from fish that were not fed with plant extract

ii)Bacteria isolated from skin versus bacteria isolated from kidney of infected fish

Scanning Electron Microscope (SEM)

Elizabethkingia meningoseptica in methanol extract was fixed with 2.5% glutaraldehyde in 0.1M Tris Buffer (pH7.3, 4˚C, 1 h) according to [23]. Sample was rinsed two times in Tris Buffer with 5% sucrose and fixed in 1.0% osmium tetroxide solution in the same buffer for 1

h. Afterwards, sample was rinsed with 0.1 M Tris buffer and dried out by successive extractions with 50 %, 70 %, 80 %, 90 % and 95 % ethanol (10 min each), 100 % ethanol twice for 15 min. Lastly, sample was air-dried by CO_3 critical-point dying technique, gold glazed, and was examined using Scanning Electron Microscope (SEM).

RESULTS

Bacterial Isolation

The diseased fish showed symptoms of increased respiration and lethargy, skin lesions such as white discoloration, shallow hemorrhagic ulcers or deep ulcers with exposed underlying muscle. Some fish showed marked hemorrhages on the base of the fins and vent. Others were dropsy, kidney congestion and enlargement, pale liver and gills, or gall-bladder enlargement with the accumulation of yellowish fluid in the body cavity. Bacterial isolates were obtained from kidney (K1) and skin lesion (S1) that grown on Shieh and enriched Anacker and Ordal's agar (EAOA). Colonies were small (1 to 2 mm diameter), smooth surface, convex, circular and shiny with complete edges on EAOA and Shieh agar. Colonies were yellowish colored on EAOA agar and creamy colored on Shieh agar. They were Gram negative, non-motile, straight, single short rods approx. 2 ¼m in length and 1 ¼m in width. They were then subject to identification using conventional, commercial kits and 16S rRNA PCR. BBL results showed that Isolates K1 and S1 were 99 % similar to *E. meningoseptica*. On the other hand, API 20E identified isolates K1 and S1 as *E. meningoseptica* with 95.4% similarity. All 16S rRNA gene sequences showed more than 97% homology to sequences deposited in GenBank. The isolates were identified as *E. meningoseptica*. Blast result and electropherogram were showed. All 16S rRNA gene sequences showed more than 97% homology to sequences deposited in GenBank. The isolates were identified as *Elizabethkingia meningoseptica* and depositedinGenBank with accession numbers for our nucleotide sequences:BankIt1604905 KC757123BankIt1611391 KC757124.

Validity of analysis

All of the samples were successfully analyzed using sample preparation and LC/MS Q-TOF analysis methods. Example of the

Table 1. List of metabolites that were differentially expressed in bacteria isolated from fish feeding with plant extract versus bacteria isolated from fish without feeding with plant extract

Compound	Regulation Treated Bacteria VS. Untreated	Function
Tricosanedioic acid	Down	Phosphocholine
2-Heptenedioic acid, 4-oxo-	Up	Bacterial inhibitors
I-Methyl-4-nitro-5-thio-imidazole	Up	Healing
Dirithromycin	Up	Antimicrobial
14,15-EpETrE	Up	fatty acid
Cuscohygrine	Up	Antibacterial
Cetrimoniumchlorid	Up	Disrupting micro-organisms'cell
1-hexadecanoyl-sn-glycerol	Up	Fatty alcohol

Table 2. List of metabolites that were differentially expressed between bacteria isolated from skin versus bacteria isolated from kidney

Compound	Regulation Skin VS. Kidney	Function
4-Hydroxytacrine	Down	Cholinesterase inhibitor
Nonoxynol	Down	penetration enhancer
5-hydroxyfluvastatin	Down	Inhibitor on(ROS)
12,13S-epoxy-9Z,11e-octadecadienoic	Down	Fatty acid
3,9,15-Docosatriynoic acid	Down	Fatty acid

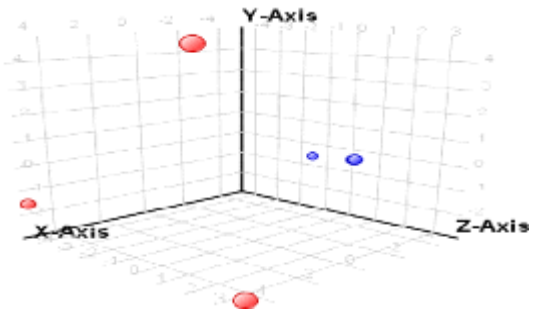

Fig. 1. The PCA 3D analysis for metabolites identified in bacteria isolated from fish feeding with plant extract versus bacteria isolated from fish without feeding with plant extract

chromatogram and spectrum profile is shown in Appendix 1. Diverse classes of metabolites were effectively detected and identified including lipids, peptides, and small molecules which indicate that the extraction methods are consistent for metabolomic analysis using LC/MS platform.

Determination of metabolites in bacteria isolated from fish feeding with plant extract versus bacteria isolated from fish without feeding with plant extract.

The profile of metabolites between bacteria isolated from fish feeding plant extract versus bacteria isolated from fish without feeding

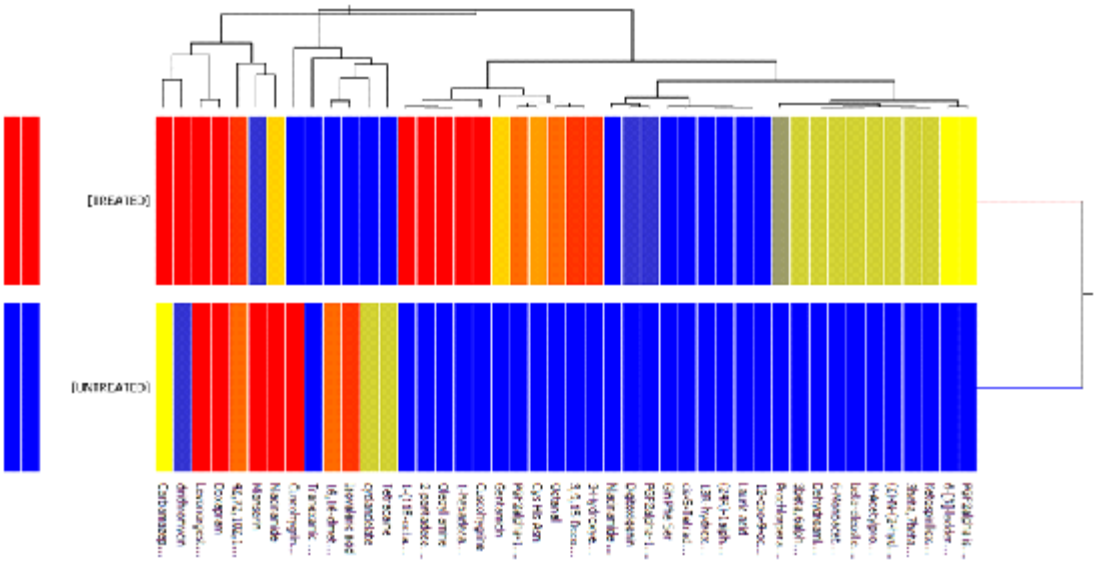

Fig. 2. Dendogram tree clustering analysis for metabolites identified in bacteria isolated from fish feeding with plant extract versus bacteria isolated from fish without feeding with plant extract

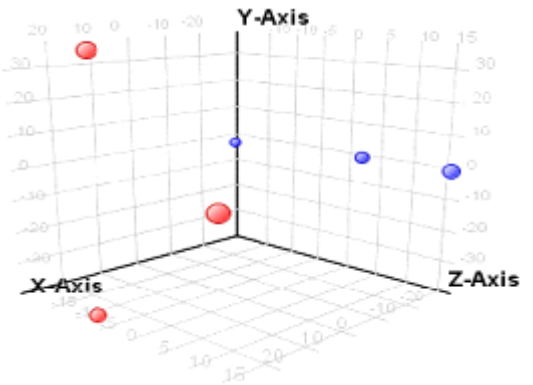

Fig. 3. The PCA 3D analysis for metabolites identified in bacteria isolated from skin versus bacteria isolated from kidney.

plant extract intake was compared in this set of analysis. From 82 compounds that were initially identified, only 75 compounds satisfy the *P-Corr*cut-off point of less than 0.05 and at least 2-fols change. Chemometric technique for data analysis (PCA) becomes an important tool for detecting differences in the metabolic profiles and it can be clearly seen from the PCA 3D analysis that all groups were well detached and there were biggest variation in the data shown that the two components are different and partially separated from each other (Figure 1). The regulations of metabolites expression are as shown in the dendogram tree in (Figure 2), while the summary of metabolites is shown in (Table 1)

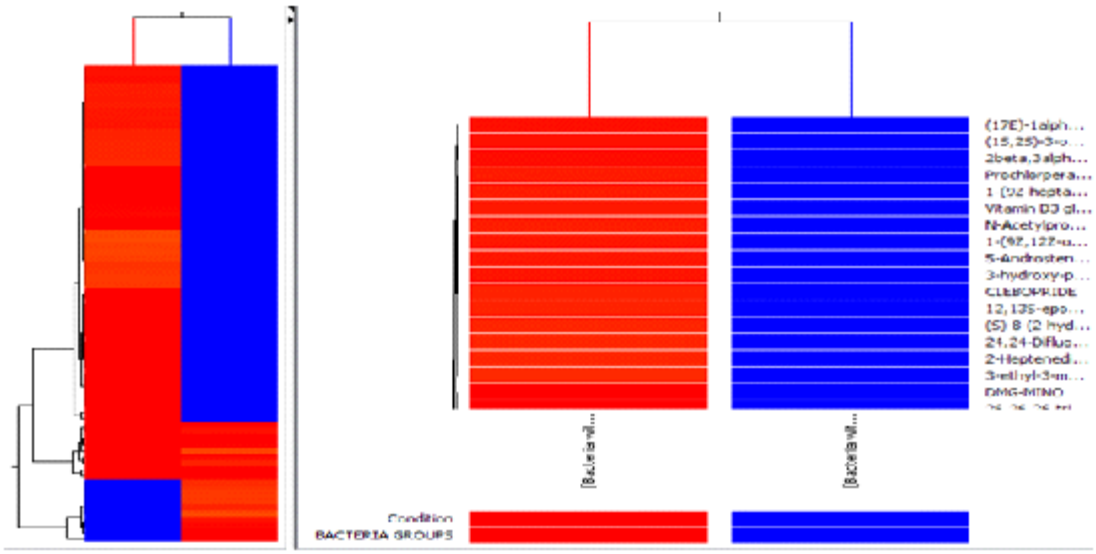

Fig. 4. Dendogram tree clustering analysis for metabolites identified in bacteria isolated from skin versus bacteria isolated from kidney

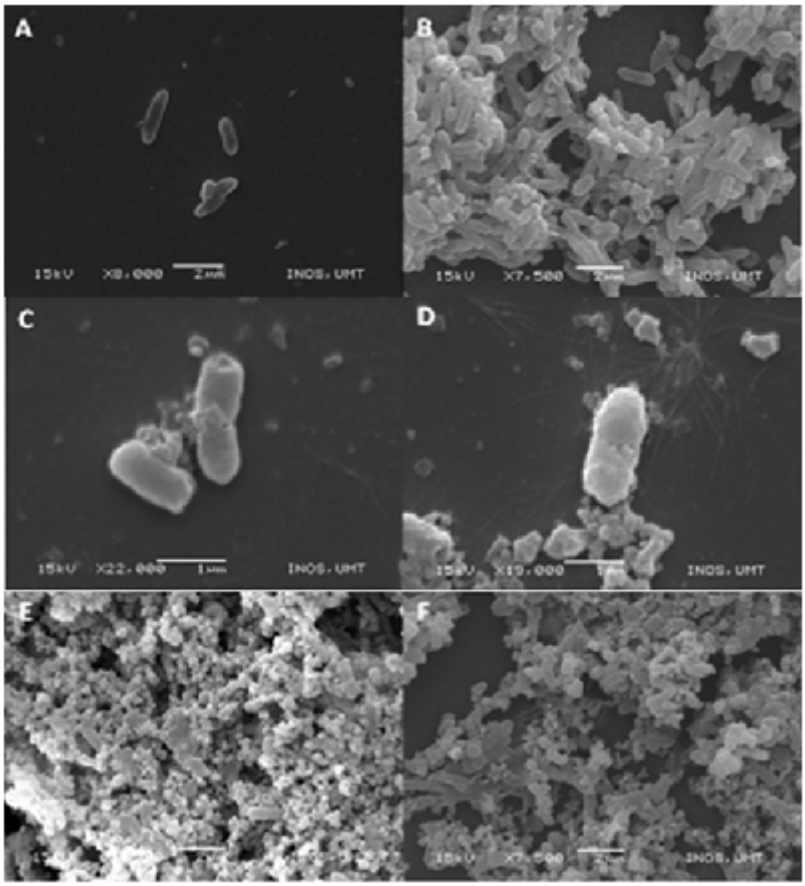

Fig. 5. SEM images of *Elizabethkingiameningoseptica* cells in different concentrations of *Excoecariaagallocha* crude leave extract

Determination of metabolites in bacteria isolated from skin versus bacteria isolated from kidney.

In bacteria isolated from skin and kidney, initially a total of 240 metabolites were identified. However, only 79 metabolites satisfied the *P-Corr*cut-off point of less than 0.01 and at least 2-fold change. Chemometric technique for data analysis (PCA) becomes an important tool for detecting differences in the metabolic profiles and it can be clearly seen from the PCA 3D analysis that all groups were well detached and there were biggest variation in the data shown that the two components are different and partially separated from each other (Figure 3).The regulation of metabolites expression is as shown in the dendogram tree in (Figure 4). While the summary of metabolites and their functions are shown in (Table 2).

Scanning Electron Microscope (SEM)

Normal (A). At the lowest concentration of 0.78 mg/ml (B), bacteria appeared normal. At 1.56 mg/ml concentration(C), the bacteria cell membrane showed signs of swelling, and severe swelling was noted at 3.125 mg/ml (D). Bacteria cell was ruptured at 6.25 mg/ml (E) Concentration, and destroyed in the highest concentration of 12.5 mg/ml (F) (Figure 4.4).

DISCUSSION

Bacterial resistance to antimicrobials is a public health problem. Therefore, new strategies to control bacterial infections are highly desirable[24]. The use of plant extracts appears as an attractive alternative to antibiotics. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illness since ancient times[25]. Measurements of low molecular weight metabolites have been increasingly incorporated in the characterization of cellular physiology, qualitative studies in functional genomics, and stress response determination using LC-MS methods to describe the analysis of microbial metabolomes[26]. As shown in (Table 1), 5 metabolites; Tricosanedioic acid, 2-Heptenedioic acid 4-oxo-, I-Methyl-4-nitro-5-thio-imidazole, dirithromycin, and 14, 15-EpETrE were differentially expressed in bacteria isolated from fish feeding Plant extract versus bacteria isolated from fish without feeding Plant extract may be related to the effects of *Excoceria agallochia* extract which particularly create bioactive molecules that enable them to react with other organisms in the environment, resulting in the inhibition of bacterial and fungal growth[27].

Tricosanedioic acid is phosphocholine derivative; some bacteria like pneumococci have an absolute nutritional requirement for choline. Choline is incorporated as phosphocholine (PCho) into lipoteichoic (LTA) and teichoic acid (TA). The PCho residues are required for transformability, the activity of autolysins, and the separation of daughter cells after cell division and for anchoring a family of surface proteins which play important roles in pneumococcal infection[28]. Although, Tricosanedioic acid was lower levels in group fed with plant extract as uptake by bacteria compared to the group that was not given plant extract,this may be related to the antimicrobial activity of *Excoceria agallochia* extracts[29]. 2-Heptenedioic acid, 4-oxo was up-regulated in group fed with plant extract as uptake by bacteria compared to the group that was not given plant extract, its function as inhibitors of bacterial dihydrodipicolinate syntheses [30]. I-Methyl-4-nitro-5-thio-imidazole has antiulcer activity and can be used in the treatment of peptic ulcers and other pathologies caused or stimulated by gastric acidity [31].Dirithromycin is antibacterial interfering with cell-wall biosynthesis [32]. On the other hand, 14, 15-EpETrE, belong to a monounsaturated fatty acid, derived from rapeseed oil, wall flower seed, or mustard oil. These metabolites was found naturally in many green plants had antimicrobial activity against gram-positive bacterium, *Staphylococcus* aureus; gram-negative bacterium, *Escherichia coli*; mold, *Penicilliumnotatum*, and yeast, *Candida utilis*. All compounds inhibited at least one of these organisms [33]. Cuscohygrine is a pyrrolidine alkaloid found in coca. Pyrrolidine is also found naturally in the leaves of tobacco and carrot and can be extracted from plants, like *Withania somnifera* belongs to family Solanaceae which contains cuscohygrine and has antibacterial activity against different strains of pathogenic bacteria[34]. Cuscohygrine was high levels in group fed with plant extract as uptake by bacteria compared to the group that was not given plant, this finding might be related to the effect of *E.agallochia*

extracts to inhibition the growth of *E.meningoseptica*. Despite little information is available about the mechanisms of action of antimicrobial compounds in bacteria, however, researchers suggested the mechanisms include membrane damage, changes in intracellular pH, membrane potential, and ATP synthesis[35,36]. Recently, the study on the mechanism of antimicrobial activity of plant against *Vibrio cholerae* by [37]concluded that the mechanism of extracts of edible and medicinal plants occurs through the changes in membrane integrity, membrane potential, internal pH and ATP synthesis of cell bacteria and cause damage to the membrane of *V. cholerae*, exerting profound physiological changes that lead to bacterial death.Cetrimonium chloride, this metabolite is quaternary ammonium compounds and has a variety of physical, chemical, and biological properties such as antimicrobials and antiseptic activities [38]. The compound 1-hexadecanoyl-sn-glycerol is fatty alcohols, derived from natural fats and oils produced from vegetable oils such as palm oil and coconut oil. Fatty acids are important biocompounds which take part in complex metabolic pathways[39]. Plans are known to produce certain chemicals which are naturally toxic to bacteria and the mechanism of antimicrobial action appears to be loss of control over cell membrane permeability [40]. As shown in (Table 2), three metabolites; 4-Hydroxytacrine, I-Methyl-4-Nanoxynol and 5-hydroxyfluvastatin, were differentially expressed in bacteria isolated from skin as compared to bacteria isolated from kidney. These metabolites were in lower level in bacteria isolated from skin but in high level in bacteria isolated from kidney. The compound 4-Hydroxytacrine is an inhibitor of enzyme acetyl cholinesterase and its immunomodulatory effect is based on inhibition of cholinesterase in the peripheral nervous system[41]. *Elizabethkingia meningoseptica* is ubiquitous and environmental contamination could occur readily[42]. Most of the infections occur in immune-compromised patients and premature neonates suffering from meningitis [43]. Therefore, higher levels of 4-Hydroxytacrine in bacteria isolated from kidney may be revealed the virulent of *E. meningoseptica* in an infected fish. On other hand, disappear of this metabolites in bacteria isolated from fish feeding plant extract may be indicated to the antimicrobial activity of *Excoecaria agallocha*.Other compound is Nonoxynol used as the penetration enhancer and can be found in many common spermicidal. Nonoxynol works by damaging sperm cell membranes by breaking these outer barrier membranes, it immobilizes and kills sperm. Adherence-enhancing effects of nonoxynol-9 were evaluated against vaginal and uropathogenic bacteria such as *E. coli* and *lactobacilli spp*[44]. Higher levels of Nonoxynol in bacteria isolated from kidney may enhancement of adherence and easily penetrated the host barrier.Third compound is 5-Hydroxyfluvastatin which is the metabolite of Fluvastatin and had inhibitory effects on the formation of several reactive oxygen species (ROS). Reactive Oxygen Species (ROS) have long been known to be a component of the killing response of immune cells to microbial invasion [45]. The levels of 5-Hydroxyfluvastatin metabolite were found to be lower level in bacteria isolated from skin but higher level in bacteria isolated from kidney and may be referred to the virulent effect of *E. meningoseptica* to destroyed host defence. These compounds12, 13S-epoxy-9Z, 11e-octadecadienoic and 3, 9, 15-Docosatriynoic acid, were fatty acid with medium to long-chain saturated and unsaturated monocarboxylic acids. Fatty acids play an essential role in metabolism, providing the cell with a concentrated source of energy, and form the structural foundation of the cell membrane. Fatty acids also play a crucial role in growth and development. Cellular fatty acids play a role as a good biomarker of changes in the physiological status of microorganisms caused by external factors [46]their main function in membranes may be to increase the fluidity of lipids as an alternative to double bonds, which are more liable to oxidation. It is very important for the barophilic or barotolerant bacteria to maintain the physiological functions of cell membrane due to many vital functions are involved in the membrane. Therefore, the characterization of membrane at high pressure is the initial task to clarify the biochemical adaptation of bacteria to the environment [47]. Bactericide compound commonly inhibits growth of bacteria through wall irritation cell, protein coagulation, and hydrolysis of cell plasma resulting in osmotic pressure imbalance [48]. The peptide compound from mangrove *E. agallocha* extract inhibits the growth of bacteria through irritate cell and coagulate

protein of the bacteria [49]. Scanning Electron Microscope (SEM) was adopted to observe morphological alteration of *E.meningoseptica* after treated with *E. agallocha* extracts, [50]. Meanwhile, the direct exposure of *E.meningoseptica* to extract of *E. agallocha* may causes a remarkable modification of the *E.meningoseptica* cell form, as displayed by Scanning Electron Microscope (SEM) imaging. Hence, untreated bacteria with *E. agallocha* extract showed rod-shaped with blunt ends having a smooth phenotype. However, the bacteria after being disposed with *E. agallocha* extract exhibited elongated shape. At concentration 6.25mg/ml of *E. agallocha* extract on *E.meningoseptica* exhibited the following characteristics; elongation, rough surface coat with discrete wart-like structures. While at concentrations 12.5 mg /ml of *E. agallocha* extract on E.meningoseptica exhibited a range of significant abnormalities with a deep roughened on the cell surface of *E.meningoseptica*. However, at concentration 25 mg /ml of *E. agallocha* extract on *E.meningoseptica* its look like to have lost their content, although the overall cell shape was still recognizable and bacteria exhibited elongated resulted in changes of bacteria surface structure all these alteration in agreement with [51]. Recently, study on the mechanism of antimicrobial activity of plant against *Vibrio cholerae* by[37]reported that the mechanism of extracts of edible and medicinal plants occurs through the changes in membrane integrity, membrane potential, internal pH and Adenosine triphosphate (ATP) synthesis of cell bacteria and cause damage to the membrane of *V. cholerae*, exerting profound physiological changes that lead to bacterial death. Another research done by[52]concluded that the potential of natural compounds may have direct inhibition on virulence gene expression in *Vibrio cholerae*. Furthermore, this inhibitory mechanism may be inhibited the secreted cholera toxin (CT) or the growth of bacteria. Our investigating may be similar to the study of [11] suggested that the potential mechanisms of tea extract components on *Streptococcus mutans* may be related to the alteration in bacteria cell wall structures and inhibition of bacterial proteins.

CONCLUSION

Metabolomics is able to illuminate the activities of a cell at the functional level. In present study them etabolic responses of *E. meningoseptica* using non-targeted mass spectrometry via LC/MS QTOF for screening of compounds with potential to be developed into biomarkers to observe various clinical conditions in fish**.** We also investigated the effect of the extracts on the morphological changes of *E.meningoseptica* under scanning electron microscope, this study demonstrated the effect of *E.agallocha* on the surface morphology of *E.meningoseptica* this affect appear through irregular and inhomogeneous in the shape of bacteria , may be related to the biochemical alternation in the structure of the cell wall.Plant extracts have shown inhibitory effect on the growth of the bacteria. Further study will helpful to understand molecular mechanism using gene expression.

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